The Dissertation Committee for Devendra Shrikant Pade Certifies that this is the approved version of the following dissertation:

USE OF *IN SILICO* PREDICTORS, SOLUBILITY AND PERMEABILITY TO SELECT BIOAVAILABILITY AND BIOEQUIVALENCE MARKERS IN HERBAL SUPPLEMENTS

Committee:

Salomon A. Stavchansky, Supervisor

Jennifer S. Brodbelt

Allan B. Combs

James W. McGinity

Robert O. Williams III

USE OF *IN SILICO* PREDICTORS, SOLUBILITY AND PERMEABILITY TO SELECT BIOAVAILABILITY AND BIOEQUIVALENCE MARKERS IN HERBAL SUPPLEMENTS

by

Devendra Shrikant Pade, B.Sc; M.Sc.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2007

Dedication

To my mother

Acknowledgements

First of all, I would like to thank my research advisor, Dr. Salomon Stavchansky for his patience, guidance, encouragement and support throughout my graduate training and research. I would also like to extend my sincere appreciation to all the members of my doctoral committee, Dr. James McGinity, Dr. Robert Williams, Dr. Jennifer Brodbelt, and Dr. Allan Combs for their guidance and review of this manuscript.

Special thanks go to Dr. Robert Pearlman and the Laboratory for Molecular Graphics and Theoretical Modeling of the College of Pharmacy at The University of Texas at Austin for the in valuable help and support extended to calculate the various *in silico* descriptors.

I would like to thank Ms. Charlotte Berndt for the assistance in conducting Caco2 cell transport experiments and Kinetana Inc., Alberta, Canada for the help with SimBioDAS® permeability.

I would also like to thank all my colleagues and friends for their support and help through out my graduate career.

Finally, the love, encouragement and support of my entire family specifically my parents Jaya and Shrikant Pade, and my uncle and aunt, Ramakant and Nirmala Mhatre, who have been instrumental in the successful completion of my work.

USE OF *IN SILICO* PREDICTORS, SOLUBILITY AND PERMEABILITY TO SELECT BIOAVAILABILITY AND BIOEQUIVALENCE MARKERS IN HERBAL SUPPLEMENTS

Publication No._____

Devendra Shrikant Pade, Ph.D

The University of Texas at Austin, 2007

Supervisor: Salomon A. Stavchansky

Due to their rising popularity, herbal supplements have created a specific niche for themselves between the food and the drug industry. Due to their categorization as dietary supplements, they lack scientific seriousness where as on the other hand they act like unregulated drugs with potential effects. Finding scientific data of questionable accuracy for herbal supplements is not uncommon, which is usually designed to sell products rather then provide unbiased information. Hence, development of performance standards based on the bioavailability of the active components of herbal extracts promises to be an attractive solution towards regulating the inflow of meaningful products in the herbal supplement market. Solubility, partition coefficient and permeability are the fundamental properties for studying drug absorption. Top selling herbal extracts from the United States that included Kava, Ginkgo biloba, Milk thistle, Ginseng, Black cohosh, Garlic, Valerian, and Echinacea were selected and *in silico* descriptors such as CLogP, minimal cross-sectional area, polar surface area and *in vitro*

permeability using the Caco-2 cell model and SimBioDAS® of their active components, determined. Based on the interparameter relationships between the minimal cross sectional area, CLogP, polar surface area and the *in vitro* permeability of the active components, bioavailability/bioequivalence markers were predicted for Kava, Ginkgo biloba and Milk thistle. Kawain was predicted as a marker for Kava, Ginkgolide B for the ginkgo terpenes and quercetin for the flavonol glycosides in Ginkgo biloba and silycristin as a marker for Milk thistle (silymarin). Silymarin comprising of isomers silycristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B was selected as a representative extract for further confirmation of marker prediction. Equilibrium solubility, experimental octanol-water partition coefficient values, and assay and *in vitro* dissolution profiles were determined for each of the active isomers in extract and market products respectively. The pharmacokinetics and absolute bioavailability of each of the active isomers was determined in male Sprague Dawley rats following intravenous and oral administration of the silymarin extract. Equilibrium solubility values indicated that all the silymarin isomers were practically insoluble, and silycristin and silydianin had relatively higher solubility values as compared to the other isomers. Experimental partition coefficient values correlated with the predicted partition coefficient (CLogP) with an r^2 of 0.834. Based on their equilibrium solubility and the partition coefficient (experimental and predicted) the active isomers were classified according to the Biopharmaceutic Classification System (BCS). Thus, isomers silybin A, silybin B, isosilybin A and isosilybin B were classified as Class II compounds (High Permeability-Low Solubility) where as silydianin was classified as a Class IV compound (Low Permeability-Low Solubility). Silycristin was classified as a intermediate between Class II and Class IV. Absolute bioavailability (F) for silycristin was found to be the lowest (0.15±0.1), followed by silybin A (0.20±0.04) followed by silybin B (0.62±0.08).

Silycristin being one of the least permeable and bioavailable component, was selected as a marker for silymarin, further confirming its prediction based on the correlations between the *in silico* descriptors and *in vitro* permeability. Pharmacokinetic parameters such as area under the curve, half life, volume of distribution, clearance and F for the components suggest significant differences between not only the silymarin isomers but also diastereomers of silybin (A and B) and isosilybin (A and B). Selection of bioavailability-bioequivalence markers, based on their least permeability/bioavailability, proves to be the most conservative and meaningful approach towards standardization of complex mixtures such as herbal extracts and supplements.

Table of Contents

List of Tables

List of Figures

STATEMENT OF OBJECTIVES AND SIGNIFICANCE OF RESEARCH

This dissertation is a result of an effort to establish the inter-relationships between *in silico-in vitro -in vivo* parameters that may help predict and select bioavailability and bioequivalence markers for herbal supplements.

The study was initiated with the following specific aims:

1. Selection of herbal extracts based on highest retail sales in the United States, whose active components would collectively span a wide range of molecular weight and show structural diversity.
2. Estimation of molecular descriptors such as the polar surface area, minimal cross sectional area and predicted octanol water partition coefficient for each of the active components in the selected herbal extracts.
3. Determination of *in vitro* permeability of the active components using the Caco-2 cell model or SimBioDAS,® both of which are epithelial cell based permeability assays.
4. To qualitatively predict markers for select herbal extracts (Kava, Ginkgo biloba, Milk thistle), that could have the least intestinal permeability by studying the inter parameter relationships between the *in silico* descriptors and the *in vitro* permeability of all the active compounds. Selection of a representative extract (Milk thistle) for the verification of marker prediction by *in vitro* and *in vivo* experiments.

5. To determine the solubility, apparent octanol-water partition coefficient and assay for content uniformity , *in vitro* dissolution profile of the active isomers in Milk thistle extract and select market formulations, respectively.
6. To determine the pharmacokinetic parameters and absolute bioavailability of the isomers after intravenous and oral administration of the extract in male Sprague Dawley rats.
7. To select the least bioavailable compound as a bioavailability and bioequivalence marker for that herbal extract, in an effort to verify the prediction obtained from the inter parameter relationships between the *in silico* descriptors and *in vitro* permeability.

The steady increase in sales on one side, and new reports, and experiments questioning their safety, efficacy and regulation on the other side, herbal extracts and supplements now demand scientific attention similar to synthetic drugs. Herbal extracts are complex mixtures with more than one active component, the pharmacologic effect of which is usually synergistic in nature. This is further complicated by large variations in the amounts of active components present in market formulations of the same extract having the same label claim. Therapeutic efficacy is proportional to the amount of drug at the action site within the therapeutic index, and drug reaching the systemic circulation (bioavailability) is an estimate of the amount of drug reaching the action site. Conducting routine bioavailability/bioequivalence tests for each active compound in a herbal extract is a tedious or a near impossible task. Thus, selection of a marker that represents the bioavailability of its extract is necessary for the biopharmaceutic

characterization of any herbal extract. Solubility, partition coefficient and intestinal permeability are the fundamentals of oral absorption and use of *in silico* and *in vitro* methods to predict intestinal permeability gives a certain directional focus in the process of marker selection. Selection of a compound that has the least intestinal permeability or bioavailability assures bioavailability of the rest of the active compounds in the extract and hence is the most conservative approach towards the biopharmaceutical characterization of herbal supplements. If implemented in practicality by Federal Law, bioequivalence studies based on marker selection can make a significant impact on the inflow of safe and biopharmaceutically relevant herbal supplements in to the American market.

CHAPTER 1: HERBAL SUPPLEMENTS: A Brief Background

1.1: HISTORY

"Life is short, the art long, the opportunity fleeting, the experiments treacherous, and judgment difficult."

–*Aphorisms i.1*, Hippocratic Corpus

Hippocrates (460-377 B.C)

The above quote by Hippocrates holds so much meaning when we think of standardization of herbal extracts and herbal drug products. According to the Hippocratic Corpus, Hippocrates himself is known to have worked on nearly 200 herbs in his life time. Herbal medicine or herbalism dates back over 5,000 years to the Sumerians, who described well established medicinal uses for plants such as laurel, caraway, and thyme. The first known Chinese herb book, dating back to about 2700 B.C., lists 365 medicinal plants and their uses, including ma-Huang, the shrub that introduced the drug ephedrine to modern medicine. There is evidence from the Shanidar Caves in Iraq that suggests Neanderthals living 60,000 years ago used medicinal plants. A body unearthed there had been buried with eight species of plants that are still widely used in ethno-medicine around the world. [1, 2] Medicinal herbs found amongst the personal effects of an "Ice man" whose body was frozen in the Swiss Alps for more than 5,300 years, appear to have been used to treat the parasites found in his intestines. [3]

Herbal remedies have become a major component of individual health care, and botanicals like Ginseng, Ma-Huang, Ginkgo, St. John's Wort, and many more have become household names throughout the world. Herbalism is a significant category in alternative medicine which includes other treatments such as acupuncture, folk medicines, mind-body techniques, homeopathy, psychic healing, and many more. The use of herbs to treat disease is almost universal among non-industrialized societies. Millions of people in the third world have used and will continue using herbal medicines because they believe in them and regard them as "their" medicine in contrast to allopathy, introduced to them from the outside world. These beliefs are further strengthened by the notion that herbs are natural and thus safe, and without any adverse effects. They are usually available locally and prescribed by traditional practitioners of medicine who are part of the community and in whose presence the patient feels comfortable. On the European and American side, use of herbal medicines is increasing rapidly mainly because of a belief that powerful synthetic agents can exert unwanted side effects, and are often used indiscriminately and irrationally.

1.1.1: Complimentary and Alternative Medicine

The National Centre for Complimentary and Alternative Medicine (NCCAM), a part of the National Institutes of Health (NIH), classifies complimentary and alternative therapies into five different categories or domains:

1. Alternative Medical Systems
2. Mind-Body intervention

3. Biologically Based Therapy
4. Manipulative and Body Based Methods
5. Energy Therapy

Aside from being more educated and more likely to report their health status, a majority of alternative medicine users appear to turn to it not so much because they feel dissatisfied with conventional medicine, but largely because they find these health care alternatives more congruent with their own values, beliefs, and philosophical orientations toward health and life.

According to a survey by Eisenberg et al. [4] and Barnes et al. [5] published in 1998 and 2002 respectively, the following are the most commonly used complementary therapies:

Table 1.1: Most commonly Used Complimentary Therapies in the United States

1997-98	**2002**
Prayer	Prayer
Mind-Body Techniques	Herbs and Supplements
Herbs and Supplements	Mind-Body Techniques
Massage	Chiropractic
Chiropractic	Yoga
Diet Therapies	Massage
Energy Healing	Diet Therapies
Acupuncture	Acupuncture

The survey conducted by Barnes et al. involved 31,044 adult participants (>18 years of age) and they reported that 36% of these participants used one or more than one complimentary and alternative medicine (CAM) therapy (prayer excluded) and 62% used one or more than one CAM therapy prayer included. [5]

The following is a brief description of the various therapies mentioned above:

Mind-Body Techniques: Mind-body techniques or medicine focuses on the interactions between the brain, mind, body, and behavior, and on the powerful ways in which emotional, mental, social, spiritual, and behavioral factors can directly affect health. [3] It regards as fundamental an approach that respects and enhances each person's capacity for self-knowledge and self-care, and it emphasizes techniques that are grounded in this approach. Mind-body medicine focuses on intervention strategies that are thought to promote health, and typically include techniques such as:

- Relaxation
- Hypnosis and Visual Imagery
- Meditation: A conscious mental process using techniques, such as focusing attention or maintaining a specific posture, to suspend the stream of thoughts and relax the body and mind.
- Yoga: A practice from Ayurvedic medicine that combining breathing exercises, physical postures, and meditation that is intended to calm the nervous system and balance the body, mind, and spirit.

- Biofeedback: Use of electronic devices to help people learn to control normally subconscious body functions, such as breathing or heart rate with the intent of promoting relaxation and health improvement.
- Tai-chi, Qi-Gong: A component of traditional Chinese medicine that combines movement, meditation, and controlled breathing. The intent is to improve blood flow and the flow of qi.
- Cognitive-behavioral therapies, Group support, Autogenic training, Prayer and Spirituality

Mind-body interventions constitute a major portion of the overall use of Complimentary Alternative Medicine (CAM) by the public. In 2002, mind-body techniques, including relaxation techniques, meditation, biofeedback, and hypnosis, were deemed to be used by about 17 percent of the adult U.S. population. Prayer was deemed to be used by 45 percent of the population for health reasons. [5]

Acupuncture: There are over 350 acupoints on the meridians of the body. Selected points are stimulated by inserting fine acupuncture needles to improve the flow of chi in the meridians and to restore balance and healthy functioning to the internal functioning of the body. Treatment points are selected on the basis of pulse and tongue diagnosis, examination and questioning and categorized according their effects on specific body systems and organs. The insertion of needles is quick and virtually painless and often a comfortable and relaxed feeling follows. [6]

Chiropractic Medicine: Chiropractic, derived from two ancient Greek words meaning "manually effective" is technically defined as the diagnosis, treatment and rehabilitation of conditions that affect the neuromusculoskeletal system. The technique was invented by Daniel Palmer in 1895, when he treated his office janitor for deafness, by realigning some small bones in his spine. Through a series of special examination and manipulative techniques, chiropractors can diagnose and treat disorders associated with nerves, muscles, bones and joints of the body. [6]

Diet Therapies: Diet therapy has been practiced for centuries dating back to Hippocrates, who wrote extensively about the therapeutic use of diet. Most alternative therapists believe that everybody can benefit from dietary self help for both prevention and treatment of disease. Diet-based therapy uses a variety of diets in order to improve health and longevity, to control weight, and to treat specific health conditions such as high cholesterol. Some different types of diet therapies are: Breatherian, Fruitarianism, Vegan, Ovo-lacto-vegetarian, Low-fat diet, Low-carb diet, Okinawa diet etc. [6]

Energy Healing: Energy healing or energy therapies are alternative treatments that involve the use of purported energy fields. Some examples of this therapy are: Magnet therapy, Medical Qigong, Reiki, Shiatsu, Therapeutic Touch, and The W.I.S.E. Method (Wholistic Integrated Spiritual Energy).

1.1.2: Dietary supplements

In the United States, a dietary supplement is a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet. The "dietary ingredients" in these products may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. Dietary supplements can also be extracts or concentrates, and may be found in many forms such as tablets, capsules, softgels, gelcaps, liquids, or powders. They can also be in other forms, such as a bar, but if they are, information on their label must not represent the product as a conventional food or a sole item of a meal or diet. Whatever their form may be, DSHEA places dietary supplements in a special category under the general umbrella of "foods," not drugs, and requires that every supplement be labeled a dietary supplement. [6, 7]

The European Union has a similar definition for dietary supplements, but with the implementation of the food supplements directive, the European Union Directive on dietary supplements now reclassifies vitamin supplements as medical drugs and mandates low dosage levels of certain vitamins which are essential only in small quantities (eg. Vitamin A). Consequently, only those supplements that have been proven to be safe may be sold without prescription. [8]

1.2: HERBAL SUPPLEMENTS

1.2.1: Definition and Overview

According to Congress, a herbal supplement is considered as a dietary supplement consisting of an herb, or concentrate, constituent, extract, or combination of any botanical intended for ingestion as a tablet, capsule or liquid and is not represented for use as conventional food or as a sole item for a meal or diet and is labeled as a dietary supplement. [7] Scientifically, herbal supplements are crude extracts or semi-purified extracts manufactured to contain a definite amount of a particular constituent or a group of constituents, which are called marker compound(s).

According to the European pharmacopoeia, herbal drugs are mainly whole, fragmented or cut plants or parts of plants, algae, fungi, lichen in an unprocessed state, usually in a dried form but sometimes fresh. Certain exudates that have not been subjected to a particular treatment are also considered to be a herbal drug. Herbal drugs are precisely defined by their botanical scientific name according to the binomial system. [9]

In Germany, there are two reasons pytopharmaceuticals are of a high standard and are classified primarily as conventional drugs by law. One reason being that during the last 50 years traditional medicine has been kept alive by both medicinal practitioners and the increasing interest of patients in herbal drugs. The other reason is that the German pharmaceutical industry, shortly after World War II, specialized in and relied on herbal drugs. It also developed and supported projects aimed at optimizing the quality of herbal

drugs by standardization and basic scientific research. This development was paralleled by an intensified evaluation of herbal drugs and a search for the active principles of phyto-preparations.[8]

In the United States, one hundred years ago, herbal drugs were very well established as medicines. [10] They were widely listed in the United States Pharmacopoeia and herbal tinctures and extracts were materia medica of the day. Then in 1938 the Food and Drug Administration was given the authority to set regulations and enforce drug safety standards. The passage of Food, Drug and Cosmetic Act gave the FDA the responsibility to prosecute the adulteration or misbranding of foods, drugs and cosmetics. Herbal preparations soon gave way to single entity chemical drugs. World War II created a demand for more powerful drugs of all kinds particularly antibiotics and trauma treatment agents. The federal government urged companies then largely botanical crude drug houses, such as Merck, Lily and Parke-Davis to invest in new synthetic chemistry based research. Single entity chemicals were more consistent, easier to measure and judged more specific in their therapeutic focus than botanical preparations. [10]

In Germany, between 1970 and 1985, the first phase of herbal drug investigations was completed which involved proving the quality of these phytopreparations and a thorough search for the various active principles. This phase was followed by clinical trials that initially focused on 10 herbal drugs or preparations that were already on the market good clinical data. This wave of clinical trials provided new findings that can be summarized below:

- Phytopreparations are applicable primarily for the treatment of moderate or moderately severe diseases. They can also be used as adjuvants in combination with a strongly acting chemosynthetic drug. (This finding would later on be checked for herb-drug interaction)
- Clinical trials indicated that several phytopreparations showed full therapeutic equivalence with chemotherapeutics and had no adverse effects.
- Many phytopreparations exihibit no immediate pharmacologic or therapeutic effects and achieve their optimal efficacy only after long term treatment.

Even though these claims were made, the new findings needed a scientific explanation for the mechanism of action and possible interactions involved with other drugs. [8]

In the United States, during the 1970's the FDA began to apply the food additive provisions of the Food, Drug and Cosmetic Act to botanicals. Under these provisions, food additives already on the market in 1958 were accepted without FDA review. However substances added to the food supply after this date were required to gain FDA approval prior to marketing, unless they were considered GRAS (generally regarded as safe). A fair number of herbs that were included on the list of GRAS food additives that had been prepared by the Flavor and Extract Manufacturers Association as flavorings for alcoholic beverages. However, the FDA viewed several other commonly used herbs as unapproved food additives and therefore subject to FDA approval prior to marketing.

This interpretation led to a series of bitterly fought court cases and several herbs being taken off the market.

Congress passed the Nutrition Labeling Education Act of 1990 (NLEA) to reform food labeling and to allow for the first time, a new class of health claims based on disease-nutrient relationships. For the most part his legislation did not apply to botanicals because of the way it was written and the way it was interpreted by the FDA. With lawsuits between herbal manufacturers and FDA common place, a group of leading herb companies met with Senator Orrin G. Hatch (R-Utah) and Congressman Bill Richardson (D-New Mexico) who drafted a legislation that became the Dietary Supplement Health and Education Act of 1994. This law was passed by Congress and signed into law by President Clinton on October 25, 1994. This was the first time a U.S law defined the term herb or botanical. [10]

The most extensive surveys on the use of complimentary and alternative medicine in the United States revealed that approximately 12% of the population used herbal medications in 1997, representing a 380% increase since 1990. [11] Patients who undergo surgery appear to use herbal supplements more frequently than the general population. A study conducted by Moss et al. on the relation between herbal medicines and perioperative care, reported that more than 70% of the patients failed to disclose their herbal medicine use during routine preoperative assessment. Explanations for this lack of disclosure included patient held beliefs that physicians are not knowledgeable about herbal medications or are prejudiced against their use. Some patients may fear admitting to their physicians their use of unconventional therapies. Others possibly neglect to mention that they are taking herbal medications when they are using them for reasons

perceived as unrelated to their medical care. Still other patients do not consider these substances as medications do not report them during preoperative questioning. Thus, for these reasons it becomes necessary for physicians to specifically seek out a history of herbal medicine use in presurgical investigations. [11] In a survey conducted by Blendon et al. in 2001, on Americans' view on the use and regulation of dietary supplements (n>1000), it was found that 48% of the participants used at least one or more than one dietary supplement regularly while 44% of the users believed that their physician knows "little" or "not much at all" about the dietary supplements products used by their patients. [12] The study also revealed that 81% of the users would require evidence of efficacy, safety and FDA approval prior to allowing the sale of that product, but 72% of the users would continue using their dietary supplement, even if a government scientific study showed negative results for that product. [12]

Surveys reveal the fraction of the U.S population using some kind of herbal therapy for a continuous of 12 months or more, increased from 2.5% in 1990 to 12.1% in 1997, to 14% in 2000 and to 18% in 2002. [4, 5, 13, 14] Table 1.2 shows the Global Dietary Supplement Market estimates for 2005 (Source: Nutrition Business Journal, Chart 73 c. 2005 Penton Media Inc)

Table 1.2: Global Dietary Supplement Market Estimates for 2005

Product Category	Estimate
Vitamins and Minerals	$27.5 Billion
Herbs/Botanicals	$19.7 Billion
Nutritionals	$19.4 Billion
Total	$66.6 Billion

1.2.2: Dietary Supplement Health and Education Act (DSHEA)

With reference to herbal supplements, the DSHEA defines the term dietary supplement as a herb or other botanical concentrate, constituent, extract, or combination of any botanical that is intended for ingestion as a tablet, capsule or liquid, is not represented for use as conventional food or as a sole item for a meal or diet and is labeled as a dietary supplement. This includes new drugs that were marketed as botanicals prior to 1994; it does not include a botanical approved as a new drug or authorized for investigation as a new drug and not previously marketed as a dietary supplement. Botanicals are thus not classified as food additives.

The DSHEA amends the Food, Drug and Cosmetic act of 1938 and alters the way dietary supplements are regulated and labeled. DSHEA was enacted by the Congress based on 15 findings, one of which was "improving the health status of United States citizens ranked at the top of the national priorities of the Federal government." Other Congressional findings supporting the adoption of DSHEA included the increasing reliance of the use of "unconventional" health care providers to avoid the increasing costs of conventional medical treatments and the belief that consumers should be able to make choices about preventive health programs based on information obtained from scientific studies of health benefits related to particular dietary supplements. [15] The DSHEA influences the FDA's regulatory practice in four primary areas by:

i. Placing the burden of proof of safety on the FDA in actions against the manufacturer of dietary supplements.

ii. Limiting the FDA's ability to require pre marketing information about product safety and efficacy.

iii. Establishing a minimal pre-marketing notification for certain new dietary ingredients not marketed in the United States before October 15, 1994.

iv. Specifying acceptable claims that could be made without triggering requirements for classification as drugs.

1.2.2.1: Lack of Pre Market Controls

One of the most misunderstood aspects of DSHEA is the issue of FDA's ability to protect the public from unsafe dietary supplements. Once the supplement is on the market, the FDA must prove that it is unsafe before imposing restrictions on its use. The burden of proof has thus shifted from the manufacturer to the FDA to prove that a substance poses an imminent health hazard. This issue of regulation, herb safety and the impact on general public can often be mischaracterized and exaggerated in the media to the extent that even the FDA officials have misstated the agency's authority.

Consumer and professional confidence in herbal preparations and other dietary supplements underwent a considerable degree of erosion during the late 1990's as various news organizations and independent groups reported that many of these products failed to meet a variety of label claims related to content of certain ingredients, standardization markers, or other elements. Thus, lack of pre market controls leads to:

i. Undeclared toxic contaminants

ii. Undeclared or high level of impurities

iii. Incorrect label amounts

iv. Microbial contamination

v. Lack of bioavailability

1.2.3: Role of the U.S Pharmacopoeia

Not surprisingly, herbs have had a long history of use as medicines in North America. Native Americans had medicinal uses for at least 2,582 species of plants including such uses as analgesics, contraceptive, laxative, sedatives, remedies for cold, tuberculosis and cancer. Until about 1930, herbs and herbal products constituted a significant portion of the materia medica of North America during the 17th, 18th, 19th and early 20th centuries. The first edition of the United States Pharmacopoeia (USP) in 1820 contained 425 botanical substances (67%) of all entries. Herb and herb product monographs reached their peak of 636 botanical substances (66%) in USP V in 1870, but by the time USP X was published in 1926, the total number of botanically related monographs had dropped down to 203 botanical substances (36%). This reflected the trend that botanical medicine was widely used in the United States until about 1920 when herbs began to be replaced with pharmaceutical drugs, not because they were determined to be unsafe and ineffective, but because their actions were generally not as pharmacologically dramatic and they were not as economically profitable as the new synthetic drugs. In 1888, the American Pharmaceutical Association published the National Formulary (NF), a compendium of formulae of primarily herb based medicines. In 1975 the publication of the NF was taken over by the USP and combined with the USP to create the USP-NF in 1980. In the 1990's the USP renewed its efforts to address the issue of establishing quality standards for conventional dietary supplements and in 1995,

after the passage of the DSHEA, USP began the development of monographs defining identity and purity standards for many of the best selling herbs in the U.S market. From 1995 to 2002, the USP has published 86 standard monographs on 25 herbs. [16]

This dissertation, in part, is also a result of the USP's efforts to develop meaningful bioavailability and quality control performance standards for herbal supplements.

1.2.4: European (Germany) Regulatory Status

In the countries of the European Union, especially Germany which accounts for nearly 50% of the total OTC sales, herbal medicines enjoy relative parity with conventional medicines. Herbal medicines are generally sold in pharmacies as licensed non prescription or prescription medicines. According to the EU directive, 65/65/EEC (European Economic Community) all phytomedicines are treated as drugs. Registrations based on quality, efficacy and safety are required. Exceptions include the Netherlands and the United Kingdom where botanicals are still sold as food supplements or dietary supplements. [17]

In the European Union herbal medicinal products are "medicinal products containing as active ingredients exclusively plant material and/or vegetable drug preparations." An herbal drug or preparation is thereof regarded as "one active ingredient in its entirety whether or not the constituents with therapeutic activity are known. However chemically defined isolated constituents or their mixtures are not considered herbal medicinal products. Other substances such as solvents, diluents, or preservatives may form part of the vegetable drug preparation. These substances must be indicated".

One of the driving forces that has resulted in mainstream acceptance of phytomedicines in Germany is the inclusion of phytotherapy in the medical and pharmacy school curricula. In the opinion of several medical groups, "modern phytotheraly is not perceived as alternative medicine, but as a part of so called traditional medicine." Since 1993 all medical school students in Germany must successfully complete a portion of their board examination in phytotherapy as a precondition for practicing medicine.

Rational phytotherapy in Germany is based on four basic rules for phytomedicines also known as the phytopharmaca:

i. Dose-Response relationship: Phytomedicines in the therapeutic arena can be applied in a dose effective manner. Possible dose dependent reversals of effects should not be interpreted as homeopathic effects- a reference to the observation that homeopathic medicines produce symptoms at higher potencies in healthy individuals. This means, sometimes a variation in the dosage of a phytomedicine can produce a different effect than a higher or a lower dose of the same herbal drug and this cannot be dismissed as homeopathic.

ii. Efficacy-Constituent relationship: Efficacy or effectiveness can be deduced from specific ingredients. They are codetermined for effectiveness, which means, in most cases not one but more than one different plant constituents are responsible for the observed effectiveness. For example: St. John's Wort used in the treatment of mild to moderate depression, is believed to be attributable to at least three types of substances: hypericins, hyperforins, and flavonoids.

iii. Total extracts vs. isolated constituents: Typically phytomedicines that are standardized extracts consisting of primary active components, secondary components and accompanying compounds manifest better effects and a greater therapeutic range of activity than indivisual isolated compounds.

iv. Pharmaceutical Quality: Phytomedicines with a high level of pharmaceutical and medical quality are the basic requirement for successful phytotherapy.

An interesting study conducted in Germany (Table 1.3), comparing the use of phytomedicines by German consumers for curing common ailments in 1970 and 1997 revealed that even after so many new drugs are being available in the market, the use of phytomedicines for common ailments has either remained constant or increased during the years 1970 to 1997.

Table 1.3: Phytomedicine Use Among German Consumers.

Condition	1970 Poll (% users)	1997 Poll (% users)
Common cold	41	66
Flu	31	38
Digestive complaints	24	25
Headache	13	25
Insomnia	13	25
Stomach ulcer	21	24
Nervousness	12	21
Circulatory Disorders	15	17
Bronchitis	12	15
Skin diseases	8	12
Fatigue & Exhaustion	8	12

Table reproduced from [17]

1.2.5: WHO: Regulatory Status

The World Health Organization (WHO) in 1991 published its "Guidelines for the Assessment of Herbal Medicines" in recognition of world wide growth in the usage of herbs in both official and unofficial medicine. Its main purpose was to aid member nations in establishing appropriate regulatory criteria and procedures to evaluate the quality, safety and efficacy of herbal medicines. These guidelines call for recognition of

the fact that long term historical use of a botanical in traditional medicine constitutes a presumption of safety unless contradicted by modern scientific research. [18]

1.4: SAFETY AND EFFICACY

1.4.1: Safety: Risks and Benefits

In general it would appear that there are fewer adverse event reports (AERs) in the United States, on a per capita basis, for herbs than for conventional pharmaceutical drugs. On the other hand it is also possible that lower incidence of AERs for herbs are a result of poor reporting mechanisms or because many herb users simply do not report minor events such as a gastrointestinal upset or a headache or because many herb users consider themselves outside the medical mainstream and may have a bias against making a report. All these explanations are equally plausible; i.e. most commercially available herbs are gentler and safer than conventional drugs, and there needs to be a better reporting mechanisms for herb-related adverse events. [19]

There are several voluntary reporting systems for herb and dietary supplement related AERs in the U.S. One is the American Association of Poison Control Centers in Washington D.C, whose results are available on a fee for service basis, usually for companies who check reports on their specific products. Another is the FDA's MedWatch system for drugs that is used primarily by health professionals, it is however considered controversial, on account of incomplete documentation and inadequate information. A new improved system at the FDA for documenting AERs related to dietary and herbal supplements is managed by the Centre for Food Safety and Nutrition

(CFSAN) and is known as CFSAN-Adverse Event Reporting System (CAER). [20] CAER replaces the older and less reliable Special Nutritional/Adverse Event Monitoring System (SN/AEMS) that was created in 1998.

The German Commission E review process is only slightly comparable to the OTC review process of old drugs conducted by the FDA. When lacking controlled studies, the safety and efficacy of known substances can be determined on the basis of well documented review articles, older clinical trials and sound knowledge of their traditional use. According to the Commission E monographs, approved and unapproved herbs are divided into separate sections into positive and negative monographs. Positive monographs always show the approved use and dosage. If no approved uses are given, if there is no dosage listed, and if the efficacy of the plant has not been efficiently proven, or there are risks that outweigh the documented benefits, the assessment becomes a negative monograph.

As mentioned earlier, the U.S. FDA deals mostly with mild acting herbs and herbal supplements. Unlike the FDA, Commission E approves relatively powerful, pharmacologically active and also potentially toxic herbs such as *Hyoscyamus niger* [Henbane], *Rauvolfia serpentina* [Indian Snakeroot] and *Urginea maritima* [Squill] all of which are treated as 'prescription only' drugs. Thus, the existence of a positive Commission E monograph does not imply that the herbal drug is sufficiently harmless to be treated as an OTC drug. [17, 21] With amendments in the risk-benefit assessment, if a certain herbal drug emerges with new data that deems it toxic, the existing positive monograph is not revised, but replaced with a negative monograph. [17] Thus, new data indicating potential risks that outweigh possible benefits attests to the rationality of the

Commission E process. This constant vigilance by Commission members regarding what has been termed the "doctrine of absolute proof" to assess safety is a significant characteristic of the Commission E evaluation process.

1.4.2: Standardization and Quality Control

1.4.2.1: Standardization

Standardization is probably one of the most controversial terms used to describe herbal supplements. Most people would agree that the goal of standardizing herbal products is to provide product consistency and thus a reliable health benefits. But standardization can also mean establishment of consistent biological effect, a consistent chemical profile or simply a quality assurance program for production and manufacturing. [10, 22, 23] The process of standardization depends partly on whether the active constituents in an herbal extract are well established. This can be done by classifying a botanical product into three categories:

(i) those containing constituents (single compound or a family of compounds) with known and acknowledged therapeutic activity that are solely responsible for the efficacy of the extract;

(ii) those containing chemically defined constituents possessing relevant pharmacological properties which are likely to contribute to clinical efficacy;

(iii) those in which no constituents have been identified as being responsible for the therapeutic activity.

Standardization of Therapeutic Activity: Standardization of a botanical extract based on therapeutic activity has been followed for over a century. Before sophisticated chemical analytical methods were available, and the active principles of an extract unknown, herbal extracts were standardized based on biological activity measurements in animals or animal tissues. As the active components were profiled with the advent of new analytical techniques, bioassays were replaced first by the measurement of representative chemical constituents of an extract (e.g silybin as a measure of silymarin in milk thistle) and then by measurement of each of the active components in the extract (measurement of all six isomers in silymarin).

Standardization of a botanical product can also be established by adjusting the preparation to contain a defined level of an active component or group of components in a single dosage form. For example Milk thistle preparations contain silymarin, the active group of components comprising of six isomers. Hence, the dosage form can be standardized to contain a certain variable amount of dried extract containing a fixed amount of silymarin (e.g. "X" mg of extract containing 140 mg of silymarin) or fixed amount of extract containing a fixed amount of silymarin (e.g. 175 mg of extract containing 140 mg of silymarin).

The standardization method in the above example is not complete. It states that the dose/extract contains 140 mg of silymarin; but silymarin itself comprises of six active isomers, wherein the proportion of each isomer can vary from extract to extract. Thus, it is necessary to quantify the composition of each isomer in the extract regardless of whether the isomer does or does not exhibit a pharmacologic effect. Standardization based on therapeutically active components can ensure consistency between lots from the

same manufacturer, but in a larger scenario, it is just a small step forward when it comes to comparing proprietary preparations from different manufacturers for the same botanical. Even if the claimed level of constituents is accurate, the inherent variability in the undefined portion of the extract must be considered. Thus, different formulations or routes of administration are bound to give variable results and affect bioavailability studies, which in itself is a completely different paradigm.

Standardization Based on Chemical Characterization: Botanical products can be standardized to a norm that may or may not relate to the expected biological activity of the product. This norm is a level of a constituent chemical or group of chemicals called marker compounds. The concept of determining levels of marker compounds was developed because it was not feasible to test for all compounds in an extract and finalize a formula for content and consistency. This gave rise to 'Markers' which are chemically defined components of an herbal extract, ideally characteristic to the herb, of interest for quality control purposes, and independent of whether they have any therapeutic activity. By providing product characterization marker compounds can be used to facilitate botanical identification and detection of adulteration. They can also be used as indicators of consistency throughout the manufacturing, handling, and storage process. Thus, setting minimum limits for marker compounds can be a useful indicator of quality in preparations in which there is little or conflicting knowledge regarding the active constituents. Thus, determinations of marker consistency in finished products should be done using specific and sensitive validation methods. Analytical methods can be used to measure either individual compounds or a collective class of compounds. It is important

to realize that no global consensus has yet been made regarding standards or test methods for herbal products although attempts are being made in this direction.

1.4.2.2: Quality Control

Standardization based on quality control is best described as a quality assurance program. It is well established that intrinsic and extrinsic factors including plant species differences, organ specificity, diurnal and seasonal variation, environment, field collection and cultivation methods, contamination, substitution, adulteration, processing, and manufacturing practices greatly affect botanical quality. [23-27] Plants are inherently dynamic living organisms, each of which is capable of being genetically influenced to differ slightly in their physical and chemical characteristics. For example a study on the accumulation of hypericin in H. *perforatum* showed that narrow leafed populations have greater concentrations than the broader leaf variety. [28, 29] Such variations can be due to diurnal, seasonal and other intrinsic factors as well as extrinsic factors such as soil, light, water, temperature and nutrients. For e.g., silymarin content in milk thistle was found to be highest in the fruits of plants grown under 60% water/field capacity (1.39%) and nitrogen level of 100 (1.46%) per acre. [18] Contamination by microbial and chemical agents (pesticides, herbicides and heavy metals) as well as by insects, animals, animal parts, and animal excreta during any of the stages of source plant material production and collection can lead to lower quality and unsafe source material. [30] Heavy metal contamination can occur at cultivation, post harvest treatment, or product manufacturing stages. Lead and thallium contaminations have been reported in multi-

component botanical mixtures and cases of lead, thallium, mercury, arsenic, gold and cadmium poisoning from the consumption of contaminated products has been documented. [31, 32]

Botanical product quality and safety can also be influenced by regulatory status, which varies from country to country. As discussed earlier, in countries like Germany botanicals are regulated as medicines and are subjected to mandated standards of quality, where as in the United States a majority of botanicals are marketed as dietary supplements. Good manufacturing practices (GMP's) are required in the production of prescription and OTC drugs, but regulatory provisions under DSHEA provide little assurance of identity, quality or purity for botanical supplements. Hence, these products have not been subjected to mandated QA/QC standards as in the case of prescription and OTC drugs. Although the FDA is taking steps in the direction of implementing strict GMP's in the labeling and manufacturing of botanical supplements, such rules have yet to be imposed. [33]

1.4.3: Bioavailability

Many consumers use botanical products in a holistic manner, due to obvious reasons implying to empirical and traditional applications. Evidence based use of botanical products, known as 'rational phytotherapy' is in complete contrast to the holistic traditional medical herbalism. Hence, in order to bring this contrast to a minimum, all botanical products must meet acceptable standards of quality, safety and efficacy. The topic of safety and efficacy brings along with it the pharmacologic effect of

herbals which can be further divided into pharmacodynamic (PD) and pharmacokinetics (PK). Comparing the PK with the PD of botanicals, it is seen that, there is sufficient and steadily increasing PD data for botanicals as compared to PK studies being undertaken. This is mainly because the study of herbal PK is extraordinarily complicated due to the complex nature of herbal extracts. Herbal extracts are multicomponent complex mixtures containing several chemical constituents in a vegetative matrix. Most botanical products are formulated after a certain extraction-purification process in order to guarantee sufficient efficacy. PK profiling of a certain herbal extract would require the analysis of each of the active component present in the extract. As a consequence, analytical methods determining bioavailability and PK of herbal products have to be sufficiently sensitive. To further complicate the matter, in many of the herbal extracts the active constituent is often unknown, implying that the compound being detected in body fluids may not necessarily be the active constituent. Natural compounds are often prodrugs that are metabolized in the digestive tract. Sometimes, herbal extracts may contain polar molecules that might be expected to have poor or highly variable/unpredictable bioavailability. [26]

Bioavailability of a substance depends on several factors like the type of formulation, solubility, permeability, partition coefficient of the molecule, gastrointestinal factors, first pass effect, interaction with food and individual factors in the patient such as pathological conditions. Besides these factors, bioavailability of components in an herbal extract can also be influenced by other components of the extract, which are not active themselves but play a major part in positively influencing the stability, solubility, bioavailability or half life time of the active compounds. A good

example is the concentration of Kawain and Yangonin (active components of the herb Kava) in mouse brain samples is higher after administration of the Kava extract (*Piper methysticum*) than after administration of the purified single compound in the same amount. [34] Similarly the oral bioavailability of Kawain from the kava extract is 10 times higher than pure Kawain. [26]

Factors influencing the ease and importance of conducting bioavailability studies include the type of therapeutic activity and the extent to which the active constituents have been identified. Thus, bioavailability and bioequivalence studies are important for botanicals with immediate and strong activity. Bioequivalence is important when one product is being substituted for another and hence should be performed for all immediate release products intended for systemic action unless there is sufficient *in vitro* data. This concept originally developed for synthetic drugs can be transferred to herbal extract formulations without modifications. In principle two parameters from the *in vitro* data are essential in this context: Solubility (predicted by *in vitro* dissolution) and permeability through the intestinal wall (as predicted by the Biopharmaceutic Classification System-BCS) [35] If both the solubility and permeability of a substance are good, the probability of bioavailability problems diminishes. However if one or the other is poor, bioavailability can be compromised. Hence, if solubility is good and permeability is poor, absorption from the gastrointestinal tract becomes the rate limiting step. On the other hand, if the permeability is good and the solubility is poor, the bioavailability will depend on the *in vitro* dissolution of the drug making dissolution the rate limiting step for bioavailability. Excepients can impact both the solubility and permeability of the active components impacting their bioavailability.

For extracts where the active components are not defined clearly and the pharmacologic effect is a result of the presence of a group of undefined components, chemical assays would not predict the pharmacological activity of the extract. In such cases bioassays that measure biological activity may be more suitable endpoints. Recently biochemical assays have been proposed for several herbal extracts like St. John's Wort, Ginkgo, Ginseng, Echinacea and Saw Palmetto. [33] An ideal bioassay would be a measurement that correlated with the therapeutic activity of the extract. This ideal is very difficult to achieve and in most cases bioassays do not reflect therapeutic effect as a whole but only selected pharmacological aspects caused by individual active markers. A disadvantage of bioassays is that they are generally not as reproducible as chemical assays and hence variability in results may complicate the evaluation of bioavailability studies. Even then, bioassays may play an important role for future pharmacokinetic approaches if new intelligent strategies are developed.

Although the study of herbal pharmacokinetics appears to be difficult, the information derived from such investigations will become an important issue to link pharmacological/chemical assays to clinical effects. In particular, a better understanding of pharmacokinetics and bioavailability of natural compounds can help in designing rational dosage regimen and it can help to predict potential botanical product-drug interactions.

1.4.4: Herb-Drug Interactions

The body cannot discern between a natural chemical emanating from a garden and a synthetic chemical generated from a laboratory and consequently deals with both in the same way. The body thinks of these chemicals as foreign (xenobiotics) as they cannot be exploited beneficially by the body and hence tries to eliminate them. A large number of drugs that reach the systemic circulation tend to be lipophilic and hence difficult to excrete, as a result of which the body converts them into hydrophilic analogs by metabolism to ease excretion and eliminate them through the biliary route. A series of enzyme systems that are efficient in breaking down drugs to excretable polar metabolites are present throughout the body, but principally found in the liver, and are intracellulary localized on the endoplasmic reticulum and in the cytosol. [36] In order for a pharmacologic response to occur, the drug must be able to gain access or interact with the specific receptor, but when a drug is metabolized the biological activity in most of the cases is terminated. The most popular and well studied enzyme system involved in drug metabolism is the cytochromes P450 (CYP450). This enzyme system displays unprecedented substrate specificity being able to metabolize a wide range of structurally dissimilar drugs. This system of enzymes is also responsible for the metabolism of herbal components. Thus, potential herbal-drug interactions should be studied to prevent therapeutic failures.

Herb drug interactions leading to changes in CYP450 activity become crucial when drugs with a low therapeutic index are concerned and plasma levels have to be maintained within a narrow concentration range to obtain optimum benefit with

minimum adverse effects. Elevated CYP450 activity translates into higher metabolic rates which may result in decreased blood plasma concentrations leading to sub therapeutic levels and loss of pharmacological effect. Conversely, suppression of CYP450 may trigger a rise in drug plasma concentrations leading to undesirable and elevated pharmacologic effect leading to toxicity.

The best researched clinically relevant interaction between diet and drugs involves grapefruit juice. Simultaneous consumption of grapefruit juice with drugs that are subject to first pass effect, results in elevated plasma levels of that drug leading to unwanted high bioavailability potentially resulting in adverse therapeutic events. [37] The effect becomes more pronounced with repeated intake of grapefruit juice, but of particular concern is that this effect is observed for at least three days following the last consumption of grapefruit juice. Grapefruit juice contains furanocoumarins which have been shown to inhibit intestinal CYP3A4 activity, which is responsible for the presystemic metabolism of these drugs, thus allowing higher than desired concentrations of the drug to reach systemic circulation leading to toxicity.

Herbal supplements are taken not only by healthy people, but also by those suffering from life threatening diseases, those about to undergo surgery or post surgery, a situation in which they are most likely to receive more than one medicinal drug. [11, 37] Herbal medicines may interact with a drug at the absorption site, modifying the pharmacologic profile of the drug. Herbal components may interact with the ATP dependent transporter proteins such as the intestinal P-glycoprotein (P-gp) and other multi-drug resistance proteins that facilitate the efflux of drugs. Phytochemicals thus have the potential to compete with traditional drugs for transporter proteins. Several

cases have been reported where a number of herbal products (Chamomile, Feverfew, Ginger, Garlic, Ginkgo, Ginseng, St. John's Wort) have known to interact with anti coagulants such as warfarin, though it is not clear whether the underlying mechanisms are competitive based or pharmacokinetic in nature. [37, 38] Warfarin is a highly protein bound drug and the displacement from its binding site may cause an undesired increase in its anticoagulant effect.

In essence, interactions between herbals and drugs involve the same pharmacokinetic and pharmacodynamic mechanisms as drug-drug interactions. Pharmacokinetic interactions may involve alteration in absorption, distribution, metabolism or excretion of the affected drug or herbal compound. Pharmacodynamic interactions on the other hand alter the relationship between drug concentration and the pharmacological response for a drug or herbal product. Though most research has been focused on the adverse effect of herb-drug interaction, not all such types of interactions have a negative effect. Animal studies have shown that the combination of the Chinese medicinal plant *Tripteriygium wilfordi* and cyclosporine, significantly increased the heart and kidney allograft survival compared to cyclosporine alone. The effective cyclosporine dose required for 100% kidney allograft survival was reduced by 50% to 75% in the presence of the herbal extract. [39]

Garlic-Drug Interactions: Garlic is promoted for its antiplatelet activity, lowering cholesterol and hypertension, delay artherosclerotic processes and improve circulation. Reports suggest that concomitant use of warfarin and garlic was followed by an increase in the INR (International Normalized Ratio) leading to increased plasma levels of warfarin in the systemic circulation increasing the risk of bleeding, especially

during post surgical recovery period. An isolated clinical trial suggested that garlic changes some pharmacokinetic variables of acetaminophen after 1-3 months of garlic treatment. [40]

St John's Wort (SJW)-Cyclosporine Interaction: Interaction between SJW and clyclosporine is one of the most serious, potentially fatal and hence the most studied. [37] Cyclosporine is one of the most widely used immunosuppressant drugs following transplantation, but it is essential that blood levels are maintained within a narrow concentration range. A drop below the therapeutic index ratio may result in the rejection of transplanted tissue or organ where as an increase can be associated with kidney and hepato toxicity. Most of the cases reported indicate a drop in the blood cyclosporin levels of the patient after the patient has been stabilized with the immunosuppressant post organ transplantation leading to tissue rejection. One report refers to 30 kidney transplant patients whose plasma cyclosporin levels were nearly halved after taking SJW, though the dose of SJW was not specified. Another case reports a female kidney transplant patient was maintained successfully on cyclosporin for 25 years, but suddenly blood levels dropped following a 4 week medication course of SJW. Cyclosporin blood levels increased only after the intake of SJW was stopped. Another heart transplant patient was stabilized on cyclosporin for 11 months. Three weeks before admission to a hospital for elective endomyocardial biopsy, self medicated himself with SJW-900mg/day in three doses. The biopsy revealed acute cellular transplant rejection. Cyclosporin levels for that patient on investigation and analysis were found to be below 100μg/L when the

desirable effective therapeutic range was between 200-350μg/L. SJW was discontinued and cyclosporin levels increased to subtherapeutic levels.

In vitro studies indicate that methanolic and ethanolic extracts of SJW act as inhibitors of CYP3A4 activity showing that components of SJW can interact with this enzyme. Exposure of healthy volunteers to SJW (3 x 300mg/day) for 2 weeks, resulted in increased expression of duodenal P-gp and CYP3A4 and hepatic CYP3A4. This combined upregulation is expected to severely limit the bioavailability of drugs that rely on these proteins for their absorption and metabolism respectively. Cyclosporin is metabolized through hydroxylation and N-demethylation by CYP3A4 to a large number of pharmacologically inactive products. This clearly suggests that SJW doses that have been reported to lower plasma cyclosporine concentrations to subtherapeutic levels are sufficient to stimulate CYP3A4 activity in the liver and the intestine and increase expression of P-gp. [37]

1.5: PERFORMANCE MARKERS

1.5.1: Need for Markers

While many conventional drugs or their precursors are derived from plants, there is a fundamental difference between administering a pure chemical and administering a chemical in a plant matrix. It is this issue of the possible advantage of chemical complexity which is rejected as having no basis in fact and avoided by most researchers as introducing too many variables for comfortable research. Multiple variables in a

matrix give rise to the concept of synergy which is a very common and important in medicinal use of botanicals. Just like biologicals, botanicals are crude extracts or semi-purified extracts manufactured to contain a definite amount of a particular constituent or a group of constituents, which are called marker compound(s). Since potency requires biological assessment of an extract, the presence of marker compounds does not guarantee the potency of an extract. Even if the marker compound demonstrates bioactivity the biological activity depends on the composition of the rest of the extract. Hence, other components even those showing no direct physiological effect can influence the absorption, distribution, metabolism and excretion of the active compounds. Such a background matrix can also lead to a difference in solubility, oil-water partitioning, permeability and bioavailability of any single compound in the given extract. Thus, it becomes necessary to know the physicochemical and biopharmaceutic properties of each of the compounds present.

Botanicals contain a group of constituents which act together to exhibit a synergistic pharmacologic effect. The basic advantage of synergism is the presence of an active constituent in the plant matrix which enhances its solubility and bioavailability properties. Consequently, if that particular constituent was administered alone, it would have much lesser bioavailability leading to sub therapeutic levels, as compared to when the constituent is administered as an extract.

Herbal extracts consisting of a range of active compounds are ascribed with pharmacological activity with often unresolved synergistic effects between individual compounds. These active constituents are complex labile compounds that traditional analytical techniques had difficulty in extracting and quantifying. As sophisticated

analytical techniques were introduced, more active constituents were discovered and designated as marker compounds which actually signified the presence of active constituents. Though the chemical profiling of extracts has advanced only to a certain extent, bioavailability and pharmacokinetic profiling of individual constituents still poses a major hurdle in the characterization of the extract.

Also due to the presence of an array of compounds in each extract, it is still difficult to physically isolate each component on a preparative scale, leading to lack of pure reference standards. Progress has been slow concerning the characterization and selection of markers for an extract due to lack of conclusive evidence for the activity of specific compounds, multiple active constituents in an herbal product and probable synergistic effects and to add to it, the reluctance of health authorities to accord recognition to medicinal herbs as valid therapeutic agents. When an individual component is chosen as a marker for activity in a herb there must be meaningful consistent relationship between the amount present and a quantitative therapeutic benefit.

1.5.2: Classification of Markers

Since herbal extracts consist of a wide array of active constituents, all of which are usually not characterized for pharmacologic effect, chemical profiling or bioavailability. Even if each of the constituent was characterized, non active constituents in the extract cannot be neglected since therapeutic effect exhibited by the extract is synergistic. Hence, it should be an accepted fact that a particular constituent, active or non active, is an integral part of standardization and marker development for a botanical.

Conducting bioavailability/routine bioequivalence studies for each likely component in the extract is not only an uphill task, with the non availability of pure reference standards, almost impossible. Thus, selecting a performance marker that represents the chemical composition of the extract for a quality control assay or a bioavailability marker that represents the bioavailability or bioequivalence of an extract would definitely be helpful in regulating a botanical product. Thus, performance markers for an extract can be classified in the following 5 categories:

1. ***Performance Markers based on Active Principles:*** These markers can be used when the active principle of the extract is known. Such a type of marker selection is analogous to the usage of botanicals during the olden days when extracts were recognized or characterized according to their therapeutic effect. This was an obvious approach during that time period mainly due to lack of scientific data concerning an extract. Thus, even today there are certain botanicals where data is available only for the therapeutic effect and individual constituent profiling is not available. In such cases the marker assigned is based on a therapeutic scale where in the mechanism of therapeutic effect is accurately known and assigned a certain magnitude.

2. ***Pharmacologically Active Markers:*** These markers are based on the pharmacologically active constituents of an extract. An extract may contain pharmacologically active and non active markers, but as explained earlier, due to synergism these non active markers cannot be neglected. Thus, an assay for a pharmacological marker can be based on the extent of pharmacologic affect exhibited by

the particular constituent or a group of constituents (where individual profiling is not possible), wherein the assay can be a bioassay. If data on individual constituent profiling is available a decision can be made on the selection of a particular constituent as a pharmacological marker for that extract. Assigning pharmacological markers can be relatively easy since there is sufficient data on many, if not all, of the herbal extracts. The major drawback with assigning pharmacological markers can be the non reproducibility of pharmacological assays and the inability to validate them.

3. ***Negative Markers:*** A particular herbal extract can be profiled based on the presence of certain toxins or allergens present. An extract can be standardized and assigned safe or unsafe based on the amount of a particular unwanted constituent. Selection and characterization of such negative markers requires highly sensitive and specific analytical assays that can measure minute quantity of toxin present in the extract. Examples of negative markers can be Ginkgo toxin (4-o-methylpyridoxine) which is a neuro toxin present in ginkgo biloba, known to cause loss of consciousness, tonic/clonic seizures and sometimes death. [41] Other examples are the valepotriates (valtrate/isovaltrate and dihydrovaltrate) in valerian which are known to be cytotoxic to the gastrointestinal mucosal cells after oral administration of the extract. [41]

4. ***Analytical Markers:*** Analytical markers are selected based on the constituents that are characteristic of the extract. These constituents potentially provide a finger print towards the identity of the extract. Analytical markers can be detected by the use of sensitive chromatography techniques which can be highly specific and validated for

reproducibility. It should be noted that a particular selected analytical marker need not be necessarily pharmacologically active, and in some cases, measurement of such a marker can give a false sense of safety and efficacy. Hypericin, which is present in St. John's Wort can be a good example of an analytical marker for the extract, since it is characteristic of the extract, unlike quercetin which can be found commonly in more than one herbal extract. Hypericin though being an analytical marker, is not pharmacologically active component of the extract.

5. ***Bioavailability and Phytoequivalence Markers:*** Bioavailability or phytoequivalence (bioequivalence of herbal products) markers can be regarded as one of the most meaningful class of markers because the efficacy and therapeutic effect of an extract finally depends on the rate and the extent of active constituents reaching the systemic circulation (an estimate of the amount of drug at the site of action). It is not wrong to assume that a majority of the herbal products are designed for oral administration and given the complex nature of contents of an extract, assigning a bioavailability marker for an extract would render the measure of efficacy and therapeutic effect to a much simpler level. When primary research is conducted on proprietary preparations or extracts, if a researcher/manufacturer is able to document bioavailability/phytoequivalence for each of the active constituents, in addition to the chemical profile, it gives rise to a strong data base as regards to the *in vivo* behavior of the compounds. In terms of phytoequivalence, these results get transferable to a claim for a preparation and no new pharmacological or clinical studies are required. Now once the constituents are characterized for bioavailability, marker selection is based on the most

conservative approach, by choosing the least bioavailable constituent as a measure for the bioavailability and phytoequivalence of that extract. Selecting the least bioavailable constituent ensures that if the selected marker reaches the systemic circulation to a certain extent, the remaining constituents in the extract would be easily bioavailable.

1.5.3: Criteria for Marker Selection

Before the criteria for marker selection are discussed in this section, it is necessary to give the reader a very skeletal and brief idea of the work undertaken for this project. This dissertation consists of three types of experimental studies where in:

a: The qualitative permeability of the active constituents of selected herbal extracts is predicted using a semi empirical model based on the *in silico* descriptors (surface areas and physicochemical descriptors such as CLogP) and *in vitro* cell permeability of the extract constituents.

b: The experimental physicochemical properties of the active constituents of a representative herbal extract are determined.

c: Finally the pharmacokinetics and absolute bioavailability of each of the active constituents in the selected representative extract are determined using an animal model.

The criteria for marker selection are applied right from the very beginning when the qualitative predictions regarding the permeability behavior of the active constituents are made.

Thus, while selecting a bioavailability/phytoequivalence performance marker for a particular herbal extract, the following criteria were maintained:

I: The selected marker should have the least permeability/bioavailability in its group of compounds in the particular herbal extract.

II: The marker should be easily available as a reference standard, at a reasonable cost and acceptable purity for routine analysis.

III: The proportion of the selected marker in the extract should be sufficient for its precise quantitative determination in biological fluids after oral administration.

1.6: SELECTION OF HERBAL EXTRACTS TO PREDICT PERFORMANCE MARKERS

Based on a survey conducted in 2002 which reported the top fifteen selling herbal supplements in the United States (Table 1.4) [42] 8 herbal extracts comprising of 37 active compounds were selected for the qualitative prediction of permeability based on the estimation of their *in silico* descriptors.

Three herbal extracts comprising of 20 active constituents, were analyzed and a performance marker was selected for each of the three extracts. The 3 selected extracts were Kava, Ginkgo biloba and Milk thistle.

After performance markers had been predicted for each of the three extracts, Milk thistle was selected as the representative extract for the verification of predicted properties and for the determination of pharmacokinetics and absolute bioavailability. Milk thistle was the choice of extract mainly for two reasons:

1. Milk thistle is composed of six isomers from which 4 are diastereomers of each other. The presence of isomers and diastereomers has always presented a challenge in the chemical and bioavailability profiling of the extract. Further among the three selected herbal extracts, Milk thistle is the one for which least clinical and pharmacokinetic studies have been conducted. Pure reference standards are yet not commercially available for a few of the active isomers. Thus, it was thought that Milk thistle would present a sufficient and interesting challenging during the process of standardization and performance marker selection for the extract.

2. Kava and Ginkgo biloba are among the most studied herbal extracts in terms of pharmacokinetics and bioavailability. There is a abundant experimental data available concerning the physico-chemical and permeability properties of ginkgo and kava.

Table 1.4: Top selling Herbal Supplements in the United States

(Retail Sales-2002, 2005) [42, 43]

Rank 2002	Herb	Retail Sales 2002 ($US. Million)	Rank 2001	Rank 2005
1	Garlic	34.5	3	1
2	Ginkgo	32.9	1	4
3	Echinacea	32.44	2	2
4	Soy	28.25	5	5
5	Saw palmetto	23.05	6	3
6	Ginseng	21.68	4	7
7	St. John's wort	14.96	7	9
8	Black cohosh	12.33	10	8
9	Cranberry	11.85	9	6
10	Valerian	8.12	8	12
11	Milk thistle	7.76	12	10
12	Evening primrose	6.02	13	11
13	Kava	4.42	11	--
14	Bilberry	3.38	15	14
15	Grape seed	3.05	14	15

1.6.1: Kava (*Piper methysticum*)

1.6.1.1: Introduction and Regulatory Status

Kava, also referred to as Kava kava is the dried rhizome and roots of *Piper methysticum* a large shrub largely cultivated in many Pacific islands from Hawaii and Tahiti to New Guinea. [38] It has large heart shaped leaves and is propagated exclusively by root cuttings. The extract of Kava consists of six known kava lactones (≥3.5% in exract) known as kava pyrones: Kawain (1.8%), Dihydrokawain (0.6%), Methysticin (1.2%), Dihydromethysticin (0.5%), Yangonin (1%) and Desmethoxy-yangonin (1%).[44, 45] (Figure 1.1) The kava extract is prepared traditionally by water extraction of the root, and commercially by ethanol extraction (~30% kava lactones) or by acetone extraction (~70% kava lactones). Other constituents of Kava include 2 chalcones, flavokawains A and B [46] which are known to be responsible for the cause of dermopathy in heavy kava users. The process of supercritical fluid extraction of kava lactones from its roots has been patented by the Schwabe company which also claims to have negligible amounts of chalcone content.

The kava pyrones have a centrally muscle relaxing, anti-convulsive and anti spasmodic effects. The herb also contains hypnotic/sedative, analgesic and psychotropic properties contributing to its use for anxiety and insomnia.

Kava was first mentioned in scientific records in 1886 and began gaining popularity for soothing the nerves and inducing relaxation and sleep. In 1914 it was listed in the British Pharmacopoeia and entered into the U.S Dispensary in 1950 for the treatment of gonorrhea (‘Gonosan’) and nervous disorders (‘Neurocardin’). During the

last two decades, kava preparations have made their way into the European market and in the USA, standardized as kava lactones to provide a daily dose in the range of 60-120 mg. In 1990, kava was approved in Germany as a non prescription drug for conditions such as anxiety, stress and restlessness, to be banned later on for its adverse effects. Kava is banned for sale in Switzerland, Germany and Canada. There has been considerable protest from the scientific community as well as ardent kava users and followers suggesting that the ban on kava sale in Germany is an exaggerated safety measure and that more safety data should be collected through research for the study of kava toxicity. [47, 48] There are news that Germany has reversed the ban on kava, but there are still no kava products on sale in the German market as kava manufacturing licenses have been suspended. [48] The oral route is the prevalent route of administration for kava dosages. Kava doses can be obtained in various denominations from 100 mg-500 mg per dose in the form of comminuted rhizome filled in capsules as a galenic preparation. Oral doses of kava are also available as tea, tincture and infusion.

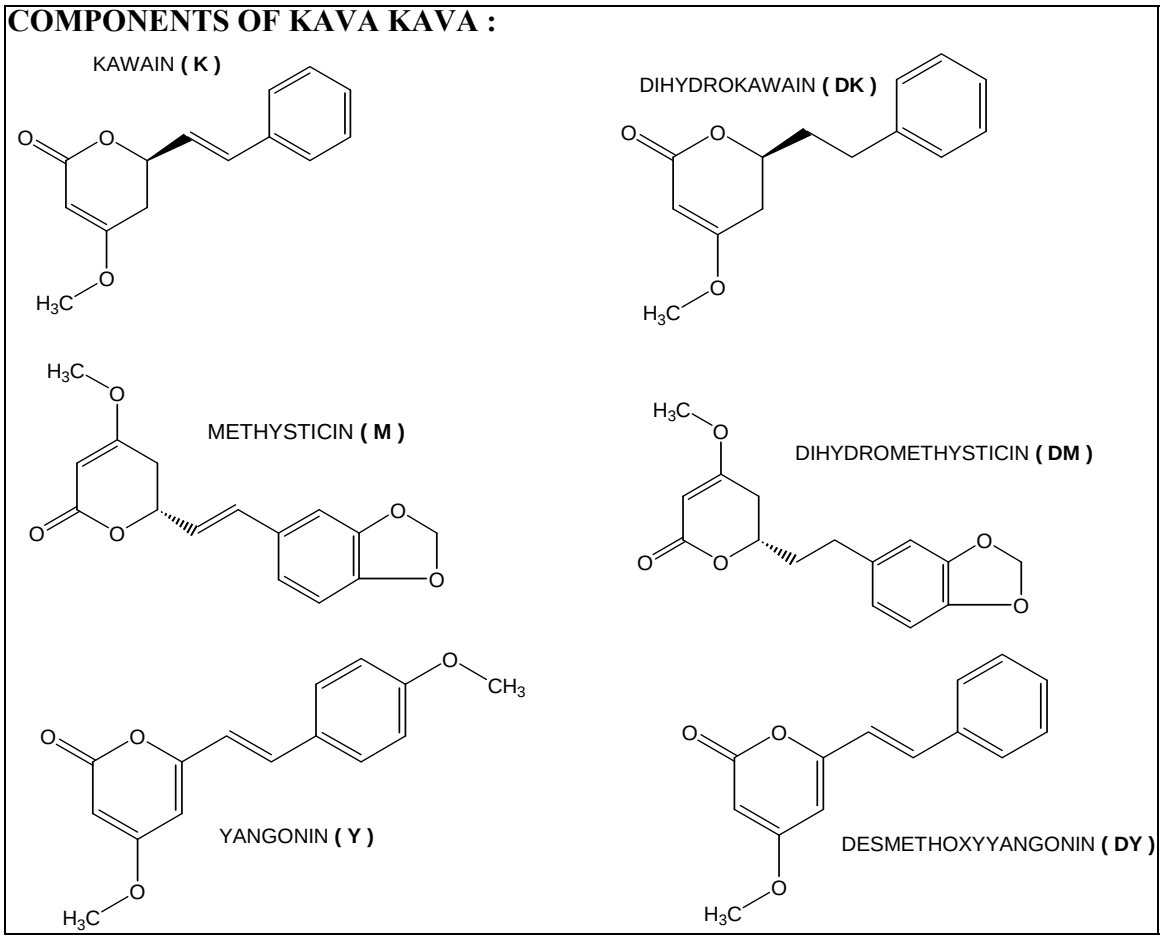


Figure 1.1: Structures of Kava Components

1.6.1.3: Pharmacology

Kava pyrones have a centrally muscle relaxing, anticonvulsive and antispasmodic effects. The herb is primarily used as an anti anxiety drug or as a mild sedative for insomnia due to its hypnotic, sedative and psychotropic properties. The pyrones are lipophilic and better absorbed with food. Also, the pyrones show greater absorption and

higher activity when administered as an extract as compared to when administered as single pyrones. This suggests synergism, indicating that the background matrix (kava resin) though not pharmacologically active, aids in the physiological processes to enhance the pharmacologic effect. The exact mechanism of action of the kava pyrones is not yet exactly known and research data indicates that concentration of the lactones, type of preparation (dosage form) and the variety of kava species used influences the pharmacologic effect. Some experiments suggest that the sedative effects may be due to an increase in the number of GABA (γ-amino butyric acid) binding site. Other preliminary evidence suggest that the sedative effects might be a result of dopamine antagonism, particularly by the Yangonin constituent. The kava pyrones, methysticin and Kawain also inhibit the uptake of noradrenalin which are suspected to contribute to the psychotropic actions of kava. Kava does not affect the benzodiazepine receptors. Also the effects of kava are not reversed by naloxone and hence analgesic effect does not occur by the opiate pathway. At high doses, kava does not impair cognitive function and sedation effect of kava occurs without respiratory depression. Consumption of kava results in an overall feeling of being sociable, tranquil and generally happy, similar to the feelings after sociable alcohol drinking in the West. The kava pyrones desmethoxyyangonin and methysticin competitively inhibit monoamine oxidase B. [49-51]

Adverse Effects: On oral administration kava can cause gastrointestinal upsets, headaches, dizziness, enlarged pupils, oculomotor equilibrium and accommodation disturbances, dry mouth and allergic skin reactions. Dopamine antagonism effect is

thought to be responsible for the twisting movements of the head and trunk. Being considered as sedative, normal doses of kava can impair the ability to drive or operate machinery. Cases of DUI ("driving under the influence") have been registered against individuals driving after drinking large quantities of kava at kava tea parties.

Hepatotoxicity had been observed in individuals after 3-4 weeks of continuous kava usage, accompanied by yellowing of skin due to jaundice, fatigue and dark urine. Further continuous use of kava for 4-8 weeks can cause elevated hepatomegaly (chemical driven liver damage) leading to the onset of encephalopathy. In isolated cases, short term use of kava has caused acute hepatitis which required the patient to undergo a liver transplant in as little as 1-3 months of kava use. These supplements have been banned from Germany, Switzerland and Canada. [51] It is interesting to note that adverse effects in most of the cases have been observed in extracts obtained from ethanol or acetone. Aqueous extracts that are used traditionally, by the Samoans and Pacific islanders do not report these adverse effects. But this does not imply that aqueous extracts are safe and hence more data needs to be collected.

1.6.1.4: Pharmacokinetics

In vivo studies in rats and humans following intravenous and oral administration of kava extract have been reported, with the pharmacokinetic profiling being done for kawain as the marker compound. Tarbah et al. studied the pharmacokinetics of kawain and its metabolite p-hydroxykawain in humans after oral administration of 6.9 and 7.7 mg/kg of kawain. [52] The main metabolic pathway of kawain is hydroxylation, the

metabolites being excreted mainly in their conjugate form (sulfates and glucuronides). Post oral administration in human, p-hydroxykawain reached peak plasma concentrations of 13ng/mL at ~0.75 hours. Free kawain showed peak plasma concentration of 41ng/mL whereas p-hydroxykawain in the form of glucuronide reached peak plasma concentration of 53ng/mL. The half lives for kawain and its metabolites was found to be between 0.7 to 1.9 hours and the areas under the plasma concentration time curve reflected that high amounts of metabolites were present in the glucuronide and sulfate conjugate form (~45% each) where as the free form represented only 10%. Renal excretion of the metabolite p-hydroxykawain was similar in magnitude to the creatinine clearance (~7.2L/h) whereas glucuronide and sulfate metabolites showed very high clearance values. Excretion via urine continued beyond 24 hours as high amounts of all three metabolites (38.4mg/mL) were found in urine at the end of 24 hours. Thus, 4.8% of the kawain dose of 800mg appeared in urine as p-hydroxykawain during 24 hours.

Mathews et al. determined the pharmacokinetics of kawain in rats after intravenous and oral administration and 7 days of repetitive oral administration of kava extract. [53] They also studied the pharmacokinetics of kawain coadministered with the kava extract. Mean peak plasma concentrations of kawain after intravenous administration (7.19mg/kg) were found to be 7.2µg/mL and the half life was 0.62 hours. On oral administration of 96.7mg/kg of kawain, peak plasma concentration of 2.6µg/mL corresponding to Tmax of 0.88 hours was observed. The half life was found to be 1.3 hours. Plasma concentrations for kawain after oral coadministration (single and repeated doses) with the extract were not significantly different from those obtained following a single oral administration. It was also found that the oral bioavailability of kawain

increased when administered along with the extract as compared to when administered alone.

1.6.2: Ginkgo biloba

1.6.2.1: Introduction and Regulatory Status

The ginkgo is the world's oldest living tree species and it can be traced back more than 200 million years to the fossils of the Permian period. Individual trees grow up to a height of 125 feet and have fan shaped leaves. The trees can live up to 125 years. The active components of ginkgo are the flavonol glycosides of quercetin, kaempferol and isorhamnetin and the ginkgo terpenes called ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J and bilobalide (Figure 1.2). Ginkgo biloba is sold as a dietary herbal supplement in the United States and is purported to improve blood flow to the brain and to improve peripheral circulation. It is promoted to mainly sharpen mental focus in healthy adults and those suffering from dementia. It is also used for diabetes related circulatory disorder and supposedly for vertigo. Ginkgo shows positive anti-inflammatory, cognitive promoting, antioxidant and vascular effects. Ginkgo is not effective in the treatment of cocaine dependency, depression, multiple sclerosis, and uncreative colitis. If is also used in the treatment of peripheral occlusive arterial disease, platelet aggregation inhibition, increasing the coronary blood flow and vasodilation and reducing hypertension. Ginkgo inhibited platelet activating factor improves hemodynamic parameters such as blood flow by decreasing blood viscosity and erythrocyte aggregation.

Ginkgo biloba extracts are standardized to contain 24% flavonols and 6% terpene lactones. An acetone-water mixture is usually used to extract the dried and milled leaves. The dry extract powder is obtained after the solvent has been removed. Regular doses range between 40-80 mg, 3 times a day. Ginkgo is available in the form of oral dosage forms in the denomination range 30 mg to 500mg per capsule, 30 mg to 260 mg per tablet or 40 mg per 5 ml of tincture liquid. [41, 44, 54, 55]

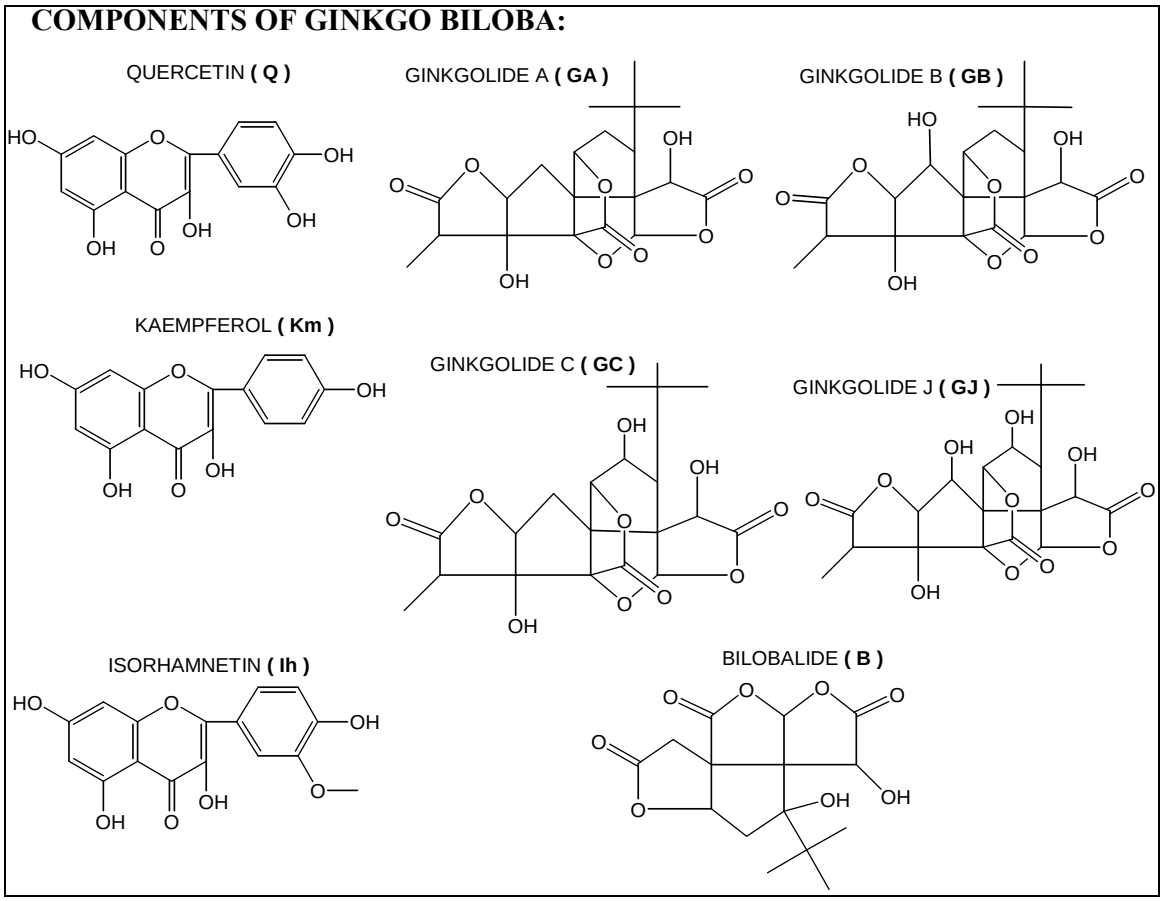


Figure 1.2: Structures of Ginkgo components

1.6.2.3: Pharmacology

The effects of ginkgo biloba are synergistic, hence attributed to the composition of the whole extract rather than any one single component. The two groups of active components are the flavonol glycosides and the terpene lactones. The mechanism of action of the ginkgo extract is only partially understood and there are several theories on how it might work in several disease states. One theory is that the extract might work by protecting the tissues from oxidative damage since the flavonoids have antioxidant and free radical scavenging properties. The flavonoids potentially seem to reduce cell membrane lipid peroxidation and decrease oxidative damage to erythrocytes. The extract is also known to protect neurons and retinal tissues from oxidative stress and injury following ischemic episodes. Protecting neurons and other tissues from oxidative damage might prevent progression of tissue degeneration in patients with dementia and other conditions. Ginkgolides in the leaf competitively inhibit platelet inhibiting factor (PAF) by binding at the membrane receptors of the respective cells. PAF inhibition decreases platelet aggregation, decreases phagocyte chemotaxis, and smooth muscle contraction. Preliminary research has also shown that the ginkgo extract inhibits formation of platelet thromboxane A2, further reducing platelet aggregation. [41, 44, 54, 55]

Adverse Effects: Ginkgo leaves and the ginkgo extract also contain a neurotoxin called the ginkgotoxin (o-methylpyridoxine, MPN) which can lead to seizures in susceptible people. Ginkgotoxin indirectly inhibits GABA. It antagonizes the activity of

pyridoxine, possibly by inhibiting enzymes such as pyridoxal kinase and glutamate decarboxylase in the brain. GABA is synthesized by glutamate by glutamate decarboxylase. Hence, by inhibiting glutamate decarboxylase, ginkgotoxin indirectly inhibits GABA. Ginkgotoxin is present in much higher amounts in seeds than in the leaf. Thus, ingestion of fresh ginkgo seeds may cause stomach ache, nausea, vomiting, loss of consciousness and shock and in some events can be fatal. *In vitro* experiments show that ginkgo extract affects several CYP450 enzymes, however in humans it does not seem to significantly affect any of these enzymes.

1.6.2.4: Pharmacokinetics

In humans, absolute bioavailability of ginkgolide A is found to be 100%, for ginkgolide B is 80-93%, for bilobalide is 70% and for ginkgolide C is very low. Peak plasma concentrations were attained within 2-3 hours for the flavonol glycosides (measured as their respective aglycones, due to the hydrolysis in the gut) after oral administration of 50mg, 100mg and 300 mg of extract. Food intake did affect the time to peak concentrations for the terpene lactones and the glycosides but did not affect bioavailability. Rat studies using radiolabeled extract have revealed a two compartment model for the distribution of the terpene lactones with extensive distribution in the glandular and neuronal tissues and in the eyes. The volumes of distribution for ginkgolide A, ginkgolide B and bilobalide were 40-60L, 60-100L and 170L. [41] The elimination half life for ginkgolide A, ginkgolide B and bilobalide was 4 hours, 6 hours and 3 hours respectively. Approximately 70% of ginkgolide A 50% of ginkgolide B and

30% of bilobalide were excreted unchanged in urine. Metabolites accounted for less than 30% of the administered dose and were not detectable in blood samples. [41, 44, 54, 55]

1.6.3: Milk thistle (Silybum marianum)

Milk thistle is an indigenous plant of Europe but now found almost all around the world. The seeds are particularly known for their medicinal benefits. The active constituents of milk thistle are a group of six flavonolignan isomers: silycristin, Silydianin, silybin A, silybin B, isosilybin A and isosilybin B (Figure 1.3). From these six isomers, silybin A and silybin B and isosilybin A and isosilybin B are diastereomers of each other. The isomers collectively are known as silymarin. Silybin A and silybin B make up almost 50% of the extract and are collectively called silybin. Silycristin is present up to 25% in silymarin. Isosilybin B and silydianin are the minor components of silymarin. The composition of the isomers in the seed is usually in the range of 1.5-3%. Orally, milk thistle is used for liver disorders including toxic liver damage by chemicals, Amanita phalloides mushroom poisoning, jaundice, chronic inflammatory liver disease, hepatic cirrhosis and chronic hepatitis. The extract is deemed safe when used orally and appropriately. Milk thistle extracts are standardized to contain 70-80% of silymarin content in market dosage forms. Numerous studies have shown that regular intake of milk thistle in recommended dose has proven to be safe with no adverse effects. [18, 44, 51, 56-60]

1.6.3.3: Pharmacology

Several activities seem to contribute to the therapeutic effect of silymarin isomers in liver disease. Silymarin seems to cause an alteration of the outer hepatocyte cell membrane that prevents toxin penetration. It also stimulates nucleolar polymerase A resulting in increased ribosomal protein synthesis, which can stimulate liver regeneration and the formation of new hepatocytes. There is also some evidence that suggests that silymarin might have anti fibrotic, anti inflammatory and immunomodulating effects that could also be beneficial in liver diseases. silymarin isomers inhibit beta glucuronidase, which might help protect against hepatic injury and possibly colon cancer. Inhibition of beta-glucuronidase is thought to reduce the hydrolysis of glucuronides into toxic metabolites in the liver and intestines. Preliminary evidence also suggests that silymarin might protect the kidney from nephrotoxic drugs like acetaminophen, vincristin and cisplatin. *In vitro* experiments show that silymarin isomers might have antiproliferative effects on androgen responsive prostrate cancer cells, triggering some interest in that area. As regards to herb-drug interaction with respect to metabolism, silymarin is not a significant inhibitor of CYP3A4. Various reports have suggested that silymarin does not affect CYP1A2, CYP2D6, CYP2E1 or CYP3A4.

Other than isolated allergic reactions and minor gastrointestinal disturbances, milk thistle does seems to be well tolerated without any adverse effects. [18, 44, 51, 56-60]

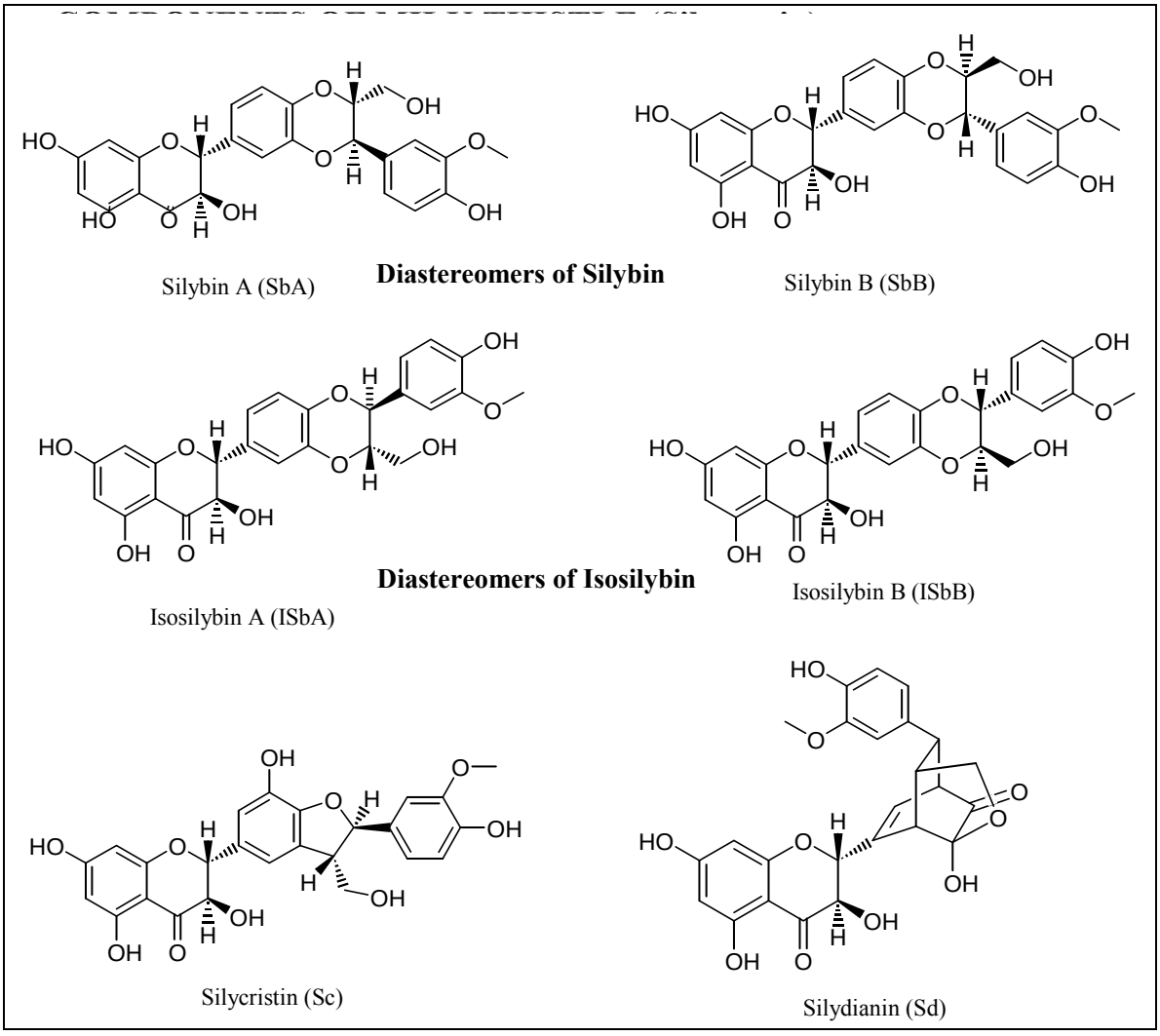


Figure 1.3: Components of Milk thistle (Silymarin)

1.6.2.4: Pharmacokinetics

A detailed and comprehensive account describing the pharmacokinetics and bioavailability issues of silymarin is given in Chapter 5 of this dissertation.

CHAPTER 2: *IN-SILICO* DESCRIPTORS AND *IN VITRO* PERMEABILITY

2.1: CHAPTER INTRODUCTION

In this chapter we attempt to establish an interrelationship or a link between the *in silico* descriptors and the *in vitro* permeability obtained for the active components of select herbal extracts. The effect of parameters such as the CLogP, polar surface area and the minimal cross sectional area on the *in vitro* permeability is studied with an aim to predict qualitatively, the least permeable component in selected herbal extract. Inter relationships are studied by plotting various *in silico* descriptors with permeability using 2D and 3D graphs. Selection of the least permeable compound from a particular extract as a bioavailability/bioequivalence marker, would be the most conservative approach towards ensuring the bioavailability of the entire extract. Apart from being the least permeable component, other criteria that need to be fulfilled for being a suitable bioavailability marker are also discussed. From the various herbal extracts under investigation, markers are selected for kava, ginkgo biloba and milk thistle (silymarin). Post selection of markers, silymarin is chosen as a representative extract, for the verification of marker prediction by solubility, partition coefficient, and *in vivo* pharmacokinetic and bioavailability experiments.

2.2: *IN-SILICO* DESCRIPTORS

The meaning of the word '*in-silico*' refers to something derived or obtained from a computer or by performing a computer simulation. The phrase has its origin from the Latin phrases '*in vitro*' and '*in vivo*' which are commonly used in biological research. *In silico* descriptors can be defined as theoretical parameters of a drug molecule that are determined by using complex computer programs. In contrast to wide spread belief, the word '*in silico*' does not mean anything is Latin but the word 'in silicio' does mean '*in silico*n'.

2.2.1: Introduction

Absorption of drugs through the gastrointestinal membrane is a complex process depending on drug molecular properties, formulation factors and physiological variables of the gastrointestinal tract. Permeability, absorption and/or bioavailability are are complex processes that depend on molecular structure and physicochemical properties of the drug as well as physiological variables of the gastrointestinal tract. [61] Thus, accurate and quantitative prediction of permeability values derived from molecular structure parameters without correlation to some permeability data is a very difficult task.

In silico descriptors are classified into 3 types mainly as one dimensional, 2-D and 3-D descriptors depending on the representation of drug molecular structure. [62] Molecular weight is a typical example of a 1-D descriptor, calculations such as CLogP (predicted log of octanol-water partition coefficient) which is based on fragment counts is an example of 2-D descriptor and molecular surface areas (polar surface area, minimal

cross sectional area) and molecular volume are examples of 3-D descriptor. [62] Estimations or results obtained from these descriptors can be of two types namely qualitative or quantitative. Qualitative estimates of a drug include high/intermediate/poor or a yes/no answer to a certain property (commonly absorption, permeability or bioavailability) where as quantitative estimates may include absolute numbers representing or estimating a certain property of the drug. Lipinski's rule-of-five is a well known example of a qualitative type of estimate which provides an approximate yes/no answer to whether a molecule can be considered as a potential drug candidate with respect to permeability and bioavailability considerations. [63, 64] Quantitative estimates can be obtained from various computer programs available, some of which can be accessed using the internet and some which are standalone. Actelion®, Advanced Chemistry Development®, ChemSilico®, Pharma Algorithms® are some of the examples of such available programs. [65, 66]

Solubility and permeability are very complex phenomena and hence there is some controversy concerning the accuracy with which these predictions are made. Further since permeability is not a completely structure based phenomena; its prediction is not only inaccurate, but probably impossible just based on molecular structure making very challenging the development of simple global structure based models to predict intestinal permeability. Models developed to predict permeability are generally applicable to transcellular passive transport. [62] Such models may involve conclusions based on correlations between *in vitro* permeability and descriptors like partition coefficient and molecular size. In smaller and simpler datasets, polar surface area also acts as a descriptor to predict the permeability of a lead compound. All these models require that the

experimental permeability values used for creating the model should be highly accurate and generated from one source.

Further as data sets become more complex with the inclusion of diverse molecular structures, model development requires more descriptors derived from structural calculations and correlation of these descriptors using complex statistical techniques such as multivariate data analysis and projection to latent structures. [62]

2.2.2: Definitions of Various *In silico* Descriptors

2.2.2.1:Predicted Octanol-Water Partition Coefficient (CLogP)

Drug lipophilicity is widely used as a predictor of membrane permeability since it is assumed that drug partitioning in the lipophilic cell membrane is a rate determining process for passive membrane permeation. Lipophilicity is traditionally expressed as the n-octanol water partition coefficient: the concentration ratio of the compound between n-octanol and water at equilibrium. n-Octanol is usually though not always the solvent of choice for the substitution of the intestinal bilayer to study drug partitioning because it best mimics the intestinal phospholipid bilayer. Transcellular diffusion requires desolvation of the compound and entry into the lipid bilayer of the cell membrane. The compound travels through the cytoplasmic aqueous phase or along the lipid membranes of the cell and crosses the cell membrane again to exit. Hence, transcellular diffusion must depend on the lipophilicity of the compound. [67]

2.2.2.2: Polar Surface Area

The polar surface area of a molecule can be defined as the surface area associated with the hydrogen bonding acceptor atoms nitrogen and oxygen, and the hydrogen atoms bound to these heteroatoms. [68]

2.2.2.3: Minimal Cross-Sectional Area

Solution phase properties such as desolvation and membrane permeabilities can be predicted using the general solute-solvent interaction (GSSI) approach. The GSSI approach is based on the principle that all solution phase processes can be modeled in terms of one or more gas-to-solution transfer processes. [69] The free energy of each gas to solution transfer process is calculated as the sum of the free energy of cavity formation and the free energy of solute-solvent interaction. The solutes contributions to the free energies are modeled on the various quantities computed from the solute's three-dimensional (3D) structure, where as the solvent's contributions are modeled by empirically determined regression coefficients. [69]

The minimal cross sectional area can be defined as that cross sectional area of the solute molecule when it is partitioned into the lipid bilayer interior and is preferentially oriented with its longest axis along the bilayer normal. (Fig. 2.1)

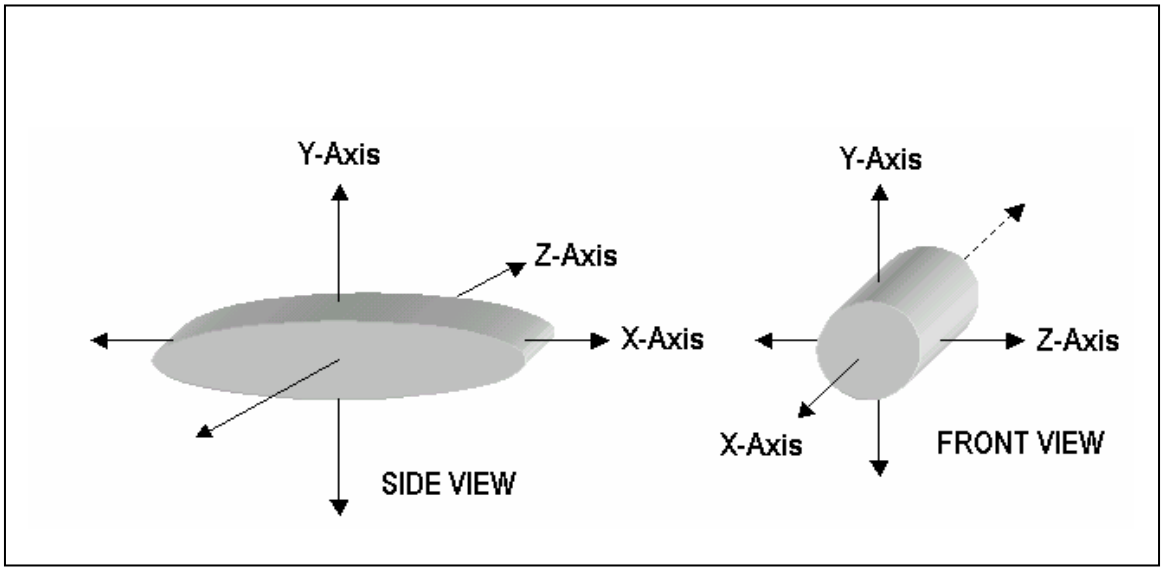


Figure 2.1. Schematic Representation of Minimal Cross-Sectional Area.

This alignment minimizes the work required to create a cavity big enough so that the solute molecule can be accommodated into the lipid bilayer. More specifically, the free energy of cavity formation is modeled on the basis of the total solvent accessible surface area of the solute. [69] Hence, the extent of permeation of the solute through the lipid bilayer mainly depends on its cross sectional area along the longest axis of the solute. Therefore transcellular diffusion occurs when a cavity or opening of free volume with cross sectional area equal to or greater than the minimum cross sectional area of the solute is created. [70]

2.2.3: Use of In Silico Descriptors to Predict Permeability

2.2.3.1: Lipinski's Rule of Five

Lipinski's Rule of Five states that poor absorption or permeation are more likely when:

1. There are more than 5 H-bond donors (expressed as sum of OH's and NH's).
2. There are more than 10 H-bond acceptors (expressed as the sums lof N's and O's).
3. The MW is over 500.
4. The log P is over 5.
5. Compound classes that are substrates for biological transporters are exceptions to this rule.

The rule of five is widely recognized as fundamental for the design of orally absorbed compounds. Though the rule is purely based on computational evaluations, it identifies the most prominent factors influencing drug absorption. [71]

2.2.3.2: Role of Calculated LogP and Polar Surface Area in Predicting Permeability

Membrane permeability is a two step process, where in first the unionized solute assumed to be in the solvated form needs to dehydrate itself from the solvent and then penetrate the lipid bilayer. Thus, 1-Octanol/water partition coefficient (LogP) and 1-Octanol/water distribution coefficient at a selected pH (LogD) are considered as key descriptors when modeling a biological process like permeability. [71] A high partition

coefficient implies greater partitioning of the drug in to the lipid bilayer indicating higher permeability. Thus, prediction of partition coefficients has had considerable success in developing quantitative structure-activity relationships during the drug discovery stage. Some of the programs that can be used to predict the octanol-water partition coefficient and distribution coefficient are:

- ACD LogP: by Advanced Chemistry Development Inc.
- ACD LogD: by Advanced Chemistry Development Inc.
- CLogP: PCModels/Daylight CIS, CLOGP/Biobyte
- MLOGP: Moriguchi LogP [72, 73]

From these above mentioned methods, CLogP is the most widely used for the estimation of partition coefficient.

During the second stage of the permeation phase when the drug penetrates lipid bilayer, after dehydrating itself from the solvent, the interactions between the bilayer and the polar parts of the molecule become important. During this process, the region of the lipid bilayer that a drug molecule interacts with first, is densely apolar and hence polarity of a drug molecule acts as a rate limiting factor towards the permeation process. Thus, higher the polar surface area of a drug molecule, lower the ability of the drug to penetrate the intestinal membrane. Conversely, it can also be implied, that higher the polar surface area of the drug, the less lipophilic the drug becomes, which means the drug tends to solvate more in a polar solvent, which in our case is water thus decreasing permeability. Hence, as polar surface area increases, which means the non polar surface area of the

molecule decreases (Non Polar Surface Area= Total surface area-polar surface area). Hence, the value of CLogP decreases as non polar surface area increases.

2.2.3.3: Minimal Cross-Sectional Area (MCSA)

The minimal cross sectional area is not a commonly used descriptor for predicting permeability when compared to partition coefficient or polar surface area. The MCSA is also termed as the Minimal Collisional Cross Sectional Area and is a directly related to the permeant size. Based on the General Solute Solvent Interaction model, the MCSA is calculated considering the solute-solute, solute-solvent, and solvent-solvent interactions. According to Pearlman et al. if the free energy of gas-to-solution transfer can be calculated accurately, the prediction of free energies of desolvation, partition coefficients, membrane permeabilities is possible. [69]

Lane et al. studied the relationship between rat intestinal permeability and hydrophilic probe size and geometry. [74] They concluded that cross sectional diameter of a molecule is a more important descriptor than molecular volume for evaluating molecular size retarded permeability.

According to Anderson et al. the minimal cross sectional area can be defined as that cross sectional area of the solute molecule when it is partitioned into the lipid bilayer interior and is preferentially oriented with its longest axis along the bilayer normal. [70]

Hence, theoretically this alignment minimizes the work required to create a cavity big enough so that the solute molecule can be accommodated into the lipid bilayer. Hence, the extent of permeation of the solute through the lipid bilayer mainly depends on its cross sectional area along the longest axis of the solute. Therefore transcellular

diffusion occurs when a cavity or opening of free volume with cross sectional area equal to or greater than the minimum cross sectional area of the solute is created.

2.2.4: Previous Related Studies

Predicting biological properties like permeability for drug like molecules is common and numerous references can be found in literature pertaining to the estimation of polar surface areas, molecular volume and predicted partition coefficient. Prediction of permeability characteristics as well as lipophilicity for herbal components is highly uncommon. Avdeef and coworkers studied the transport properties of kava lactones through filter immobilized artificial membranes laying emphasis on the effects of stirring during transport experiments. [75] They also determined predicted octaol-water partition coefficient using the program ACD LogP by Advanced Chemistry Development (Advanced Chemistry Development, Toronto, Canada; www.acdlabs.com), the calculated aqueous solubility of the uncharged species and the log of partitioning of the kava lactones in the blood-brain barrier using the program ABSOLV (Sirius Analytical Instruments, Forest Row, E. Sussex, UK; www.sirius-analytical.com). The log partition coefficients (LogP) for the Kava lactones ranged between 1.51 and 2.05, with Yangonin and Desmethoxy-yangonin being the most lipophilic and Methysticin being the least lipophilic. [75]

2.2.5: Why *In Silico* Descriptors?

Herbal supplements are crude extracts or semi purified extracts manufactured to contain a definite amount of a particular constituent or a group of constituents, which are

called marker compound(s). Since potency requires biological assessment of an extract, the presence of marker compounds does not guarantee the potency of an extract. Even if the marker compound demonstrates bioactivity the biological activity depends on the composition of the rest of the extract. Bioavailability data for these herbs or herbal supplements in humans is not readily available or difficult to obtain. If data is available it is accompanied by variation and ambiguity. [41] There have been several bioavailability studies of silymarin (a group of active markers in milk thistle), but most of them have been in terms of silybin or considering silybin as a measure for silymarin. It should be pointed out that silybin and isosilybin exist as diastereomers suggesting significant differences in their solubility and bioavailability. [76-79]

The complexity in the composition of the herbal extracts, lack of genuine *in vitro* permeability data and ambiguous bioavailability studies, *in silico* descriptors provide a preliminary economic alternative to expensive permeability and bioavailability experiments. Since in many cases, the pharmacologic effect of an herbal extract is synergistic, selection of a particular component as a marker based on *in silico* descriptors that give a certain idea about the permeability of the component becomes helpful in providing a lead for a performance and bioavailability marker. These descriptors (qualitative or quantitative) give a certain directional focus in the selection of a single marker compound from a pool of numerous active ingredients present in particular herbal extract.

2.2.6: Selected Herbal Extracts and their Active Components

Selection of herbal supplements was primarily based on their highest consumption and ranking according to retail sales. [80, 81] Since studying all the known active compounds in an herbal extract would be a tedious task, Table 2.1, gives the list of herbal extracts and their corresponding active compounds selected for investigation. The herbal extracts were selected as per their rankings based on retail sales in the United States in the years 2001 and 2002 (Table 1.4). The second criterion for selection was structural diversity which included kavalactones from kava, terpene trilactones and flavonol aglycones from ginkgo, flavonolignan isomers from the fruit of milk thistle and the ginsenosides from ginseng. All together they spanned a wide range of molecular weights and functional groups. Thus, a total of 8 herbal extracts comprising of 37 active ingredients were selected for the estimation of their *in silico* parameters.

2.2.7: Estimation of *In Silico* Descriptors

Estimation of *in silico* descriptors included the calculation of:

i. Polar Surface Area (PSA)

ii. Minimal Cross Sectional Area (MCSA)

iii. Predicted Octanol-Water Partition Coefficient (CLogP)

Table 2.1: Selected Herbal Extracts and their Active Constituents for the Estimation of *In Silico* Descriptors

Herbal Extract	**Active Compounds**	**Ranking**		
		1997	1998	2001
Kava	Kawain (K), Dihydrokawain (DK), Methysticin (M), Dihydromethysticin (DM), Yangonin (Y), Desmethoxy-yangonin (DY).	13	17	11
Milk thistle	Silybin A (SbA), Silybin B (SbB), Isosilybin A (ISbA), Isosilybin B (ISbB), Silycristin (Sc), Silydianin (Sd), Taxifolin (Tx)	12	13	12
Ginkgo biloba	Ginkgolide A (GA), Ginkgolide B (GB), Ginkgolide C (GC), Ginkgolide J (GJ), Isorhamnetin (Ih), Kaempferol (Kf), Quercetin (Q)	1	1	1
Ginseng	Ginsenoside Rb1(GRb1), Ginsenoside Rb2 (GRb2), Ginsenoside Rc (GRc), Ginsenoside Rd (GRd), Ginsenoside Re (GRe), Ginsenoside Rf (GRf), Ginsenoside Rg1 (GRg1), Ginsenoside Rg2 (GRg2).	2	3	4
Black Cohosh	26-deoxyactein (dAcn), 26-deoxyacteol (dAcl) Actein (Acn)	17	N/A	10
Echinaceae	Caftaric acid (CfA), Chicoric acid (CcA)	4	5	2
Garlic	Alliin (Aln), Deoxy-alliin (dAln)	3	4	3
Valerian	Hydroxy-valerenic acid (HVA), Valerenic acid (VA)	10	2	10

2.2.7.1: Molecular Structures

Conventional molecular structures when viewed on a computer screen are in the 2-D format, the file names typically having .mol or the .cdx form of a file extension. In order to estimate molecular descriptors such as polar surface area, molecular volume and minimal cross-sectional area, it is necessary that the compounds are represented as geometrically optimized 3-D structures. Hence, we used CONCORD® for the conversion of 2-D or crude 3-D input structures to accurate and geometrically optimized 3-D structures. [82]

2.2.7.2: Generation and Selection of Conformers

Since a molecule now exists in 3-D, it gives rise to numerous conformers with varying energies. CONFORT,® a powerful conformational analysis tool, based on a novel algorithm for conformational searching, performs exhaustive and rapid analysis of 3-D drug sized molecules.[83] We used CONFORT® to identify various global energy and maximally diverse conformers. Only conformers having a global conformational energy less than 250 kilo calories per mole were selected for the estimation of the polar surface and the minimal cross sectional areas. The conformer with the maximum non polar surface area was selected for the calculation of PSA and the conformer with minimal cross-sectional area was identified for the calculation of the MCSA.

2.2.7.3: Calculation of Polar Surface Area and Minimal Cross-sectional Area.

In order to calculate the PSA and the MCSA of a solute molecule, it is necessary to calculate the accessible surface area (van der Waal's surface area) of the molecule. Solvent accessible surface area of a solute can be described as that surface created by the centre of a probe or solvent sphere, when the probe is rolled over the entire van der Walls surface of a solute molecule (Fig. 2.2) [84] The accessible surface area was determined using SAVOL3, a widely distributed program for calculating the mentioned molecular descriptors.[85] The algorithm computes the molecular (or solvent accessible) surface area by summing the non occluded surface area of each atom in the molecule. This can be done by imagining a slicing plane (passing through the two poles of the sphere) being rotated incrementally about its axis, thereby cutting the sphere into many double lunar segments (imagine, for example, two slicing planes intersecting at the centre of the sphere at an angle of one degree from each other, the resulting pair wise spherical segments are what is referred to as double lunar segments).[84, 86] The non-occluded surface area of each double lunar segment is calculated by analytically summing up portions which are not contained in the van der Walls sphere of a neighboring atom. The precision of the surface area depends on the angle of increment used for rotating the slicing plane.

SAVOL3 was incorporated along with the GSSI [69, 86] (General Solute Solvent Interaction) model for the calculation of the minimum cross sectional area of the solute. The GSSI model is based on a semi-empirical approach to enable the prediction of solution phase properties (free energies of desolvation, partition coefficients, and membrane permeabilities).

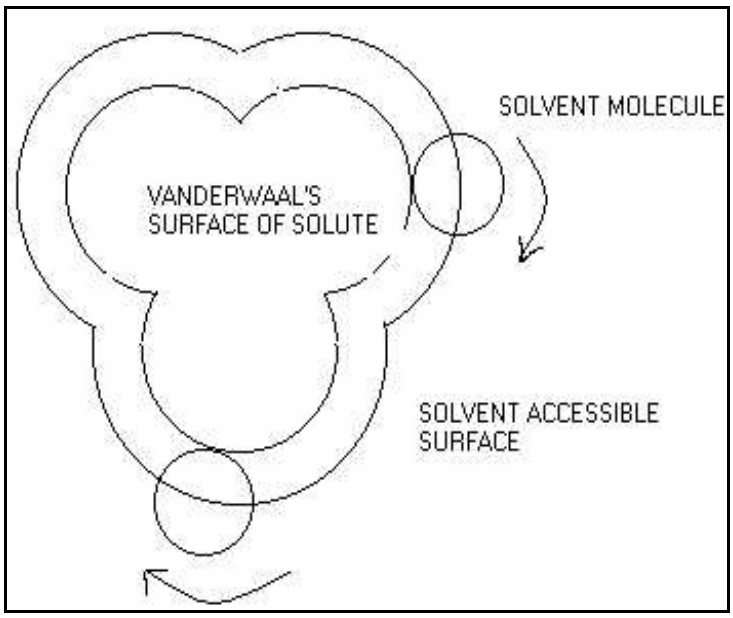


Figure 2.2. van der Waal's Accessible Surface

2.2.7.5: Determination of CLogP

In the following work SYBYL6.8® which incorporates the program CLogP was used to predict the octanol-water partition coefficient of the molecules. [87] The program divides the molecule into basic fragments and calculates the log $P_{o/w}$ by the summation of hydrophobic contributions of these fragments. [88] CLogP has been tested on an elaborate database which includes nearly 8000 compounds and yields good results (r^2 = 0.970). [89]

2.2.8: Results

The following tables 2.2-2. describe the calculated molecular descriptors (CLogP, PSA and MCSA) for the selected 39 active herbal constituents.

Table 2.2: Data for Kava constituents:

Compound	Mol. Wt.	C Log P	PSA (Å^2)	MCSA (Å^2)
Desmethoxy-yangonin	228.24	2.79	89.232	92.250
Dihydrokawain	232.28	2.15	83.790	107.726
Dihydromethysticin	276.28	1.71	136.049	113.722
Kawain	230.26	2.04	92.568	106.461
Methysticin	274.27	1.61	145.773	105.66
Yangonin	258.27	2.71	108.388	97.242

Table 2.3: Data for Milk thistle constituents:

Compound	Mol. Wt	C LogP	PSA (Å^2)	MCSA(Å^2)
Isosilybin A	482.44	1.94	350.239	123.707
Isosilybin B	482.44	1.94	350.593	133.146
Silybin A	482.44	1.95	349.809	146.32
Silybin B	482.44	1.94	348.589	125.89
Silycristin	482.44	1.38	410.998	131.682
Silydianin	482.44	-0.39	368.061	157.108
Taxifolin	304.25	1.02	347.423	101.285

Table 2.4: Data for Ginkgo biloba constituents:

Compound	Mol.Wt.	CLogP	PSA(Å^2)	MCSA (Å^2)
Bilobalide	326.30	-2.64	271.405	139.435
Ginkgolide A	408.40	-1.55	283.058	128.28
Ginkgolide B	424.40	-1.45	313.477	132.547
Ginkgolide C	440.40	-2.48	309.311	134.402
Ginkgolide J	424.40	-2.58	326.152	134.414
Isorhamnetin	316.26	1.75	282.536	109.498
Kaempferol	286.24	1.90	307.353	96.256
Quercetin	302.24	1.30	353.742	111.565

Table 2.5: Data for Ginsenosides (Ginseng)

Compound	Mol. Wt.	CLogP	PSA (Å^2)	MCSA (Å^2)
Ginsenoside Rb1	1109.29	4.544	647.745	224.914
Ginsenoside Rb2	1079.27	5.232	603.788	232.126
Ginsenoside Rc	1079.27	4.299	622.799	282.806
Ginsenoside Rd	947.15	5.726	556.208	273.938
Ginsenoside Re	947.15	3.869	623.308	295.768
Ginsenoside Rf	801.01	4.726	417.294	209.152
Ginsenoside Rg1	801.01	4.932	438.223	245.672
Ginsenoside Rg2	785.01	4.758	350.105	209.695

Table 2.6: Data for Black cohosh, Echinaceae, Garlic and Valerian:

Herb	Compound	Mol. Wt.	CLogP	PSA (Å^2)	MCSA(Å^2)
Black cohosh	26-deoxyactein	660.83	6.124	186.216	152.839
	26-deoxyacteol	600.78	6.781	184.475	164.142
	Actein	676.83	5.419	269.289	182.592
Echinaceae	Caftaric acid	312.23	-1.540	420.790	104.414
	Chicoric acid	474.37	0.139	516.139	167.085
Garlic	Alliin	177.22	-2.708	215.180	83.821
	Deoxy-alliin	161.22	-1.198	202.245	83.847
Valerian	Hydroxy-valerenic acid	250.33	2.650	172.723	116.400
	Valerenic acid	234.33	4.737	117.788	124.200

2.3: IN VITRO PERMEABILITY

2.3.1: Introduction

Assessment of the absorption of a given compound from the gastrointestinal tract simply means establishing the permeation of this compound across the wall of the gastrointestinal tract. The gastrointestinal absorption process can be influenced by factors such as the dissolution properties of the drug, which may be modified due to physiological parameters such as gastric juice and bile resulting in precipitation of the

drug at the absorption site, adsorption of the drug to components in the gastrointestinal tract, chemical and bacterial degradation of the drug and metabolism of the drug in the lumen, and the brush border or in the intestinal wall and others. There are no *in vitro* methods that can assess all these influences simultaneously during the permeation process unless these studies are done in intact humans or animals. Conducting permeability transport experiments in intact humans or animals limit the number of compounds that can be screened and also present ethical and significant technical difficulties. There are a wide variety of *in vitro* methods that are used to study only the translocation of drug molecules across the intestinal membrane after oral administration, but none of these methods take into account other factors that influence the actual bioavailability of the drug.

2.3.2: Factors Affecting Drug Permeability

During permeation after oral administration, the drug in order to be able to cross the gastrointestinal membrane has to overcome two big hurdles: (i) Release of the drug from the formulation matrix and dissolution in the gastrointestinal fluids. This mainly depends on the physicochemical properties of the drug; (ii) Transport itself across the gastrointestinal membrane and into the blood stream, with minimal or no degradation. This mainly depends on the anatomic and physiologic conditions present in the gastrointestinal tract. The rate at which the drug reaches the systemic circulation is determined by the slowest step in the sequence which is known as the rate limiting step.

2.3.2.1: Physicochemical Properties of the Drug

When a drug is administered orally, it must be transferred from the dosage from to the systemic circulation to be bioavailable. Thus, bioavailability is a measure of the amount (extent) of drug entering the blood stream and the rate at which it enters. Bioavailability of a drug absorbed from the gastrointestinal tract is influenced by the anatomic and physiological properties of the gastrointestinal system and the physicochemical properties of the drug or the drug product. Physicochemical properties include solubility, rate of dissolution, ionization, particle size, surface area, crystal form and stability of the drug or the drug formulation.

2.3.2.1.1: Solubility and Dissolution Rate

For a drug to undergo passive diffusion, it is required to be released from the formulation matrix and enter into solution form. Thus, for an orally administered drug in the form of a capsule, tablet or a suspension, the rate of absorption is governed by the rate of dissolution of the drug into the biological fluids.

2.3.2.1.2: Particle Size and Surface area

A drug dissolves more quickly when the surface area is increased. Therefore a faster dissolution rate can be achieved by reducing the particle size of the drug.

2.3.2.1.3: Crystalline Form

Drug molecules can exist in more than one crystalline form, thus giving rise to polymorphism. Polymorphs of a drug may differ from each other in physical properties such as density, solubility and dissolution rate, and melting point. The crystalline from of a drug is less soluble than the amorphous form as high energy is required to pull the drug crystal apart and get it into solution. Many drugs may associate with solvents to produce crystalline forms called solvates. Solvate forms of a drug with organic solvents may dissolve faster than the non-solvated form. [90]

2.3.2.1.4: Stability

Acid and enzymatic hydrolysis of a drug may occur in the gastrointestinal tract causing extensive degradation of the drug leading to poor bioavailability [91, 92]. Additionally interactions between the drug or dosage form and a dietary component may lead to alterations in the rate and extent of absorption of the drug.

2.3.2.2: Physiologic Variables of the Gastrointestinal System

The physiological factors include mesenteric blood flow, gastrointestinal motility patterns, volume, viscosity, and flow rate of gastrointestinal contents, presence or absence of food, pH and membrane permeability.

2.3.2.2.1: pH

The gastrointestinal barrier favors the permeability of uncharged, lipid soluble solutes. Thus, the proportional ratio of the ionized to unionized species of the drug

highly depends on the pH of the solution. Hence, pH at the site of absorption is an important factor during drug absorption.

2.3.2.2.2: Gastrointestinal Motility

Gastrointestinal motility involves the movement of material mainly food through the gastrointestinal tract. The stomach is composed of two main layers of smooth muscle. The outer longitudinal muscle layer is in continuity with the duodenal muscle layer while the inner circular layer extends up to the pylorus. The arrangement of muscles allows the stomach to produce coordinated movements of the gastric contents. The vagus and the splanchnic nerve connect the stomach to the central nervous system. Stimulation of these nerves produces contractions in the stomach. The stomach also possesses two main plexuses of the nervous system: the mesenteric plexus and the submucosal plexus. The major hormones that affect the stomach are secritin, gastrin, cholecystokinin, and glucose independent insulinotropic peptide (GIP) and enteroglucagon. This hormonal and nervous network together regulates and optimizes the behavior of the gastrointestinal tract. The stomach exhibits two pattern of motility:

(i). Fasted Pattern of Motility: Gastric emptying occurs even during fasting and the pattern of electrical activity observed is markedly different to that observed in the fed state. This activity is called the interdigestive myoelectrical cycle or the migrating myoelectric complex. Migrating motor complexes are waves of activity which sweep through the gastrointestinal tract in a regular cycle during fasting state. This cycle originates in the stomach every 75-90 minutes during inter digestive phase and is responsible for the rumbling observed when hungry. These complexes trigger peristaltic

waves which facilitate transportation of indigestible substances such as bone, fiber and foreign bodies from the stomach through the small intestine past the iliocecal sphincter into the colon. It also transfers bacteria from the small intestine to the large intestine and inhibit the migration of colonic bacteria into the terminal ileum.

Each cycle is divided into four consecutive phases of activity. Phase I corresponds to 40-50% of the cycle duration, Phase II to 20-30%, Phase III to 10-15% and Phase IV to 0-5%. During Phase I a series of weak intensity contractions are observed. Phase II comprises of irregular contractions gradually increasing in amplitude culminating in the onset of discharge of gastric contents. During Phase III the contractions become regular and have the greatest amplitude. Discharge of gastric contents is completed during this phase. Finally Phase IV comprises of a reduction in the frequency and amplitude of contractions. The whole cycle repeats itself every 2 hours until a meal is ingested, which then triggers a fed pattern of motility.

Factors that can trigger a fasted pattern of motility are hungry state, alkaline buffer solutions, anxiety, lying on the right side, hyperthyroidism, metoclapromide and dopaminergic blockers. Factors that prevent this cycle are diets rich in fatty acids, mental depression, lying on the left side, gastroenteritis, pyloric stenosis, gastric ulcer, Crohn's disease and hypothyroidism. [93]

(ii). Fed Pattern of Motility: Stimulation of the parasympathetic nerves increases contractility and relaxes the pylorus, resulting in a decrease in the rate of emptying of solids and liquids. Liquid and solid emptying is controlled by the proximal and distal portions of the stomach respectively. The proximal stomach produces slow distal

contractions which are responsible for the basal pressure in the stomach which is responsible for the gastric emptying of liquids. The distal portion or antrum of the stomach has a thicker muscular wall which is concerned with the emptying of solids. The peristaltic waves initiated do not expel food from the stomach but cause the larger particles to accumulate away from the walls in a zone where the flow is reversed. Thus, the larger particles are retropulsed into the antrum where they are caught up by the next peristaltic wave. The liquid component of the meal empties exponentially while the solid component empties linearly after a variable lag time. This lag time depends on the size of the food particles in the stomach and larger the particles, longer it takes to break these particles to a suitable size. Eventually all material is emptied by the stomach into the small intestine where absorption occurs. The small intestine like the stomach exhibits two distinct patterns of motility where the fed pattern is characterized by three sequential contractions separated by 5 to 40 seconds of inactivity. This fed pattern consists of segmental and peristaltic contractions in which the segmental contractions are frequent. The interdigestive myoelectirc complex continues from the stomach to the small intestine repeating every 140-150 minutes and as one complex reaches the ileum another starts at the duodenum.

Absorption from the small intestine is known to be rapid, depending on how fast the emptying process from the stomach takes place. Absorption of drugs is significant from the small intestine and thus the emptying process from the stomach into the small intestine becomes the rate limiting step for efficient absorption of drugs. Highly statistically significant correlations have been observed between the gastric emptying half life and the time to maximum plasma concentration of acetaminophen. [94]

2.3.2.2.3: Gastrointestinal Blood Flow

Passive transport of drugs takes place over a concentration gradient from a region of high drug concentration to a region of low drug concentration until equilibrium is established. When a drug is transported through the gastric membrane to the systemic circulation, it is removed continuously by the blood flow maintaining sink conditions and continuous transport. Thus, a decrease in the mesenteric blood flow decreases the rate removal of drug from the gastrointestinal tract thus affecting the rate and extent of drug absorption. [95, 96] However normal physiological variability in mesenteric blood flow does not significantly affect absorption rate of drugs.

2.3.3: Transport Mechanisms

The biological membrane may be described as a dynamic lipoid sieve or a semi permeable lipoid membrane containing several aqueous pores or channels. It hosts carrier molecules that shuttle ions, proteins etc back and forth across the membranes. Transport of drugs across the gastrointestinal membrane can be categorized into three main sections. These are diffusion, active transport and transcytosis.

2.3.3.1: Diffusion

This is a process in which molecules spontaneously move from a region of higher concentration to a region of lower concentration, until equilibrium is established. The concentration gradient is the main driving force for this phenomenon. Diffusion is a movement due, solely, to the kinetic energy and the electrical charge of the molecules,

and the electrical field in which they exist. [97, 98] No external energy is required for this process.

2.3.3.1.2: Passive Diffusion

In this process the flux is proportional to the concentration of the solute and the rate is independent of direction.

2.3.3.1.3: Facilitated Diffusion

This is a carrier mediated transport phenomenon where the flux is saturable with increasing concentration. The mechanism is structurally selective and drugs with similar structure may act as competitive substrates for the transport system. The rate of flux in facilitated diffusion may be asymmetric. Glucose and other medium sized molecules are transported in this manner.

2.3.3.2: Active Transport

This is a carrier mediated transmembrane process characterized by transport of drug against a concentration gradient (or an electrochemical gradient), i.e from a region of low concentration to a region of high concentration [99, 100]. The mechanism involves a directly or indirectly coupled energy consuming system and the flux rate is asymmetric. The energy for active transport is usually derived from the hydrolysis of high energy phosphate bonds contained in adenosine triphosphate, creatinine phosphate or arginine phosphate. The system is substrate specific and drugs with similar structures may compete for binding sites on the carrier molecule. Further more because only a

certain amount of carrier is available, at high drug concentrations, all binding sites on the carrier molecule become saturated.

Cotransport is a variety of active transport in which the absorption into the cell against the concentration gradient is linked to the secretion of a cellular ion such as sodium down its concentration gradient. This process is important for the absorption of amino acids in the small intestine.

2.3.3.3: Transcytosis (Endocytosis or Vesicular Transport)

Endocytosis is a process whereby cells absorb material (molecules such as proteins) from the outside by engulfing it with their cell membrane. It is used by all cells of the body because most substances important to them are large polar molecules, and thus cannot pass through the hydrophobic plasma membrane. This process takes place against a concentration gradient and therefore is usually energy dependent. The opposite of endocytosis is exocytosis.

2.3.3.3.1: Absorptive Endocytosis

During this process the solute attaches itself either to the surface proteins or to the glycolipids. The mechanism often has high capacity, but can be saturable and show high competition due to non-specific receptor binding.

2.3.3.3.2: Receptor-mediated Endocytosis

The substrate has high affinity for the specific receptor and hence the process is very substrate specific, saturable and asymmetric.

2.3.3.3.3: Fluid-phase Endocytosis

This process is also known as pinocytosis where in small soluble molecules are internalized with the vesicle volume. The process is non specific and non saturable and occurs due to the fluidity of the cell membrane. Phagocytosis differs from pinocytosis in that very large particles are taken up from the lumen and released into the circulation by migrating leukocytes or macrophages. The particles can also enter the intestinal villi across the gap created by the sloughing off of the enterocytes at the tip of the villi. Pinocytosis appears to be more substrate specific that phagocytosis.

2.3.4: Transport Pathways

Passage of molecules across the epithelial cell membrane occurs either paracellularly (between two adjacent cells) or transcellularly (through the cell). The permeating solute is open to both these pathways, however the relative contribution of the observed transport pathway depends highly on the properties of the solute and the type of membrane involved.

2.3.4.1: Paracellular Transport

Paracellular transport occurs strictly by passive diffusion. Generally, polar, membrane impermeant molecules diffuse through the paracellular route which is dominated by the tight junctions. Exceptions to this are molecules that are transported actively through one or both membrane domains of the cell. The tight junction is a mesh work of continuous chains of membrane proteins. This intercellular space occupies

0.01% of the total surface area of the epithelium. The closely fitting tight junctions on the apical side gate them. In humans, the duodenum has the largest mean pore radii of intercellular spaces, which is approximately 0.8 nm. The mean pore radii o the intercellular spaces in the ileum and the colon are approximately 0.3nm. [101] As a result drugs excluded from the transcellular route are often well absorbed from the upper part of the small intestine. [102] The mean pore radius presents only a crude estimate since the pores of the paracellular pathway range between large number of small pores and small number of large pores. This explains how, for many drug molecules, even though the mean pore radius is too small to permeate through tight junctions, they can still be transported from these channels. Additionally nutrients and hormones regulate the permeability of the paracellular pathway for several drug molecules. [103, 104]

2.3.4.2: Transcellular Transport

Transcellular transport can occur through passive, facilitated or active processes. Moderate and highly lipophilic molecules diffuse passively across cellular barriers by the transcellular route. Tanscellular diffusion can occur through a number of mechanisms. [105] According to one mechanism the solute can pass through the aqueous diffusion layer and partition from the aqueous extracellular environment (including the negatively charged glycocalyx) into the cell plasma membrane. In the membrane the solute can remain mostly within the hydrocarbon domain of the, lipid bilayer and diffuse laterally around the tight junctions. The transport of the solute is then completed by desorption from the receiver side membrane or by repartitioning into the aqueous receiver compartment. Alternatively it has been proposed that the solute can partition out of the

donor side membrane into the cell cytoplasm. The solute then diffuses through the cell cytosol and repartitions into the basolateral cell membrane. The solute thus can sequentially partition its way through the cell depending on its relative affinity for different intracellular membranes. Further, there maybe specific carrier proteins within the cytosol that transfer the solute across the cell or among various subcellular compartments.

2.3.5: Mathematical Models for Drug Absorption

The amount, M of material flowing through a unit cross-section, S, of a barrier in unit time, t, is known as the flux, J (Equation 2.1). [106]

$$J = \frac{dM}{Sdt} \qquad \textbf{Equation 2.1}$$

The flux, in turn, is proportional to the concentration gradient, dC/dx: (Equation 2.2)

$$J = -D\frac{dC}{dx} \qquad \textbf{Equation 2.2}$$

In which, D is the diffusion coefficient of the permeant in cm^2/sec, C is the concentration in g/cm^3 and x is the distance of movement perpendicular to the surface of the barrier in cm. The negative sign of the equation implies that the diffusion occurs

from a region of high concentration to a region of low concentration. In other words, diffusion occurs in the direction of decreasing concentration of the permeant and hence the flux will always be a positive quantity. The diffusion constant of the diffusivity, D, can be affected by temperature, pressure, solvent properties and the chemical nature of the permeant. Hence, D should be referred to as a diffusion coefficient rather than a constant.

The schematic representation of diffusion across a semi-permeable membrane is illustrated in Figure 2.3. When C_1 represents the concentration in the membrane on the donor side and C_2 refers to the concentration in the membrane on the receiver side. 'h' represents the thickness or the width of the membrane.

The concentration gradient within the membrane is assumed to be constant for a quasi-stationary state to exist. It is also assumed that the aqueous boundary layers on both sides of the membrane do not significantly affect the transport process.

Therefore, combining the equations 2.1 and 2.2, the flux (diffusion rate) can be described as:

$$\frac{dM}{Sdt} = -D\frac{dC}{dx} \qquad \textbf{Equation 2.3}$$

$$J = \frac{dM}{Sdt} = D\left(\frac{C_1 - C_2}{h}\right) \qquad \textbf{Equation 2.4}$$

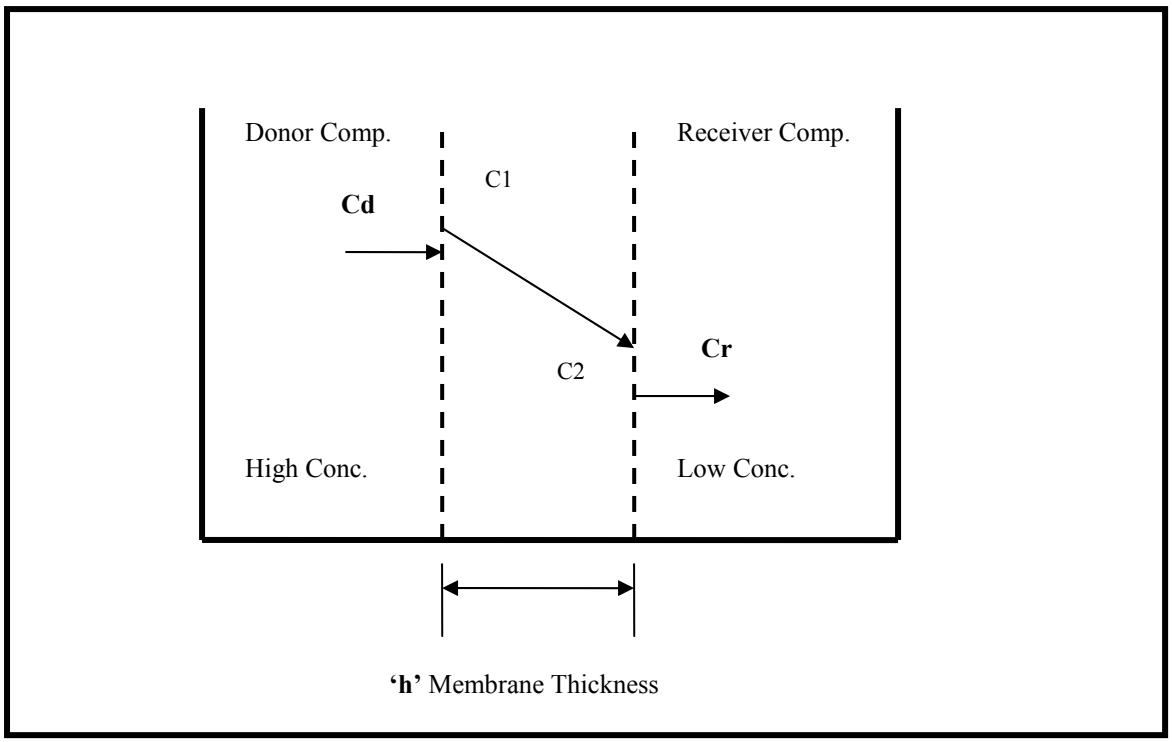


Figure 2.3: Schematic representation of diffusion across a semi-permeable membrane

Although the concentrations C_1 and C_2, within the membrane are not ordinarily known they can be related to the concentration of the permeant in the donor compartment, C_d, or the concentration in the receiver compartment, C_r, by using the intrinsic partition coefficient between the membrane, the donor and the receiver compartment. This is

$$\frac{dM}{dt} = \frac{DSk\ (Cd\ -\ Cr\ )}{h} \qquad \textbf{Equation 2.5}$$

Since the apparent permeability coefficient, P_{app}, is defined as,

$$Papp = \frac{Dk}{h} \qquad \textbf{Equation 2.6}$$

Then Equation 2.5 becomes

$$\frac{dM}{dt} = P_{app} S\left(Cd - Cr\right) \qquad \textbf{Equation 2.7}$$

Under sink conditions, Cr = 0. The the equation becomes,

$$\frac{dM}{dt} = P_{app} SCd \qquad \textbf{Equation 2.8}$$

Another method to determine drug absorption rate (the flux) has been suggested by Amidon et al. [35]

$$Jw = Pw.Cw \qquad \textbf{Equation 2.9}$$

Where Jw (x,y,z,t) is drug flux (mass/area/time) through the intestinal wall at any position and time, Pw (x,y,z,t) is the apparent permeability of the intestinal wall, and Cw (x,y,z,t) is the drug concentration a the intestinal membrane. The rate of loss of drug from the intestinal lumen assuming no luminal reactions, at any time is given by,

$$Absorption\ \ Rate = \frac{dM}{dt} = \int_0^t \iint_s Pw.Cw.dS.dt \qquad \textbf{Equation 2.10}$$

where the double integral is over the entire intestinal surface. [35]

The total mass, M, of the drug absorbed at time t, is

$$M(t) = \int_0^t \iint_s Pw.Cw.dS.dt \qquad \textbf{Equation 2.11}$$

For generality the permeability is considered to be both time dependent and position dependent. Based on the above analysis, it is seen that the two factors that are primarily involved in drug absorption are drug permeability and concentration at the absorption site. Maximum absorption would occur when the drug has the highest permeability and highest concentration (given by its saturation solubility) at the site of absorption. As a result oral absorption of a drug *in vivo* could be predicted *in vitro* based on the solubility and permeability measurements of the drug. The local mass absorbed per unit time, per unit area is therefore the basis of first principle of bioequivalence. The analysis implies that if two drug products containing the same drug have the same permeability-concentration time profile, at the intestinal membrane surface, then they will have the same rate and extent of absorption. This further implies that if two drug products have the same *in vivo* dissolution profile under luminal conditions, then they will have the same rate and extent of absorption. [35] Thus, this approach can also be used to establish bioequivalence of drug products.

2.3.5.2: pH Partition Theory

The pH partition theory proposed by Brodie and associate's states that drugs are absorbed from the gastrointestinal tract mainly in their molecular and undissociated form. [107-109] It is reasoned that the partition coefficient between the membrane and the gastrointestinal fluids is large for the unionized species and favors the transport of the molecular form from the lumen through the predominantly lipophilic gastrointestinal barrier and into the systemic circulation. In developing the pH-partition hypothesis, Brodie studied intestinal absorption by opening up rats and perfusing their stomachs or

intestines with drug solutions and simultaneously injecting drug solutions in to their blood. Thus, by doing so an instantaneous equilibrium was established between the two fluids, described by the following equation:

$$D = \frac{Cg}{Cb} \qquad \textbf{Equation 2.12}$$

where,

Cg: Total drug concentration in the gut.

Cb: Total drug concentration in blood.

Therefore mathematically D can be calculated using the following equations:

For a weak acid,

$$D = \frac{1 + 10^{\,pH_1 - pK_a}}{1 + 10^{\,pH_2 - pK_a}} \qquad \textbf{Equation 2.13}$$

For a weak base,

$$D = \frac{1 + 10^{\,pK_a - pH_1}}{1 + 10^{\,pK_a - pH_2}} \qquad \textbf{Equation 2.14}$$

where pH_1 and pH_2 are the pH of blood and the gastrointestinal fluid, respectively.

Brodie found an excellent correlation between the experimentally determined D and the mathematically determined D. However results from a large number of *in vitro* and *in vivo* experiments indicate that Brodies' theory (pH partition hypothesis) is only partly applicable to biological systems. [110] In many cases, the ionized as well as the unionized drug partition into, and is transported across the lipophilic membrane.

2.3.5.3: Alternative Approach to the pH -partition principle

An alternative physical model approach to the pH-partition theory was presented by Ho and Huguchi. This model describes the intestinal membrane as a barrier containing a lipoidal membrane interspersed with aqueous regions called aqueous pores. At the surface of this membrane is a stagnant aqueous diffusional barrier which could be rate limiting in certain conditions. When empirically verified, the model indicates that unionized species partitions into the lipoidal membrane and diffuses across whereas small ionized species and unionized species diffuse through the pores. [111, 112] Thus, this alternative approach differs from the original pH-partition theory which states that only the unionized species permeates through the intestinal barrier during the absorption process. Theoretically, a sigmoidal relationship should exist between the mathematical and experimental values of rate of absorption and drug lipophilicity. At low lipophilicity, the rate of absorption approaches a plateau with minimal rates of absorption due to diffusion through the aqueous pore pathway. At high lipophilicity the absorption rate assumes a plateau with maximal rate, and absorption in this region is limited by the rate of diffusion through the stagnant aqueous layer.

2.3.6: *In vitro* Methods to Study Intestinal Permeability

Assessment of absorption of a given drug from the gastrointestinal tract implies the determination of permeability of that drug, but there are more factors involved in the net process. Apart from permeation resulting in translocation there are factors that have a

negative influence on the absorption of the drug such as influence of the physiological components such as gastric juice and bile on the dissolution properties of the compound, precipitation at absorption site, chemical or bacterial degradation of the compound and metabolism of the drug in the lumen, at the brush border or in the intestinal wall. No *in vitro* method can assess all these aspects simultaneously with the exception of studies in intact animals or humans. Whole animal or human studies pose ethical difficulties and are unsuitable for screening large number of trial drug candidates. Certain *in vitro* methods such as brush border membrane vesicles and isolated intestinal segments are used to perform detailed studies on intestinal drug absorption.

2.3.6.1: Brush Border Membrane Vesicles

In this approach, cell homogenates or intestinal scrapings are treated with the calcium chloride precipitation method by centrifugation. The obtained pellet contains the luminal wall bound proteins and phospholipids which are responsible for the brush border enzymatic and carrier activity. Resuspension of the pellet into a buffer results in the formation of vesicles. These vesicles are mixed with permeant in buffer and filtered after a fixed time to determine the amount of permeant taken up by the vesicles. Since the precipitation-centrifugation procedure results in the isolation of only the brush border components only the apical transcellular transport can be measured by this system. [113] An interesting modification to this procedure was done by Pidgeon et al. who isolated brush border components and immobilized these components on a chromatographic system leading to the famous and well known Immobilized Artificial Membranes (IAM). The drugs under investigation are eluted with an aqueous eluent, thus enabling the

estimate of permeation capacity factors. Though this method is bound by limitations, it is more popular due to its fast and less complicated procedure. [113]

2.3.6.2: Isolated Intestinal Cells

The method of isolated intestinal cells can be divided fundamentally into to categories: an in situ procedure, in which the intestine is perfused with enzyme solutions that release the cells; and an ex vivo approach, in which the cells are treated by chelating agents or by enzymatic means. [113] The freshly isolated cells are immediately suspended in Krebs-Hanseleit buffer with 10mM glucose added and kept on ice for fifteen minutes, during which they are bubbled with carbogen (95% O_2, 5% CO_2). The exposure to glucose increases the viability of the cells even after the media has been replaced by glucose free media. During a typical experiment, the cells are separated from the primary buffer by centrifugation and resuspended in buffer under carbogen, in the presence of the permeant and shaken well. After a designated time, the cells are separated by gradient centrifugation or rapid filtration and extracted. The major drawback of this method is that it requires the permeant to be radio labeled.

2.3.6.3: Caco-2 Cell Model

The Caco-2 cell line was established by Dr. Jorgen Fogh (Memorial Slough-Kettering Cancer Center, NY) in 1974. [114, 115] However the original proposal of the Caco-2 model for intestinal absorption was proposed by Borchardt and coworkers in 1989.

The Caco-2 cells undergo spontaneous enterocytic differentiation in culture that starts when cell reach monolayer integrity (7 days) and is completed in 20 days. The cells form numerous domes spontaneously after reaching confluence. This is consistent with their ability to undertake transepithelial transionic transport. Except for the mucous layer the Caco-2 cells caontain the major permeability barriers (aqueous boundary layer, cell membranes and intercellular junctions between the cells) to drug absorption. The ability of Caco-2 cells to achieve a high degree of enterocytic differentiation than other cell lines and their spontaneous dome formation makes this cell line the most relevant *in vitro* system investigating transport processes associated with intestinal cells. Many researchers have demonstrated that Caco-2 cell monolayers can be successfully grown on microporous filters. The cells are grown in a similar way as the Madin-Darby canine kidney cells where the apical and the basal fluids are separated, which helps to carry out transport studies of drug molecules from one fluid to the other. The presence of collagen matrix on the basement (polycarbonate) membrane promotes cell growth and attachment. Therefore when cell epithelia are in direct contact with the basement membrane, cell matrix interactions are important for the regulation of cell polarity. Hilgers et al. studied the effect of collagen presence on the confluency of the Caco-2 cell monolayers and found that collagen matrix is unnecessary for the establishment of monolayers. Further they also found that collagen promotes the migration of cells through the filter during the early stages of the culture resulting in the depolarization of the Caco-cell model. [116]

2.3.6.3.1: Growth and Morphology

The Caco-2 cells develop a typically enterocytic morphology as seen in villous cells and also posses the polarity of a number of brush border enzymes. As per the experimental growth conditions, these properties are fully expressed within 15-20 days in culture. By the fourth day after seeding on uncoated polycarbonate filters (pore size 3μ), patchy clusters of actively dividing cells are seen with tight junctions between adjacent cells. The cells appear squamous in shape, containing many endoplasmic reticulum and mitochondria and no glycogen. At this stage very few immature microvilli can be seen on the apical side. By day 7 the cells reach confluence, their shape changing from squamous to cuboidal and establish desmosomes. At day 10 mature microvilli develop and large vacuoles containing unknown material are present. By the fourteenth day the cells become columnus in shape and the nucleus can be seen eccentrically located in the basolateral border of the cell. Glycogen is the major component of the cytoplasm between the nucleus and the apical brush border. Intercellular lumens expressing microvilli are seen running parallel to the polycarbonate filter. After 16 days it is seen that the Caco-2 cell monolayers are about 25-30μ in height and posses a morphology similar to that observed in the simple columnar epithelia of the small intestine. After day 21 small amounts of cytoplasm are seen extending into the filter and these keep on increasing through 28 days, but no microvilli are seen in the receiver chamber of the cell. Fifteen day old Caco-2 cell cultures have an average height of 25μ, an average width of 6μ and the tight junction between two adjacent cells has an average length of 0.1μ.

2.3.6.3.2: Cell Polarity

The degree of functional polarity achieved by the cells is determined by the specific activity and the ultrastructural distribution of the enzyme alkaline phosphatase in the brush border membrane. In normal intestinal epithelial cells this enzyme is highly polarized and located exclusively on the apical brush border membrane.

Caco-2 cells express sucrase-isomaltase, lactase, aminopeptidase, dipeptidyl aminopeptidase, γ-glutamyl transpeptidase, all brush border enzymes that are differentiation-specific markers for small intestinal enterocytes. Cytosolic phase II enzymes such as glutathione S-transferase, Sulfotransferase, and glucuronidase as well as microsomal cytochrome P450 are expressed in Caco-2 cells. [116, 117]

2.3.6.3.3: Integrity of the Monolayer

Monolayer integrity, meaning the ability of the cell membrane to act as a barrier to let/let not certain molecules through can be determined by a certain characteristic molecules known as permeability markers. The inability of horseradish (Mol. Wt 40,000 g/mole) to cross the Caco-2 cell tight junctions demonstrates that this *in vitro* permeability system is very much similar to the characteristics of the small intestine *in vivo*. Minimal leakage (<0.25%/hour) is observed for Lucifer Yellow CH (Mol. Wt. 453 g/mole), Polyehtylene glycol (Mol. Wt. 4000 g/mole), Inulin (Mol. Wt. 5000 g/mole), Dextran (Mo. Wt. 70,000 g/mole). One of the most common permeability marker is Mannitol (Mol. Wt. 180 g/mole) which is exclusively transported by the paracellular pathway and its uptake by the cell membrane is very low.

Transepithelial electrical resistance (TEER), which is the measurement of electrical resistance across the cellular monolayer, is also a very sensitive and reliable method to check the monolayer for its integrity and permeability. This measurement reflects the resistance across the cell tight junctions and not the cell membrane. An average value between 150-400Ω-cm^2 has been reported from different laboratories, indicating that under different culture conditions Caco-2 cell monolayers can demonstrate electrical properties of the small intestinal or colonic enterocytes. In one study the TEER measurements for 103 monolayers was compared and was found to be between 220-380 Ω-cm^2. TEER values of 700-900 Ω-cm^2 have been reported for Caco-2cell monolayers by Augustijns et al. It seems that high TEER values were related to the passage number of the cells as cells with passage number between 50-100 were found to have a TEER measurements between 260-900 Ω-cm^2 . Cells with a low passage number, in the range of 19-35, were found to have TEER values between 170-250 Ω-cm^2. Although these high TEER values dependent on passage number were significantly higher than what would be expected for the human small intestine. [113, 116, 117]

2.3.6.3.4: Transport and Permeability Properties

Several studies have been carried out to determine the suitability of the Caco-2 cell line to carry specific carriers that are characteristic of the small intestine. A number of specific transport systems that are capable of carrying nutrients and macromolecules such as folates, sugars, dipeptides, and cephalosporins have been seen to be functional in the Caco-2 cell system. It has been reported that the properties of apical H+/dipeptide cotransport in the Caco-2 cell system closely resembles to that of the small intestine. The

systems involved in the carrier mediated transport of bile acids and large neutral amino acids show many of the basic properties (specificity, saturability, competitiveness, unidirectionality) shown by the small intestine *in vivo*. [113, 116, 117]

One of the major advantages of the Caco-2 cell model is its application to high throughput screening (HTS) strategies in large numbers due to its simple and highly reproducible culturing techniques. Rubas et al. compared the permeabilities of a series of compounds with poor intrinsic permeabilitiy (eg. gamma interferon) in both Caco-2 cells and human large intestinal tissue and found that the cells well reflect permeability characteristics of the large intestine. This implies that flux measurements across Caco-2 monolayers may be predictive for permeabilities of the human colon and rectum. [113, 117, 118]

2.3.6.4: In vivo and In Situ Techniques

Most of the *in vivo* and in situ models for drug absorption are based in animals. Physiological conditions are maintained in both these models and the intestine maintains a constant blood supply from its own systemic circulation.

The *in situ* rat gut technique is a small closed loop system in which the drug solution is perfused through a segment of the intestine and the disappearance of the drug is measured as a function of time. The method is simple, economical, fast and perfusion studies can be performed using easily available apparatus. Data obtained from a single animal can be subjected to complete kinetic analysis and physiological variability from animal to animal is minimum. Therefore this method is used for studying drug absorption *in vivo*.

Schanker et al. and Kakemi and others have studied the dynamics of absorption using single pass perfusion. In single pass experiments drug solution is perfused through the gut and the entering and leaving concentrations are determined. It is assumed that the absorption process follows first order kinetics and the decrease in drug concentration is followed. The disadvantage of this technique is that the volume of the perfusion fluid is larger than the volume of the gut and hence at any instant of time, only a small fraction to of the perfusion fluid is exposed to the absorption site. The through and through perfusion technique developed by Ho et al. lays emphasis on the volume of the boundary layer (which can be rate limiting for highly permeable drugs) as compared to the in situ rat gut technique. Perfusion studies in humans are carried out using multiple lumen tubes for intubation. In these studies mostly open or semi open perfusion systems were used, and these methods were hampered by the fact that conditions at the absorption site could not be controlled due to the extensive penetration of the proximal/distal luminal contents. The technique also required higher perfusion flow rates than normal jejunal flow of 0.6-4.2 ml/min. A new perfusion instrument developed by Lennernas consists of multi channel tubes with two inflatable balloons. A 10 cm long segment is created between these balloons enabling perfusion of a defined and closed part of the jejunum. The technique was first tested for antipyrine and a good correlation was observed between the absorption from the intestinal perfusate and the absorption obtained by deconvulation of plasma concentrations. [113, 116, 117, 119]

2.3.6.5: Simulated Biological Dissolution and Absorption System (SimBioDAS®)

SimBioDAS® was developed by Kinetana Inc. and is an epithelial cell based *in vitro* permeability assay which can be used to predict absorption. [120] SimBioDAS® is an acronym for Simulated Biological Dissolution and Absorption System, the patent for which was issued in 2000. Although SimBioDAS® has not been published in the peer reviewed literature, the inventors have made presentations at various scientific conferences. [121, 122] The SimBioDAS® assay has two key components: a non-human cell-line and a computer model that "scales" the *in vitro* cell permeability to absorption along the intestine *in vivo*. To determine the absorption and permeability of a compound, a solution of a botanical extract is applied to the donor side and the absorbable components appear on the receiver side. Absorption is estimated by scaling the *in vitro* permeability with the use of a seven-compartment model. Permeability is estimated on the basis of the rate at which the compound is transported across the cell membrane. Kinetana has compared the performance of SimBioDAS® with that of Caco-2 cell monolayers in estimating the absorption of 35 commercially available substances, including ginkgo (*Ginkgo biloba* L., Ginkgoaceae) extracts. [122]

The schematic set up for SimBioDAS® is very similar to the Caco-2 cell set up which involves the Transwell® chamber with a polycarbonate filter membrane. The drug under investigation is applied in the upper, apical chamber and the amount of drug transported to the basal chamber, through the cell monolayer is accounted for. The cell monolayer which is non human in origin is cultured over the polycarbonate membrane and its integrity is tested by trans-epithelial electrical resistance (TEER). The monolayers of SimBioDAS® achieve differentiation within three days. During transport

studies, integrity of the cells is monitored by TEER and by using Lucifer Yellow as the permeability marker.

Three day SimBioDAS® has been directly compared to 21 day Caco-2 cells on 35 validation compounds shown in Table 2.7.

Table 2.7: List of Model Drugs for Comparison of SimBioDAS® and Caco-2 permeability

Drug	Human F_a	Mol. Wt.	Drug	Human F_a	Mol. Wt.
Testosterone	1	288	Furosemide	0.61	331
Piroxicam	1	331	Chlorothiazide	0.56	296
Glucose	1	180	Ranitidine	0.56	314
Verapamil	1	455	Atenolol	0.55	266
Naproxen	0.99	230	Metformin	0.51	129
Cefalexin	0.99	347	Methotrexate	0.51	454
Phenazone	0.97	188	Sulpiride	0.35	341
Theophylline	0.96	180	Erythromycin	0.35	734
Glibenclamide	0.95	494	Nadolol	0.34	309
Metoprolol	0.95	267	Benzylpenicillin	0.3	334
Ketoprofen	0.92	254	Acyclovir	0.215	225
Propranolol	0.9	257	Foscarnet Na	0.171	192
Saccharin	0.85	183	Mannitol	0.159	182
Gabapentin	0.74	171	Gancyclovir	0.03	255
Terbutaline	0.73	225	Cefuroxime	0.01	424
Amoxicillin	0.72	365	Raffinose	0.003	504
Hydrochlorothiazide	0.67	298	Doxorubicin	0	544
Cimetidine	0.64	252			

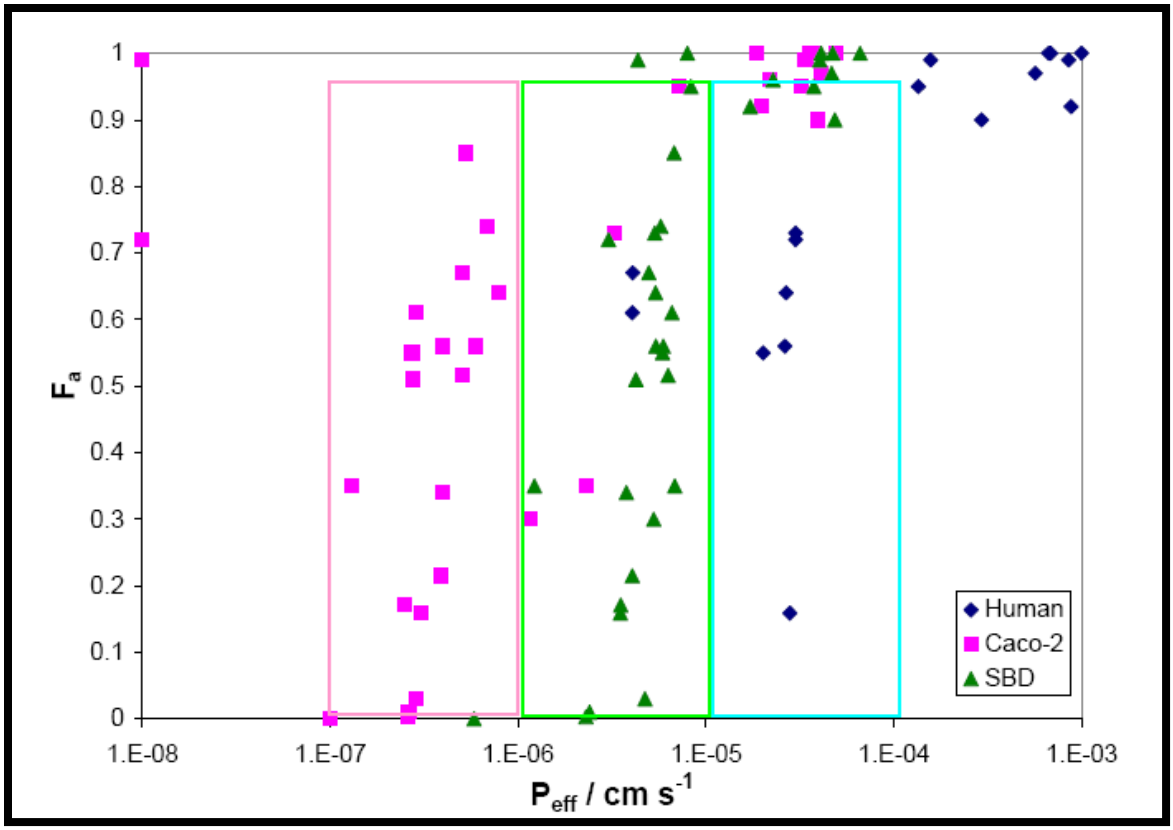


Figure 2.4: Human *in vivo* Fa versus P_{eff} of 35 model drugs in SimBioDAS® and Caco-2 monolayers.

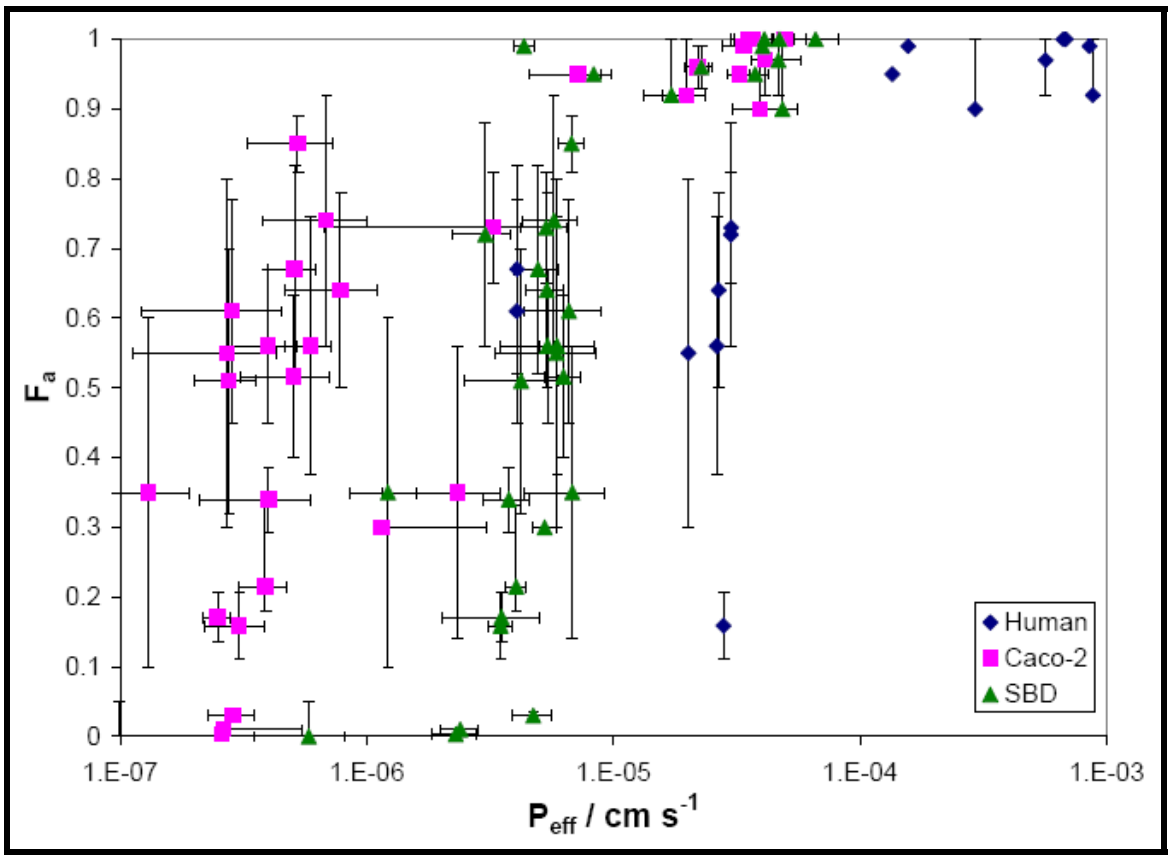


Figure 2.5: Human *in vivo* F_a versus P_{eff} of 35 model drugs in SimBioDAS® and Caco-2 monolayers with error bars (SD) for F_a and permeability

Figure 2.4 is a plot of human *in vivo* Fa versus the permeability by SimBioDAS® and Caco-2 cell monolayers for the 35 model drugs used during the comparison process. Figure 2.5 shows the same plot with error bars (SD) for the 35 model drugs. It can be seen that SimBioDAS® and Caco-2 cell permeabilities for highly permeable drugs absorbed via transcellular diffusion require only a cell membrane surface area correction (roughly 10 fold) to correlate to published human *in vivo* data. Drugs with moderate to

low Fa show less scatter with SimBioDAS® than with Caco-2. Also the magnitude of variation in permeability values observed for SimBioDAS®, Caco-2 cells and human Fa is similar and the application of a certain area correction helps in the correlation of the three properties with each other. None of the orally administered drugs tested with SimBioDAS® were found to be outliers. SimBioDAS® requires an approximate 10 fold area correction for high, moderate and poorly permeable drugs, to correlate to the published human *in vivo* data, where as Caco-2 cell permeability for moderate to poorly permeable drugs require an approximate 100 fold area correction, probably due to Caco-2's too tight junctions. Thus, the Caco-2 data may lead to an under estimation of the paracellular diffusion values of these drugs and thus an under estimation of the total passive diffusion. SimBioDAS® and Caco-2 cell permeability values for well absorbed drugs are the same.

2.3.7: Estimation of CaCo-2 cell Permeability of Silymarin Isomers (Silybin A&B)

CaCo-2 cell monolayers have been widely accepted as an *in vitro* human permeability surrogate and as a high through put permeability screening tool for new drug candidates. This section describes the CaCo-2 cell transport studies for silymarin isomers Silybin A and Silybin B. CaCo-2 cell permeability experiments were carried out only for the diastereomers due to solubility limitations and also due to non availability of pure reference standards for each of the isomers in silymarin.

2.3.7.1: Materials and Reagents

The CaCo-2 cell line used in the transport experiments was obtained from Cedra Corporation, Austin, Texas. Transwell® cell culture chambers (24.5mm diameter, 3.0 μm pore size, 4.71 cm2 growth area), six well cluster plates, and cell culture flasks (T-25, T-75 and T-162) were obtained from Corning Costar Corporation (Cambridge, MA). Dulbecco's Modified Eagle Medium (DMEM, 10x, powder) with 4.5g/liter D-glucose and 584 mg/liter L-glutamine was obtained from Sigma Aldrich (St. Louis, MO). Phosphate Buffer Saline with/without calcium and magnesium (10x solution) were obtained from Sigma Aldrich (St. Louis, MO). Non essential amino acids solution (NEAA, 100x), sodium pyruvate soloution (100mM), and HEPES (hydroxyethylpiperazine ethane sulfonic acid) were obtained from Gibco-BRL Life Technologies Inc (MD). Fetal Bovine Serum (FBS, certified) was obtained from Atlanta Biologicals (Norcross, GA). Trypsin-EDTA and D-Glucose were obtained from Sigma Aldrich. All other reagents used were of analytical grade unless otherwise specified.

2.3.7.2: Preparation of Growth Medium

CaCo-2 cells were grown in a medium containing 90% DMEM, 10% FBS, 1% NEAA, and 1% sodium pyruvate. 10x DMEM powder was dissolved in 90% of the final required volume of sterile distilled water, at room temperature (15-20°C). The growth medium was sterilized by passing through a 0.22μm membrane. All procedures were carried out under sterile conditions under a laminar flow hood and precautions were taken

to prevent any contamination. The media was stored in sterile bottles at 4°C and warmed to 37°C in a water bath prior to use.

2.3.7.3: Growing Caco-2 Cells

Caco-2 cells grow in monolayers and domes arise when the cells become confluent. Caco-2 cells were grown and expanded using the prepared growth medium in a carbon dioxide incubator maintained at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The cells were grown in a 162cm^2 T-flask and the medium was changed every other day for the first seven days and then once a day. 95% of the cells are known to be confluent by the end of seven days. The cells were then passaged.

For passaging the cells the media was removed gently by suction and then the cells were rinsed with phosphate buffered saline (PBS without Ca^{++} and Mg^{++}) solution to remove any remaining medium. The PBS was removed by suction and the cells were treated with 10ml of EDTA in Ca^{++}-Mg^{++} free PBS until the cells started to move apart from each other (~5-10 minutes). They were then incubated with 4 ml trypsin for 1-2 minutes. At this time the cells started to detach from each other and from the substratum. Addition of 10ml of DMEM to the flask inhibited the action of trypsin. The cells were then transferred to a centrifuge tube and spun at 80g (<1000 rpm) for 10 minutes. The supernatant solution was suctioned off and the cell pellet was resuspended in fresh medium. The cells were brought into ta single cell suspension by drawing them up and down in a pipette.

A 10µL aliquot of the single cell suspension was then transferred to each side of the hemocytometer using a pipettor fitted with a sterile pipette tip. The cells in one

square (or a total of at least 100 cells in one or more squares) were counted on each side of the hemocytometer and the average number of cells in each square was estimated. Each side of the hemocytometer with the cover slip in place represents a total volume of 10^{-4} cm^3. The number of cells per mL was estimated using this relationship. The cells were then seeded into a Transwell® cup (for transport experiments) or into another 162cm^2 T-flask for the continuation for the cell line. The required density for seeding a 4.71 cm^2 Transwell® chamber was 63000 cells per cm^2 and the required density for inoculation a T-162 flask was 2 x 10^5 cells per mL.

In the Transwell® chamber, 1.5 ml of the cell suspension was added to the apical chamber and 2.5 ml of medium was added to the basal chamber to promote optimal growth of cells. The Transwell® chambers were then labeled and incubated at 37°C, 95% relative humidity and 5% CO_2.

25 ml of the diluted cell suspension was added to a T-162 flask, labeled and incubated under the same conditions as mentioned above. The filtered vented flask allowed the equilibration of air within the flask with the atmosphere of the incubator.

The Transwell® system used in the transport studies consists of microporous cell culture inserts designed to fit the cell culture clusters. A schematic representation of the Transwell® system is shown in Figure 2.5.

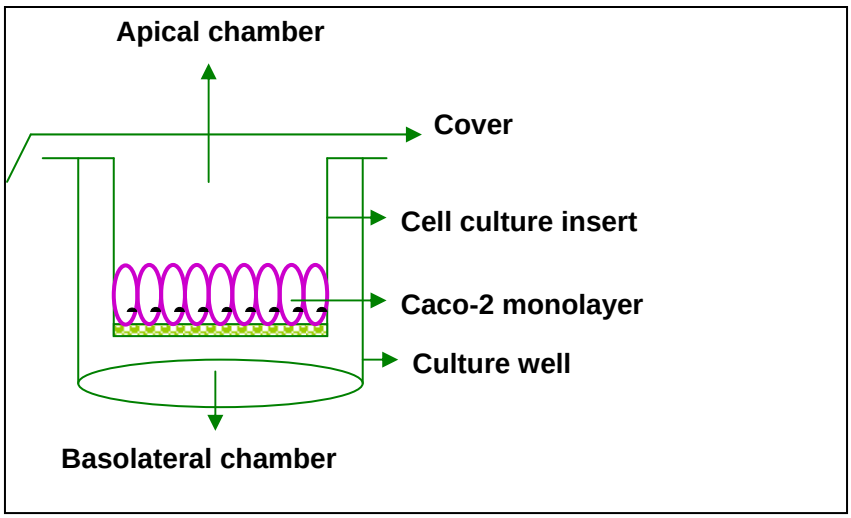


Figure 2.6. Schematic of a Transwell® System

The insert hangs from top of the well providing independent access to both the apical and the basolateral membrane of the cell. Every insert has openings for pipette tips so the media and samples can be added and removed during experiments. The thin microporous polycarbonate membrane present in the inserts provides permeable growth support for the cells. Transwell® inserts used for permeability experiments were sterile with a pore size of 3.0μ and a diameter of 24mm resulting in a growth area of 4.71 cm^2.

2.3.7.4: Assessment of Monolayer Integrity

The integrity of the Caco-2 cell monolayers was determined by measuring the trans-epithelial electrical resistance (TEER). The passage number of cells was another critical factor that was controlled during transport experiments, since repeated passaging

can lead to singling of the cells into a sub population that can have characteristics different from the parent population.

The TEER of the Caco-2 cell monolayers was measured prior to starting the transport experiments for each Transwell® chamber. A Transwell® cup without a monolayer, and filled with DMEM was used as a blank to eliminate any background signal. A pulsating current of 3μA was through two electrodes filled with 3M potassium chloride and 3% agar using a DVC 1000 voltage/current clamp. The zero adjustment was done so as to eliminate the background noise caused by the electrical system and the polycarbonate membrane. Then a Transwell® cup with the monolayer was placed and a pulsating current of 50μA was passed, and the corresponding voltage recorded. The corresponding epithelial resistance was calculated by multiplying the voltage change with the surface area (4.71cm^2) and then applying Ohms Law.

2.3.7.5: Preparation of Transport Buffer

The transport buffer was composed of Dulbecco's Phosphate Buffered Saline (D-PBS), HEPES (hydroxyethylpiperazine ethane sulfonic acid) and glucose. 10ml of 10x D-PBS in 1 liter sterile water was mixed with 15 mmol (3.575 g/liter) of HEPES and the pH was adjusted to 7.2 with 1N NaOH, giving a buffer with pH control from 6.8 to 8.2. Finally 1g/liter D-Glucose was dissolved into the buffer. Due to the high concentration of glucose in the solution, and its susceptibility to microbial contamination the buffer solution was prepared fresh prior to each experiment and stored in the refrigerator up to seven days.

2.3.7.6: Transport Experiments

Fifteen day old monolayer of Caco-2 cells grown in Transwell® chambers was used during the transport experiments. The transport of silymarin isomers, Silybin A and Silybin B was determined from the apical to the basal side at pH 7.2. Silybin solution for the transport experiments were prepared by dissolving the appropriate amount of Silybin A+B in transport buffer. Due to the limited solubility of Silybin isomers, only one concentration, of each isomer could be tested for transport experiments. The concentration of Silybin A was 32µg/mL and concentration of Silybin B was 34.31µg/mL. Prior to the experiment the growth medium was suctioned off from both the chambers and the monolayers were washed thrice with pH 7.2 transport buffer. The monolayers were then incubated with 1.5ml of transport buffer (pH 7.2) in the apical chamber and 2.5ml of transport buffer in the basal chamber for 15 minutes. Transport experiments were conducted on triplicate at 37°C in an atmosphere of 5%CO2 and 95% relative humidity. Sampling intervals for the experiment were set according to the chemical structure, lipophilicity and available pKa data of the isomers. The sampling intervals were also approximated accordingly so that not more than 10% of the drug went across the monolayers between each sampling interval. Thus, the transport experiment was carried out under sink conditions and the amount of drug diffusing back from the basal to the apical side was minimized. The sampling time intervals were 5, 10, 20, 30, 45, 70, 90, 120, 150 minutes. The amount and reason for drug loss due to metabolism, precipitation or accumulation of solute, or adsorption to apparatus was accounted for by mass balance calculations. Adsorption of drug to the Transwell® cups was also checked prior to the transport experiments, by incubating the Transwell® cups (without

monolayers) with the drug solution for 3 hours and calculating the amount of drug adsorbed, if any. Amount of drug accumulated into the cells was determined by treating the monolayers with a cell solubilizing agent (Triton-X®) and analyzing for drug content.

2.3.7.7: Quantitative Determination of Silybin A and Silybin B

The amount of Silybin A and Silybin B transported from the apical side to the basal side was determined by high performance liquid chromatography using ultraviolet detection. The complete procedure has been described in detail in the Analytical Methodology section of this dissertation.

2.3.7.8: Calculation of Apparent Permeability Coefficients

The percent mass transferred to the receiver side during each time interval was calculated using equation 2.14.

$$\text{Percent Mass Transferred} = \frac{[R]_t}{[D]_0 - [R_{cum}]_{t-\Delta t}} \qquad \textbf{Equation 2.14}$$

where, $[R]_t$ is the amount in the receiver compartment at time t, $[D]_0$ is the amount in the donor compartment at time zero, i.e. the initial amount in the donor compartment. $[Rcum]_{t-\Delta t}$ is the cumulative amount transferred until the beginning of that time interval and, Δt is the length of the time interval.

During the transport experiments the concentration in the donor compartment decreases continuously as a function of time. This is illustrated in Figure 2.7.

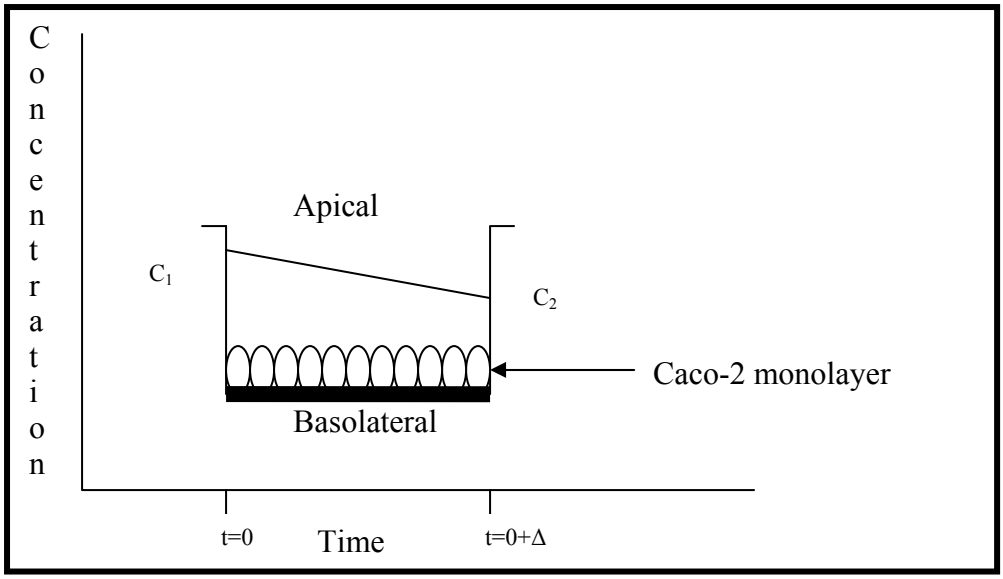


Figure 2.7. Schematic diagram illustrating the continuous decrease in donor concentration as function of time.

Since permeability across the cell monolayer remains constant, equation 2.15 suggests that decreasing concentration would result in a decrease in the flux over a period of time.

$$J = P.C$$ **Equation 2.15**

Suppose at the beginning of a time interval the amount of drug in the donor compartment is $[D]_0 - [Rcum]_{t-\Delta t}$ then at the end of the time interval, the amount of drug in the donor compartment would be $[D]_0 - [Rcum]_{t-\Delta t} - [R]_t$ It was therefore assumed that the amount of drug in the donor compartment throughout the time interval remained uniform at $[D]_0 - [R_{cum}]_{t-\Delta t} - [R]_t/2$

The percent mass transferred at each time interval was then normalized to account for the loss of drug from the donor compartment during each time interval using equation 2.16:

$$Normalized\_Percent\ Mass\_Transferred = \frac{[R]_t}{[D]_0 - [R_{cum}]_{t-\Delta t} - \frac{[R]_t}{2}} x100 \qquad \textbf{Equation 2.16}$$

The average normalized percent mass transferred during each time interval was calculated, excluding the first and the last data point to increase the precision of estimation. The percent mass transported per unit time ($\Delta\%/\Delta t$) was estimated by simply dividing the normalized percent mass transferred by the duration of the time interval. The effective permeability coefficients were then calculated using equation 3.4:

$$Pe = \frac{Vd}{A} \cdot \frac{\Delta\%}{\Delta t} \qquad \textbf{Equation 2.17}$$

Where P_e is the effective permeability coefficient measured in cm/sec, V_d is the volume of the donor compartment (1.5ml) during the apical to basolateral transport experiments, A is the surface area of the monolayer (4.71cm2) and $\Delta\%/\Delta t$, which has been previously defined.

Finally the mass balance can be estimated using equation 2.18:

$$\text{Mass Balance}\,(\%) = \frac{[R]_{cum} + [D]}{[D]_0} \qquad \textbf{Equation 2.18}$$

Where, $[R]_{cum}$ is the cumulative amount transported to the receiver compartment, D is the amount of drug remaining in the donor compartment, D_0 is as previously defined.

2.3.7.9: Results

Integrity of the Monolayers: The trans-epithelial electrical resistance (TEER) reflects the resistance across the tight junctions of the cell. The TEER for the Caco-2 monolayers was consistently between 320-380 ohms-cm2 indicating that the monolayers used in the transport experiments did not have any leaks or have any imperfectly formed occluding junctions or holes. Monolayers having values of TEER above 300ohms-cm2 are usually considered fit for transport studies, though the value may vary considerably with the increase in passage numbers.

Permeability of Silybin A and Silybin B: The effective permeability coefficients of Silybin A and Silybin B were measured in the apical to basal direction at pH 7.2. The transport of the silymarin isomers was found to be linear over the time intervals studied. The results of the permeability experiments for Silybin A and Silybin B are shown in Table 2.8.

Table 2.8: Effective Permeability of Silybin A and Silybin B

Compound	Conc. (µg/mL)	P_{eff} x 10^{-6} (cm/sec)		Mean P_{eff} x 10^{-6} cm/sec (± SD)	Log P_{eff}
		n_1	n_2		
Silybin A	32.00	1.67	1.52	1.60 ±0.106	-5.795
Silybin B	34.31	2.53	2.70	2.62 ±0.119	-5.581

P_{eff}= Effective Permeability, Conc. = Concentration, SD= Standard Deviation

2.3.8: Estimation of *In vitro* Permeability using SimBioDAS®

In vitro cell permeability values for the active ingredients of the selected herbal extracts were obtained in collaboration with Kinetana Inc. [120]

2.3.8.1: Results

Permeability results obtained using SimBioDAS® are shown in Table 2.9-2.13.

Table 2.9: SimBioDAS® Permeability Results for Kava Compounds.

Compound	Mol. Wt.	P_{eff} x10^{-6} (cm/sec)	SD x10^{-6} (cm/sec)
Desmethoxy-yangonin	228.24	21.66	1.03
Dihydrokawain	232.28	19.33	0.81
Dihydromethysticin	276.28	19.33	0.81
Kawain	230.26	20	1.09
Methysticin	274.27	20.33	0.81
Yangonin	258.27	65.66	16.84

P_{eff}= Effective Permeability, SD= Standard Deviation

Table 2.10: SimBioDAS® Permeability Results for Milk Thistle Compounds.

Compound	Mol.Wt	P_{eff} x10^{-6}(cm/sec)	SD. x10^{-6} (cm/sec)
Isosilybin A	482.44	N/A	N/A
Isosilybin B	482.44	N/A	N/A
Silybin A	482.44	1.88	1.02
Silybin B	482.44	1.86	0.99
Silycristin	482.44	1.31	0.80
Silydianin	482.44	N/A	N/A
Taxifolin	304.25	2.20	0.63

Table 2.11: SimBioDAS® Permeability Results for Ginkgo biloba Compounds.

Compound	Mol. Wt.	P_{eff} x10^{-6} (cm/sec)	SD x 10^{-6} (cm/sec)
Bilobalide	326.30	3.89	0.13
Ginkgolide A	408.40	6.20	0.26
Ginkgolide B	424.40	3.921	0.17
Ginkgolide C	440.40	4.25	0.24
Ginkgolide J	424.40	3.19	0.51
Isorhamnetin	316.26	N/A	N/A
Kaempferol	286.24	0.83	0.07
Quercetin	302.24	0.76	0.10

P_{eff}= Effective Permeability, SD= Standard Deviation

Table 2.12: SimBioDAS® Permeability Results for Ginseng Compounds.

Compound	Mol. Wt.	P_{eff} x10^{-6} (cm/sec)	SD x10^{-6} (cm/sec)
Ginsenoside Rb1	1109.29	0.16	0.12
Ginsenoside Rb2	1079.27	N/A	N/A
Ginsenoside Rc	1079.27	0.28	0.25
Ginsenoside Rd	947.15	0.20	0.06
Ginsenoside Re	947.15	0.99	0.37
Ginsenoside Rf	801.01	N/A	N/A
Ginsenoside Rg1	801.01	2.03	0.92
Ginsenoside Rg2	785.01	N/A	N/A

P_{eff}= Effective Permeability, SD= Standard Deviation

Table 2.13: SimBioDAS® Permeability Results for Black cohosh, Echinaceae, Garlic & Valerian Compounds.

Herb	Compound	Mol. Wt.	P_{eff} x10^{-6} (cm/sec)	SD x10^{-6} (cm/sec)
Black Cohosh	26-deoxyactein	660.83	11.067	2.551
	26-deoxyacteol	600.78	5.267	1.457
	Actein	676.83	2.883	0.366
Echinaceae	Caftaric acid	312.23	9.750	0.683
	Chicoric acid	474.37	11.500	0.837
Garlic	Alliin	177.22	1.333	0.308
	Deoxy-alliin	161.22	2.400	0.456
Valerian	Hydroxy-valerenic acid	250.33	5.800	1.006
	Valerenic acid	234.33	39.667	10.328

P_{eff}= Effective Permeability, SD= Standard Deviation

2.4: CORRELATION BETWEEN *IN SILICO* DESCRIPTORS AND SIMBIODAS® *IN VITRO* PERMEABILITY

This section attempts to establish a link between *in silico* descriptors and the *in vitro* permeability, the discussion of which leads to the selection of a meaningful bioavailability and performance marker. Tables 2.14-2.18 lists the *in silico* descriptors and the *in vitro* permeability for 39 actives from the various herbal extracts studied.

Table 2.14: *In Silico* & Permeability Data for Kava Compounds.

Compound	Mol. Wt.	CLogP	PSA ($Å^2$)	MCSA ($Å^2$)	P_{eff} x10^{-6} (cm/sec)	SD x10^{-6} (cm/sec)
Desmethoxy-yangonin	228.24	2.79	89.232	92.250	21.66	1.03
Dihydrokawain	232.28	2.15	83.790	107.726	19.33	0.81
Dihydromethysticin	276.28	1.71	136.049	113.722	19.33	0.81
Kawain	230.26	2.04	92.568	106.461	20.0	1.09
Methysticin	274.27	1.61	145.773	105.66	20.33	0.81
Yangonin	258.27	2.71	108.388	97.242	65.66	16.84

Table 2.15: *In Silico* & Permeability Data for silymarin Compounds.

Compound	Mol.Wt	CLogP	PSA($Å^2$)	MCSA ($Å^2$)	P_{eff} x10^{-6} (cm/sec)	SDx10^{-6} (cm/sec)
Isosilybin A	482.44	1.94	350.239	123.707	N/A	N/A
Isosilybin B	482.44	1.94	350.593	133.146	N/A	N/A
Silybin A	482.44	1.95	349.809	146.32	1.88	1.02
Silybin B	482.44	1.94	348.589	125.89	1.86	0.98
Silycristin	482.44	1.38	410.998	131.682	1.31	0.80
Silydianin	482.44	-0.39	368.061	157.108	N/A	N/A
Taxifolin	304.25	1.02	347.423	101.285	2.20	0.63

MCSA=Minimal Cross sectional Area, PSA=Polar Surface Area, P_{eff}= Effective Permeability, SD= Standard Deviation

Table 2.16: *In Silico* & Permeability Data for Ginkgo biloba Compounds.

Compound	Mol. Wt.	CLog P	PSA ($Å^2$)	MCSA ($Å^2$)	P_{eff} x10^{-6} (cm/sec)	SD x10^{-6} (cm/sec)
Bilobalide	326.30	-2.64	271.405	139.435	3.89	0.13
Ginkgolide A	408.40	-1.55	283.058	128.28	6.20	0.26
Ginkgolide B	424.40	-1.45	313.477	132.547	3.92	0.16
Ginkgolide C	440.40	-2.48	309.311	134.402	4.25	0.24
Ginkgolide J	424.40	-2.58	326.152	134.414	3.19	0.51
Isorhamnetin	316.26	1.75	282.536	109.498	N/A	N/A
Kaempferol	286.24	1.90	307.353	96.256	0.83	0.07
Quercetin	302.24	1.30	353.742	111.565	0.76	0.10

Table 2.17: *In Silico* & Permeability Data for Ginsenosides (Ginseng).

Compound	Mol. Wt.	CLogP	PSA ($Å^2$)	MCSA ($Å^2$)	P_{eff} x10^{-6} (cm/sec)	SD x10^{-6} (cm/sec)
Ginsenoside Rb1	1109.29	4.544	647.745	224.914	0.16	0.12
Ginsenoside Rb2	1079.27	5.232	603.788	232.126	N/A	N/A
Ginsenoside Rc	1079.27	4.299	622.799	282.806	0.28	0.25
Ginsenoside Rd	947.15	5.726	556.208	273.938	0.19	0.06
Ginsenoside Re	947.15	3.869	623.308	295.768	0.98	0.37
Ginsenoside Rf	801.01	4.726	417.294	209.152	N/A	N/A
Ginsenoside Rg1	801.01	4.932	438.223	245.672	2.03	0.92
Ginsenoside Rg2	785.01	4.758	350.105	209.695	N/A	N/A

MCSA=Minimal Cross sectional Area, PSA=Polar Surface Area, P_{eff}= Effective Permeability, SD= Standard Deviation

Table 2.18: *In Silico* & Permeability Data for Black Cohosh, Echinaceae, Garlic and Valerian

Herb	Compound	Mol. Wt.	CLog P	PSA ($Å^2$)	MCSA ($Å^2$)	P_{eff} x10^{-6} (cm/sec)	SD x10^{-6} (cm/sec)
Black cohosh	26-deoxyactein	660.83	6.124	186.216	152.839	11.067	2.551
	26-deoxyacteol	600.78	6.781	184.475	164.142	5.267	1.457
	Actein	676.83	5.419	269.289	182.592	2.883	0.366
Echina-ceae	Caftaric acid	312.23	-1.540	420.790	104.414	9.750	0.683
	Chicoric acid	474.37	0.139	516.139	167.085	11.500	0.837
Garlic	Alliin	177.22	-2.708	215.180	83.821	1.333	0.308
	Deoxy-alliin	161.22	-1.198	202.245	83.847	2.400	0.456
Valerian	Hydroxy-valerenic acid	250.33	2.650	172.723	116.400	5.800	1.006
	Valerenic acid	234.33	4.737	117.788	124.200	39.667	10.328

MCSA=Minimal Cross sectional Area, PSA=Polar Surface Area, P_{eff}= Effective Permeability, SD= Standard Deviation

The CLogP for the actives ranged from -2.70 for alliin to +6.7 for the highly lipophilic acteols from black cohosh. The Ginsenosides are found to be the largest molecules in terms of size and though being highly lipophilic, have low permeability mainly due to their size. (Figure 2.8, 2.9) The kava lactones from kava are found to be the most lipophilic followed by the flavonolignans from milk thistle, and the flavonol aglycones from ginkgo biloba. Though flavones are present as glycosides in ginkgo biloba, they are known to be hydrolyzed to aglycones (quercetin, kaempferol and

isorhamnetin) after oral administration of ginkgo biloba. Hence, the aglycones are taken into consideration when studying permeability. The terpene trilactones from ginkgo are found to be least lipophilic.

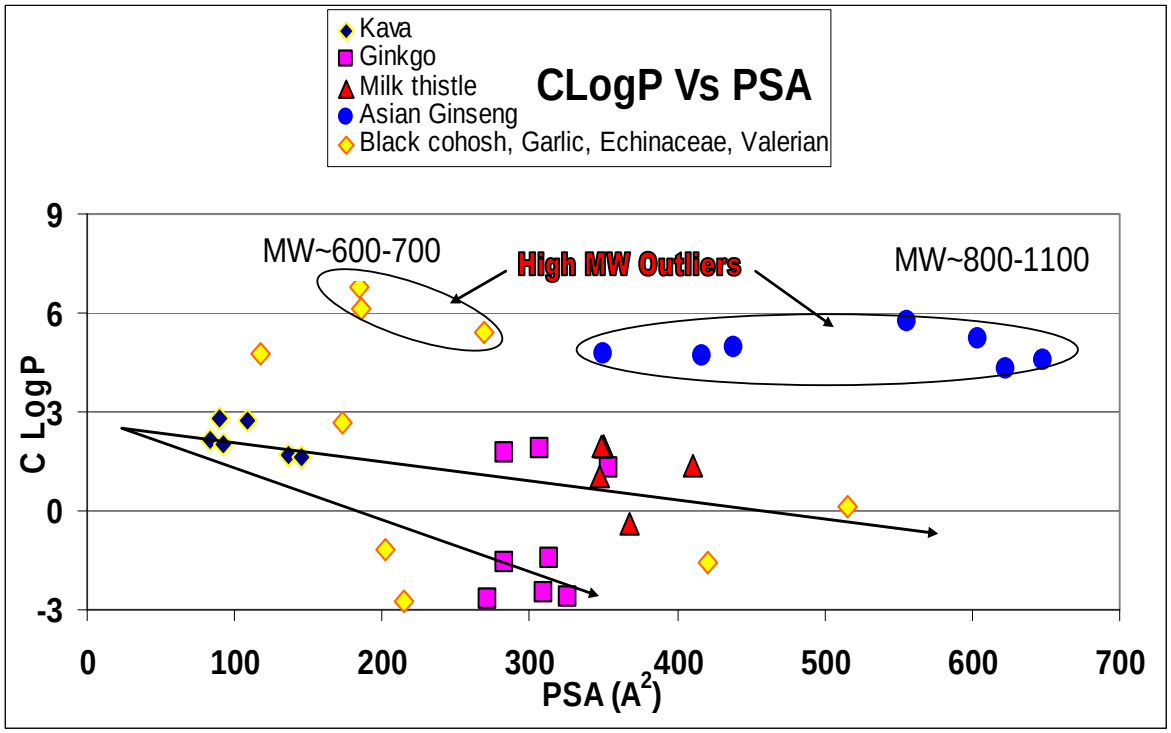


Figure 2.8. Plot of CLogP versus Polar Surface Area

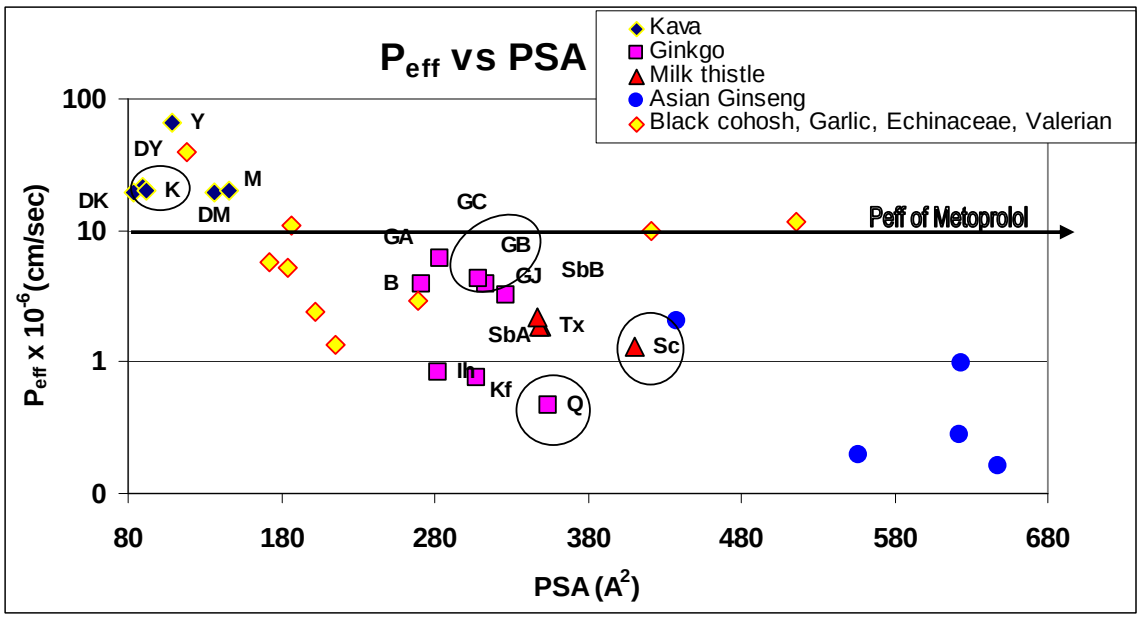


Figure 2.9. Plot of Permeability (P_{eff}) vs Polar Surface Area (PSA)

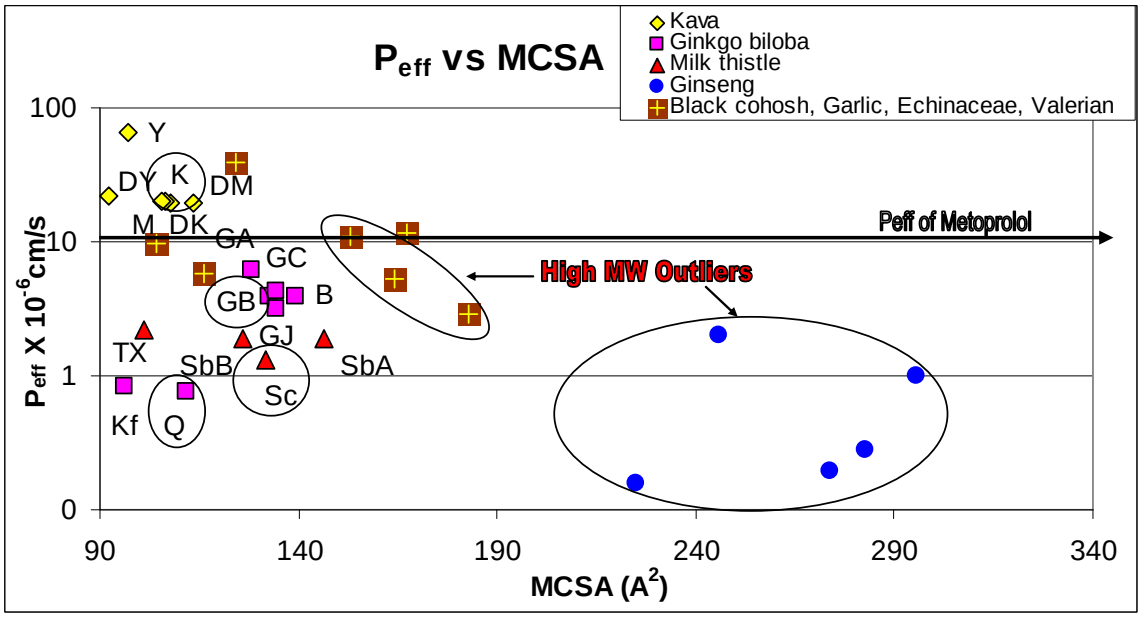


Figure 2.10. Plot of Permeability (P_{eff}) vs Minimal Cross Sectional Area (MCSA)

In this section an attempt has been made to study the interrelationships between the *in silico* descriptors and *in vitro* permeability to see whether these correlations help us select the least permeable component in select herbal extracts (kava, Ginkgo biloba and Milk thistle). Selecting the component which has the least intestinal permeability is the most conservative approach to ensure the bioavailability of a multicomponent herbal supplement.

Hence, while selecting a bioavailability marker for a herbal extract three criteria need to be fulfilled:

1. The selected marker should have one of the least permeability among its active components in the extract

2. The proportion of the selected marker in the extract should be sufficient for its precise quantitative determination in biological fluids after oral administration.(e.g. silycristin in milk thistle, kawain in kava).

3. The selected marker should be easily available as a reference standard, at a reasonable cost and acceptable purity for routine analysis.

A plot of the predicted octanol-water partition coefficient versus the polar surface area is shown in Figure 2.8. The plot depicts an overall rough trend that the polar surface area of all the components (except the outliers) increases as the components move from the lipophilic scale to the hydrophilic scale. This holds true for the kava components, all the ginkgo terpenes (GT) and 3 isomers of silymarin (Tx, Sd, Sc). The ginsenosides and the acteols from Black cohosh can be considered as outliers due to their unrealistic high values of CLogP. These unrealistic high CLogP values can be attributed to their large molecular weight (between 600-1100g/mole) and structure. It has been reported that the

efficiency of partition coefficient predicting programs such as CLogP goes down as the molecular weight or the size of the compound increases. [123] Thus, the ginsenosides and and the acteols can be considered as clear outliers. High polar surface area indicates the greater hydrophilic nature of the compound and thus a lower polar surface area implies greater lipophilicity and hence higher permeability which is again true in the case of kava compounds, which are lipophilic, have a low polar surface area and are the smallest and simplest of the molecules in terms of molecular weight and structure (Ch.1; Figure 1.1: Structure of kava components).

Figure 2.9 is a plot of the effective permeability determined using SimBioDAS® (Peff) versus the polar surface area for the active herbal components. The plot depicts a clear trend that as the PSA increases, the P_{eff} of the components decreases. If metoprolol (P_{eff} = 10 x 10^{-6} cm/sec) is considered as a cut off limit to distinguish between high permeability and low permeability compounds, we see that most of the herbal components fall into the low permeability class with the exception of the kava components. The plot depicts a linear decrease in permeability with the increasing PSA (R^2 =0.72) indicating that the decrease in permeability is probably due to the solvation of the compound and the increasing molecular size. It can be inferred from this plot that from a group of components in each extract, the one which has a low permeability and a high PSA would indicate lower intestinal permeability and can be selected as a probably marker. For the kava compounds, yangonin (Y) is the only component which has a very high permeability (P_{eff} =65.66x 10^{-6} cm/s) where as the other 5 components have permeabilities in a close range with each other (19.3-21.6 x 10-6 cm/sec). Between these low permeability components, methysticin and dihydromethysticin (M&DM) have the

highest PSA (~140 Å^2) followed by kawain (92.56 Å^2). Yangonin cannot be considered as a marker, mainly due to its high permeability. Kawain (K)is one of the major components of the kava extract, in proportion and in terms of pharmacologic effect and thus kawain is considered as a marker, for kava.

For the ginkgolides in ginkgo biloba, ginkgolide J (GJ) has the highest polar surface area accompanied with the lowest permeability, and can be considered as a marker. The proportion of GJ in the extract is very minimum as compared to the other ginkgo terpenes like GA, GB or GC and the next least permeable component among the terpenes is GB. GB is also easily available commercially as a reference standard and hence GB can be considered as a marker for the ginkgo terpenes. Similarly quercetin can be considered as a marker for the flavonol glycosides in ginkgo biloba.

Among the silymarin isomers, silydianin (Sd) has the highest polar surface area and is expected to be the least permeable compound in the extract, due to its very low lipophilicity (-0.39). Sd is one of the minor components of silymarin and also suspected to be pharmacologically inactive as compared to the silybins [56], where as the next least permeable compound silycristin (Sc) has a very high proportion in the extract and is easily quantifiable. Thus, Sc can be selected as a marker for the silymarin isomers.

Hence, based on the plot of effective permeability versus PSA we have we have kawain as a marker for kava, ginkgolide B as a marker for the ginkgo terpenes and quercetin as a marker for the flavonol glycosides and silycristin as a marker for milk thistle.

Figure 2.10 shows the plot of effective permeability (P_{eff}) versus minimal cross sectional area (MCSA). The plot depicts an approximate trend of decreasing

permeability with the increase in the MCSA which is expected, as the MCSA is the area of the molecule when it is partitioned in the lipid bilayer during the permeation process. Hence, larger the cross sectional area , the more difficult it is for the molecule to permeate in to the lipid bilayer, in this case the gastro intestinal membrane. Among the kava components, we have DM and DK with similar permeability values and increasing MCSA's. They are followed by kawain which has the next highest MCSA and a similar permeability of 20x10-6 cm/sec. As earlier, among the three, kawain is selected as a marker for kava based on its pharmacology and its major proportion in the extract. Also kawain is readily available as a reference standard.

Among the silymarin isomers, between Sc and SbA, Sc would be selected as a marker. Sd is not a choice due to its minor proportion in the extract. Even though SbA has a higher MCSA (146 $Å^2$) than Sc (131 $Å^2$) SbA is more lipophilic (1.95) as compared to Sc (1.38) and has a lesser permeability value as compared to SbA. Hence, the low lipophilicity and the low permeability value of Sc makes it a more lesser permeable candidate than SbA and hence based on this plot, Sc is selected as a marker for silymarin.

Among the ginkgo terpenes a choice needs to be done between bilobalide (B) and ginkgolide B (GB) as performance marker. The previous plot, based on Peff versus PSA predicted GB as a marker, due to its high polar surface area and proportion in the extract among the terpenes. Considering the permeability values, there is no significant difference between the permeability values of GB and B. Also GB has a moderately hydrophilic nature (CLogP=-1.45) as compared to B (CLogP=-2.64) which is highly hydrophilic. This increases the chance of GB being as the least permeable compound, as

compared to B which would not be permeable at all due to high salvation. Thus, GB is selected as a marker for the terpenes and quercetin which has a high MCSA (111 $Å^2$) and low permeability (P_{eff}=0.76x10-6 cm/sec) is selected as a least permeable marker for the ginkgo flavonol glycosides.

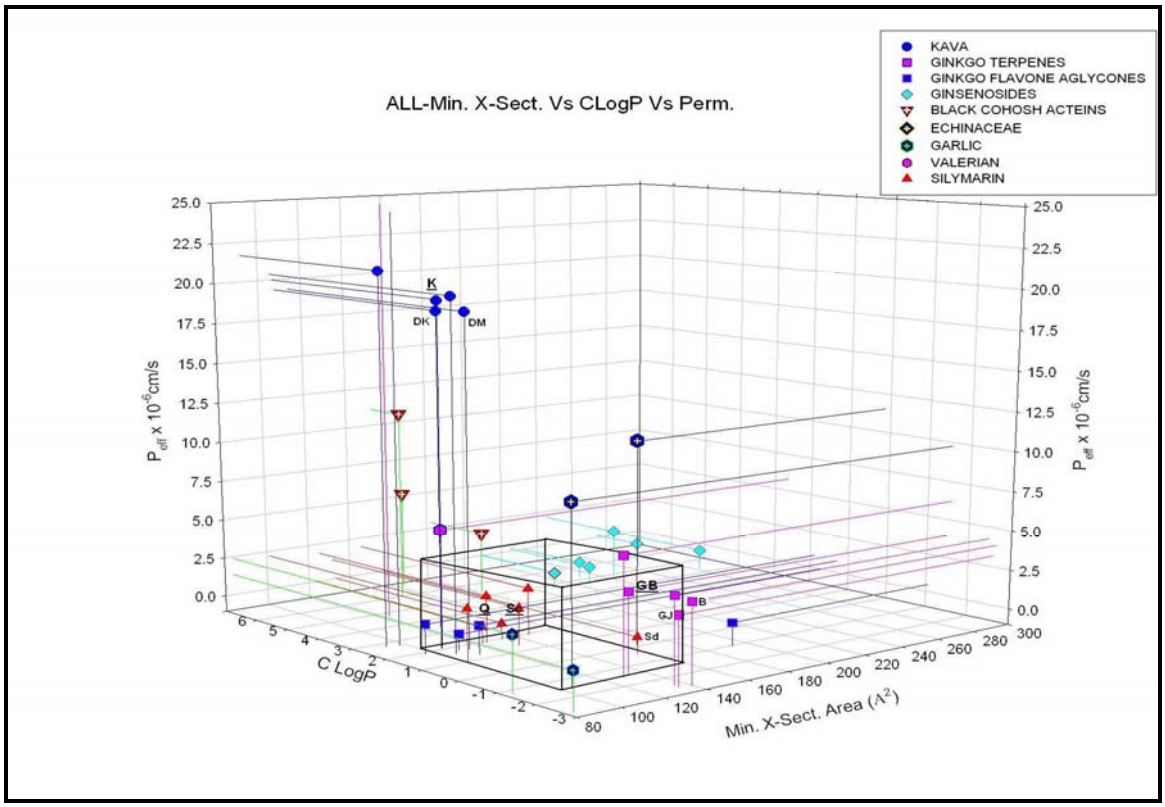


Figure 2.11. Graph of MCSA vs. CLogP vs. Permeability of all Active Components

Figure 2.11 shows a 3D plot of the MCSA vs CLogP vs Peff of the active components of the herbal extracts. This further clarifies the choice of markers predicted for the select extracts, based on the inter-parameter relationships between CLogP, MCSA

and P_{eff} of the compounds. Figure 2.11 shows the effect of MCSA and CLogP on the permeability of the compounds indicating the basic trend that most of the compounds have a low permeability (<5 x 10^{-6}cm/sec) and range in a window with CLogP from -1.5 to +2.2 and MCSA from 100 $Å^2$ to 160 $Å^2$. Thus, considering these limits for permeability, CLog P and MCSA, a rectangular box can be drawn so as to include this cluster of marker compounds.

Considering that the least permeable marker in a group of compounds in a particular herbal extract, gives rise to a theoretical marker and a practical marker for each of the three herbs. Due to obvious reasons the performance standard selected should be characteristic of the extract, the components of kava are considered to be an exceptional case due to their high permeability values. Yangonin has a high permeability and smaller MCSA among the kava components and hence does not fulfill the conditions for our conservative approach of least permeability. Dihydromethysticin and dihydorkawain have similar permeability values, but differ in the MCSA and CLogP values. Higher MCSA value indicates lower permeability and dihydromehtysticin is comparatively less lipophilic than dihydrokawain. Hence, dihydromethysticin can be assigned as the theoretical marker for kava. Ideally dihydromethysticin would be the practical marker too, but a performance standard should be easily available at a reasonable cost for daily analysis. Further the proportion of dihydromethysticin among the kava lactones is in the range of 5.2%-7.4 % where as that of kawain is around 33-35%. The individual proportion of kawain being much higher for higher sensitivity and easy analysis, the low permeability value along with an intermediate CLogP and MCSA value indicate that kawain would be an appropriate practical marker for Kava.

Considering kava as an exception, all other compounds that lie out of proposed 'box' cannot be considered as suitable markers due to their isolation from the observed cluster. These compounds either have a high permeability from its fellow compounds or even if the permeability is within range (<5 x 10^{-6} cm/sec) either the CLogP or the MCSA provide a hint that these candidates can pose as a false lead towards being a performance standard. Hence, observing the effect of two variables (CLogP & MCSA) on the third variable (*in vitro* permeability), using a 3D scatter plot proves to be extremely useful. Bilobalide from ginkgo biloba can be considered as a good example of a false lead, where it has a low effective permeability (3.891 x 10^{-6} cm/sec) and MCSA (139.435 $Å^2$) not very different from other compounds but its CLogP value (-2.64) warns us about its high hydrophillic nature and isolating it from the proposed cluster box.

Ginkgolide B, a terpene lactone from ginkgo biloba can be selected as the theoretical and practical marker due to its low permeability, moderate CLogP and MCSA values as compared to its fellow terpene lactones in the extract. Similarly among the flavonol aglycones quercetin, kaempferol and isorhamnetin, quercetin is a suitable candidate as a standard.

The study of milk thistle includes seven active flavonolignans from which 6 are isomers. The MCSA plays a major role as a deciding factor in this case as the CLog P values for the silybin and isosilybin diastereomers are similar. Silydianin can be considered as a theoretical marker for milk thistle based on its CLogP and MCSA. Though the permeability value of silydianin is not available in this study, the higher value of MCSA definitely suggests that silydianin would have the least permeability among the milk thistle compounds. The isosilybins cannot be considered as markers as the

separation of these isomers into separate compounds has not been possible to date. Also the percentage of isosilybins in the extract is very less as compared to the other components such as silybin and silycristin. Silycristin can be considered as the practical marker for milk thistle, being moderately lipophillic and having a comparatively low permeability. Further isolation of silycristin from other silymarin isomers has been possible and also the percentage of silycristin in silymarin (milk thistle extract) is very high enabling higher sensitivity.

The following three figures (Figures: 2.12, 2.13, 2.14), show the components of the individual extracts separately so as to give a clearer idea of how the predicted markers are positioned with respect to their permeability, lipophilicity and MCSA. Observation of the data by plotting them on 3D axes is necessary to interpret the effects collective influence of each descriptors (MCSA and CLogP) on permeability and vice versa.

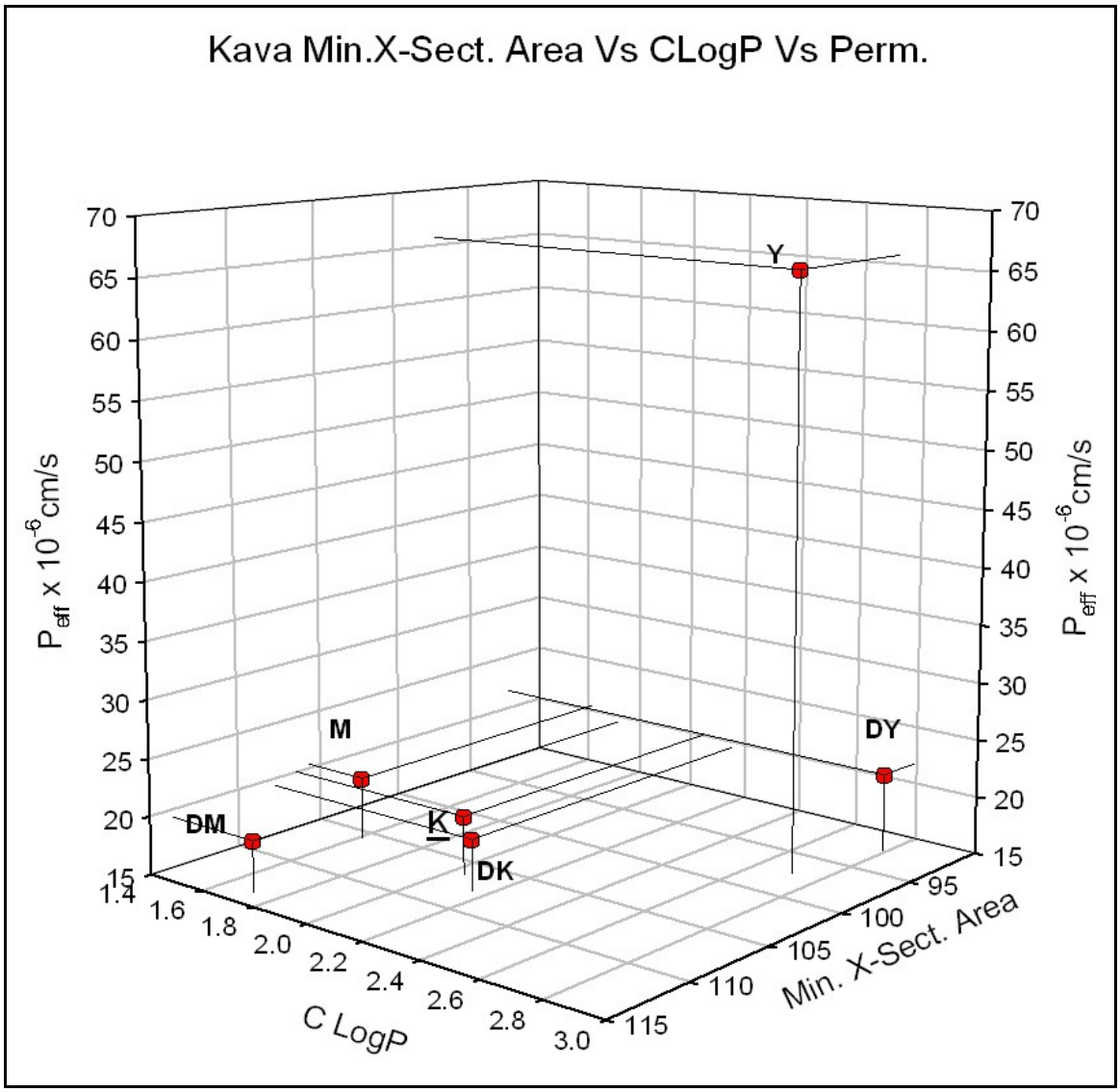


Figure 2.12. Plot of MCSA vs CLogP vs Permeability of Kava components

(Min.X-Sect Area=MCSA)

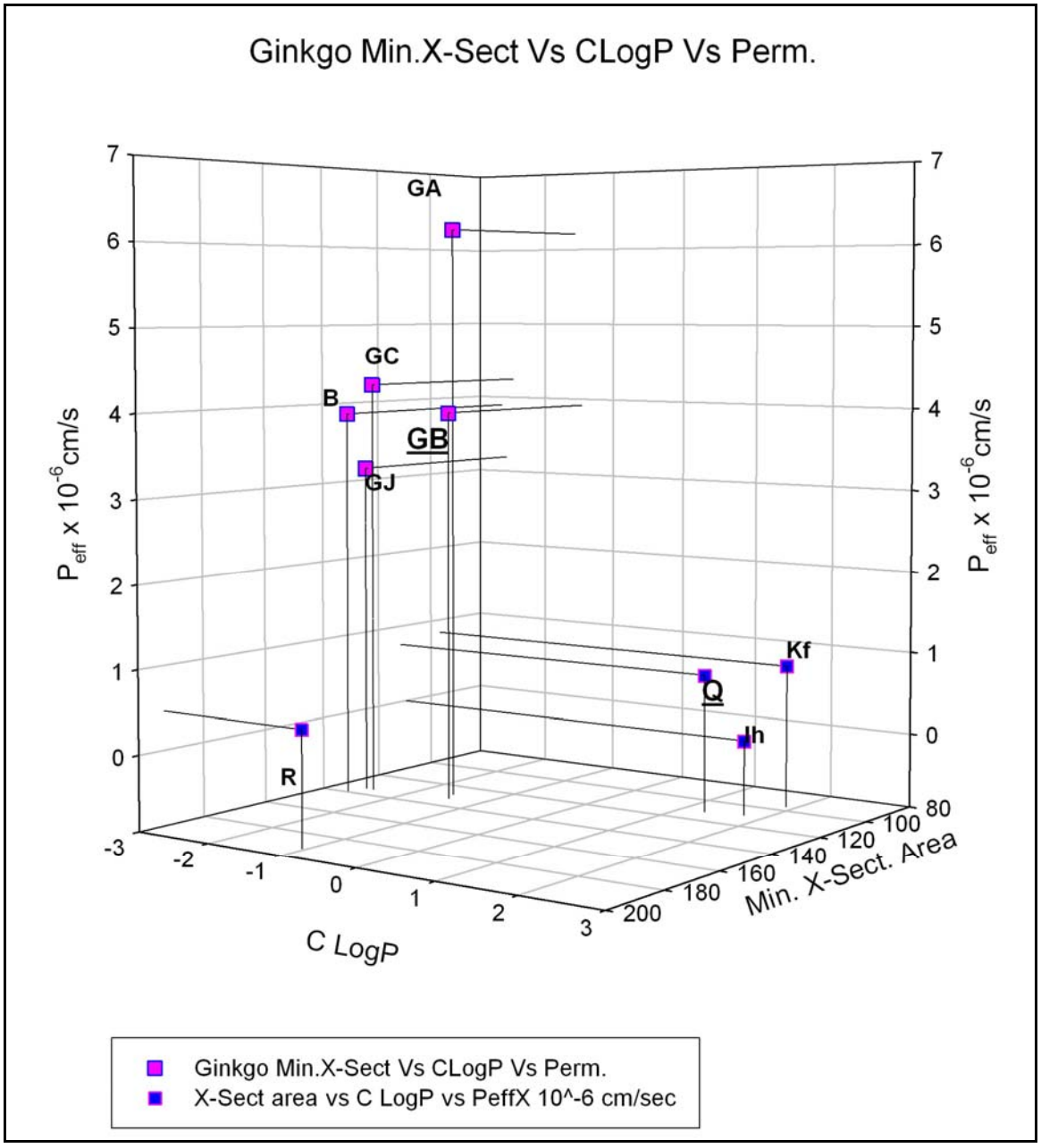


Figure 2.13. Plot of MCSA vs CLogP vs Permeability of Ginkgo components

(Min.X-Sect Area=MCSA)

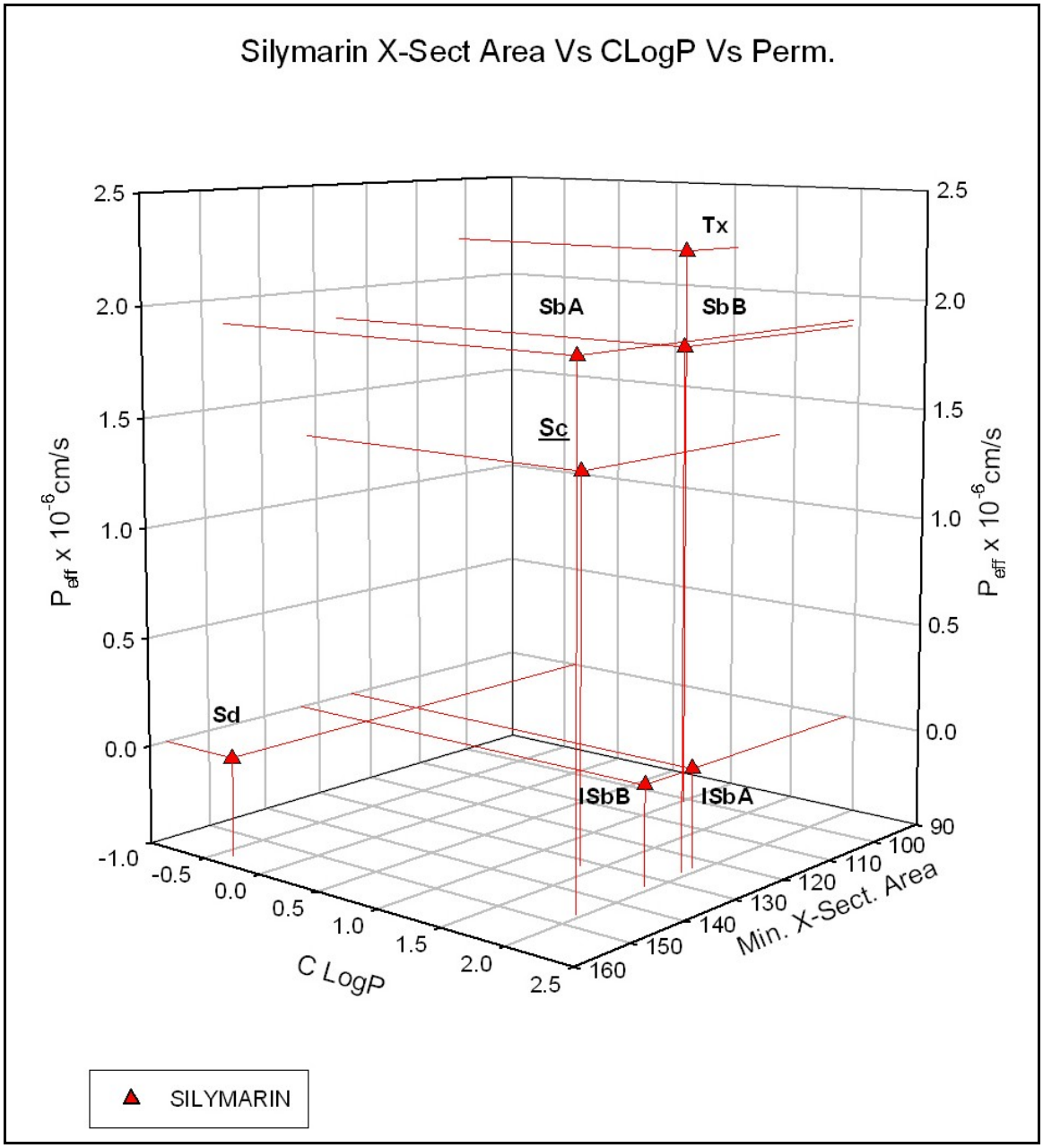


Figure 2.14. Plot of MCSA vs CLogP vs Permeability of Silymarin components

(Min.X-Sect Area=MCSA)

Thus, from the inter-parameter relationships observed between the PSA, MCSA, CLogP and the effective permeability of the compounds, and maintaining the three criteria for the selection of an appropriate bioavailability/bioequivalence marker, the following active components are selected as markers for the respective herbal extracts:

I. Kawain as a marker for the kava extract

II. Ginkgolide B as a marker for the ginkgo terpenes and quercetin as a marker for the flavonol glycosides in Ginkgo biloba

III. Silycristin is selected as a marker for the silymarin isomers in Milk thistle.

In this section, an attempt has been made to classify the active compounds from various herbal extracts so as to facilitate the selection of bioavailability markers for the three herbs: Kava, Ginkgo biloba and Milk thistle (silymarin).

2.4.1: CHOICE OF EXTRACT FOR MARKER SELECTION

From the previous section it is inferred that kawain is the best selected marker for Kava kava, ginkgolide B is a suitable marker for the terpene lactones and quercetin for the flavonol aglycosides in Ginkgo biloba, and silycristin is the best suitable marker for silymarin (Milk thistle). Each herbal extract from three being studied is unique in its own sense in terms of therapeutic effect. Each herbal extract shows exhibits a pharmacological effect in a synergistic way, due to the presence of six or more active components. From these three extracts, silymarin presents itself as one of the most interesting due to the presence of its isomers and diastereomers. silymarin consists of six active flavonolignan isomers, from which four of them exist as diastereomers. Thus,

silymarin presents a considerable analytical challenge in the separation of all the isomers. Thus, we consider silymarin as our model extract to proceed with the *in vitro* dissolution, solubility and partition coefficient experiments. Due to the presence of isomers and diastereomers in silymarin, it becomes very interesting to know how the pharmacokinetic parameters and bioavailability for each isomers differ from each other. Hence, we select silymarin as our model extract to proceed with the *in vitro* solubility and dissolution experiments as well as the *in vivo* pharmacokinetic experiments.

2.5: SUMMARY

The results in the previous section indicate that the minimal cross-sectional area is a function of molecular weight and also a good predictor of the extent of permeability of the compounds being tested. Though the investigation includes 39 selected compounds from 8 herbal extracts, each marker has to be a characteristic of its herb which is a basic requirement for the selection of a performance standard. Hence, the kava compounds which have high permeability values as compared to the other compounds, we select kawain as the marker which is one of the least permeable among its group. Similarly the selection of Ginkgolide B and Quercetin as the markers for Ginkgo biloba satisfies the proposed requirements. The MCSA proves to be very useful while explaining the selection of Silycristin as the marker for milk thistle extract, considering the fact that 6 of the 7 active compounds of milk thistle are isomers. Hence, the decision of selecting a performance standard has been done by keeping in mind primarily its permeability extent, partition coefficient, its percentage in the extract and the pure commercial availability. The conservative approach of selecting the least permeable compound as a

bioavailability marker seems logical considering the lack of quality control that is associated with the sale of these products**.** The effect of two variables, MCSA and CLogP on the permeability of the compounds explains the efficiency of a 3D scatter plot and warns the investigator about potential false leads.

Hence, selection of the least permeable compound for bioavailability testing (*in vitro and in vivo*) would enable us to develop performance standards for the selected herbal supplements ensuring their efficacy and bioavailability.

CHAPTER 3: ANALYTICAL METHODOLOGY

3.1: INTRODUCTION

This chapter discusses the development of a reverse phase binary gradient HPLC-UV method for the quantitative determination of the six silymarin isomers in various different chemical matrices and rat plasma. A new method was required for the quantitation of isomers, because all of the previous methods did not separate the silybin and isosilybin isomers into its respective diastereomers. Some of the previous methods were only focused on the separation and quantitation of silybin alone as a single isomer and did not measure the levels of any other silymarin isomers. Hence, a new method that would measure all the silymarin isomers in chemical and biological matrices, and have a simple, efficient and less time consuming, extraction procedure (mainly for the extraction of isomers from a biological matrix) was required. This chapter discusses a method that was developed and that could be used across various chemical and biological matrices with minor modifications. The complete validation of the method for the quantitative determination of silymarin isomers in rat plasma is presented in this chapter. Sample preparation methods in chemical matrices like the CaCo-2 cell transport media, capsule formulations and *in vitro* dissolution media, equilibrium solubility and octanol-water partition coefficient media are discussed. The chapter explains the standardization of the silymarin extract to yield the exact proportion of each isomer present. Sample chromatograms of the separated isomers in various chemical and biological matrices are shown.

3.2: PREVIOUS RELATED STUDIES

3.2.1: ANALYSIS OF SILYMARIN IN CHEMICAL MATRICES

High performance liquid chromatography (HPLC) coupled primarily with ultra-violet detectors, has been the method of choice ever since the discovery of silymarin isomers with initial references appearing in the 1970's [57, 60, 124-128] Chemical matrices mentioned in the following references were either the standardized extract or pharmaceutical formulations such as tablets or capsules that contained the dried plant powder along with other pharmaceutical excepients. Most common methods of extraction are of two types: (i) Dissolution of the extract or the sample powder into methanol followed by filtration to get a clear solution ready for analysis [76, 129]; (ii) Extraction of the sample powder using an appropriate solvent such ethyl acetate or ether followed by a solvent extraction procedure using a soxhelet or vortex mixer.[130, 131] Titel et al. carried out the analytical separation of silybin, silydianin, silycristin and taxifolin but their work did not give any account of the diastereomers of silybin and isosilybin. [127, 128] The work by Quercia et al. in 1983 indicated for the first time that, apart from silydianin and silycristin, silymarin consisted of diastereomers of silybin and isosilybin. [132] Even after the proven presence of diastereomers in silymarin and a surge in its analytical research in the 1990's, most of the methods still focused on the separation of isomers rather than the diastereomers. [76, 77, 130, 133, 134] This can be attributed mainly to the fact that silybin was considered as the major and important pharmacological constituent of silymarin extract which is in contradiction to the work proposed by Quercia et al. which states that there was no significant difference found in

the pharmacological activity of silybin and isosilybin against phalloidine poisoning. [132] Work by Ding et al. was one of the first ones to report the complete quantitative separation of silymarin isomers and diastereomers. [129] They reported differences in the proportion of isomers and diastereomers in extracts obtained from Germany and China, attributing the differences to differing geographical locations. Bilia et al. reported on the stability of silymarin tincture (60%v/v ethanol) and found the isomers to be stable at 25°C for up to 3 months. [133] The most recent method following the work of Ding et al. was that by Lee et al. which involved the complete separation of all the isomers and diastereomers using a HPLC coupled with mass spectrometry, but did not report any validation data for the method. [131]

3.2.2: ANALYSIS OF SILYMARIN IN BIOLOGICAL MATRICES

Isomers of silymarin have low solubility resulting in minimal plasma levels and low bioavailability. Since silybin has been considered to be the major portion of the extract quantitatively and pharmacologically, much of the *in vivo* work in human and animal models has been concentrated on the improvement of bioavailability by the formation of silybin-phosphatidylcholine complex or other modified formulations. [79, 135-138] silymarin isomers are mainly excreted as metabolites into the bile and are subject to enterohepatic circulation. [56] Thus, methods have been developed that monitor silybin along with its conjugates in the bile. [79] Chronologically Martinelli et al. first developed the separation of free and conjugated silybin (as a single isomer) in human plasma and urine [135] followed by the diastereomeric separation of silybin

(silybin A and silybin B) and its conjugates by Wehenmeyer et al. [79, 139] Wehenmeyer's method was found to be the most sensitive with a limit of detection of 2.5 ng/mL for the unconjugated isomer. Recently Lee et al. proposed that their method for the complete separation of silymarin isomers in a chemical matrix can be extended to biological matrices in human and animal models. [131] There has been only one documented report involving the analysis of silybin in rat plasma, tagged with radioactive ^{125}I. [140] Thus, the complete separation and quantification of all silymarin isomers and diastereomers has just been achieved recently with very few documented references.[131, 141]

3.2.3: PREPARATIVE SEPARATION OF ISOMERS AND DIASTEREOMERS

Availability of silymarin isomers as pure and isolated reference standards is rare, with very limited suppliers and associated with prohibitive prices. The first ever complete preparative isolation of silymarin isomers (silycristin and silydianin) and diastereomers (silybin A and B, isosilybin A and B) was achieved by Wani et al. [142] This is the only documented research on the isolation of silymarin diastereomers on a preparative scale such that detailed pharmacologic and pharmacokinetic studies can be done on individual isomers.

3.3: HPLC METHOD FOR SILYMARIN ISOMERS IN RAT PLASMA

3.3.1: QUANTITATIVE DETERMINATION OF SILYMARIN ISOMERS IN RAT PLASMA

Initial method development leading to the quantitative separation of all the silymarin isomers resulted in an analysis runtime of approximately 45 minutes. Thus, extraction of silymarin isomers from rat plasma required a quick and short method with no loss in analyte due to extraction. Due to its plasma denaturing properties, high solubility of silymarin isomers and internal standard 1-naphthol, and being a part of the mobile phase, methanol was the solvent of choice for the extraction of silymarin isomers from rat plasma.

3.3.1.1: Chromatography

During method development it was observed that pH of the sample was the most important factor influencing peak shape and resolution of the isomers. A pH of 7.0 ±0.05 had to be maintained in order to achieve optimum peak shape and response for all isomers as well as higher resolution between the diastereomers. The second most influencing factor was the flow rate at 1ml/min from 28 to 36 minutes, when the proportion of methanol increased to 55%. Any attempt to elute the peaks at an earlier time point resulted in the peak merging of the silybin and isosilybin diastereomers.

Sample chromatograms of the six isomers along with their retention times, separated in rat plasma are shown in Fig. 3.1 & 3.2. The resolution between the diastereomers silybin A and B was 1.59, between diastereomers isosilybin A and B was 1.30 and between silybin B and 1-naphthol was 2.24.

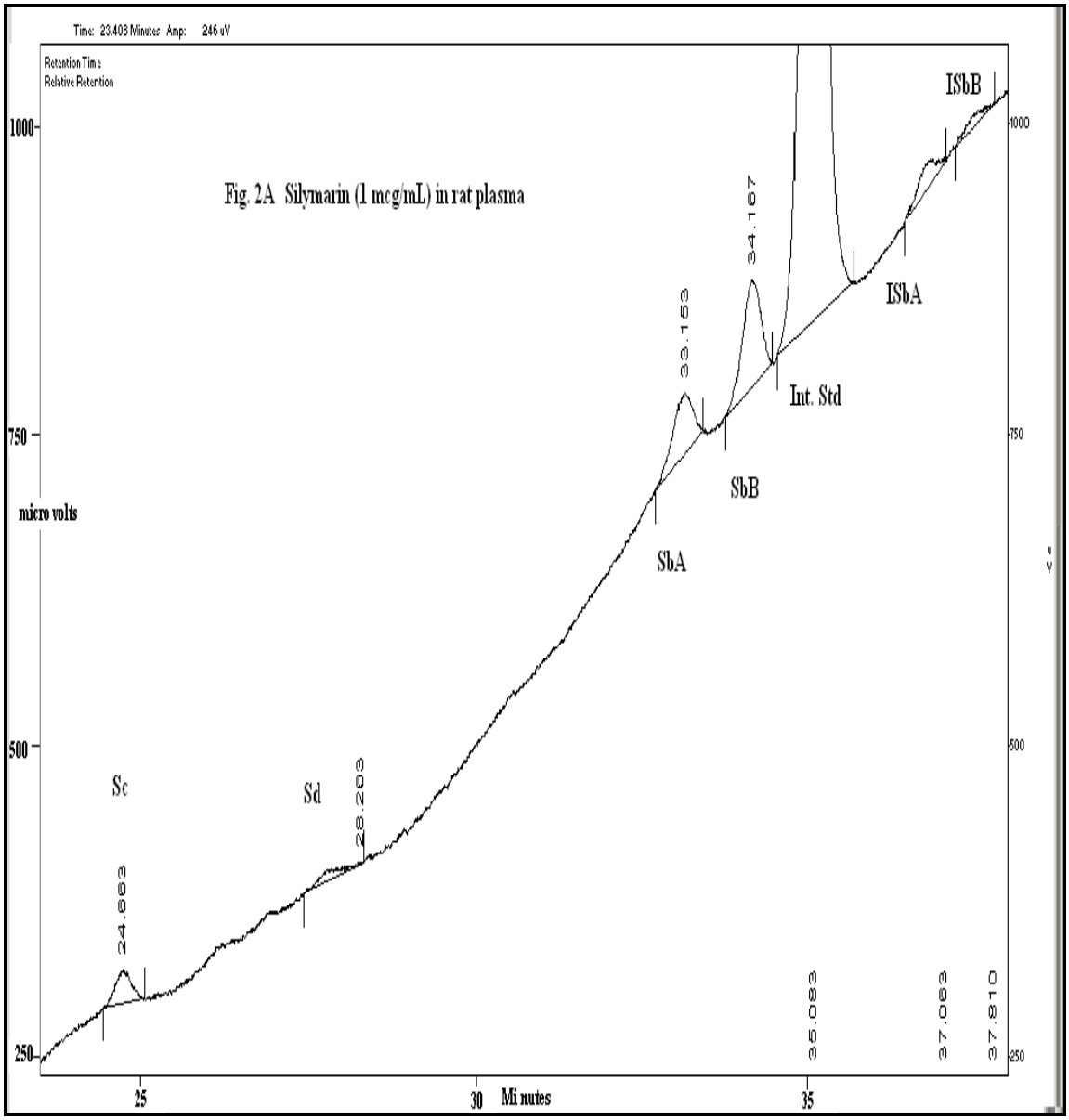


Figure 3.1. Sample Chromatogram of Silymarin (1µg/mL) in Rat Plasma Comprising of Sc: 0.24µg/mL (24.66 mins); Sd: 0.03µg/mL <LOD (28.26 mins); SbA: 0.18µg.mL (33.15 mins); SbB: 0.34µg/mL (34.18 mins); ISbA: 0.08µg/mL (37.1 mins); ISbB: 0.03µg/mL <LOD (37.8 m ins).

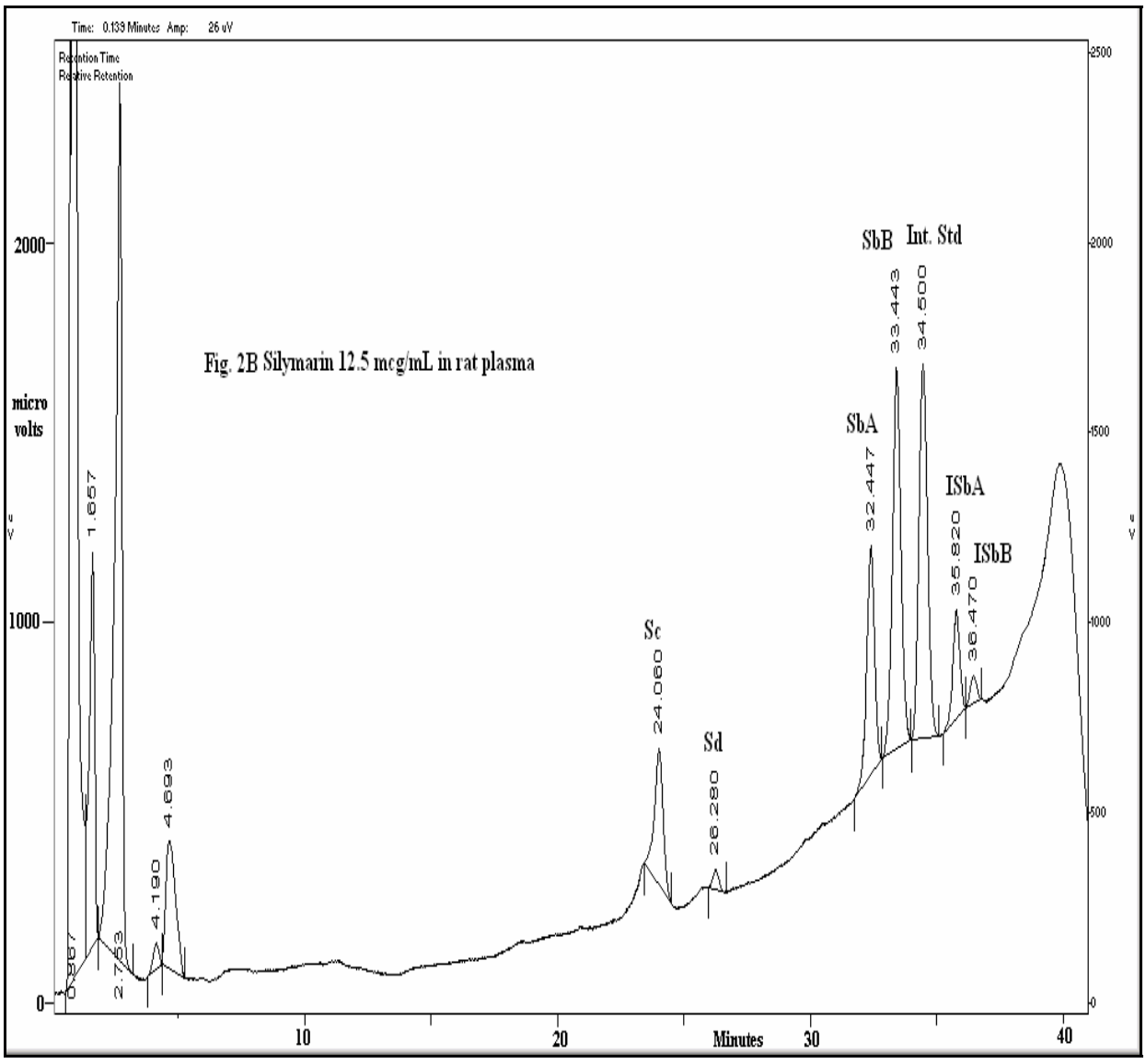


Figure 3.2. Sample Chromatogram of Silymarin (12.5µg/mL) in Rat Plasma comprising of Sc: 3.06µg/mL (24.66 mins); Sd: 0.45µg/mL (28.26 mins); SbA: 2.36µg/mL (33.15 mins); SbB: 4.32µg/mL (34.18 mins); ISbA: 1.02µg/mL (37.1 mins); ISbB: 0.32µg/mL (37.8 mins).

3.3.1.2: Sample Preparation

400 µL of rat blood was placed in a heparinized 1.5mL centrifuge tube and centrifuged immediately at 8000 rpm for 7 minutes to obtain a clear supernatant plasma layer. This was then transferred to a clean 1.5 mL centrifuge tube and frozen at -20°C until analysis. At analysis time, the frozen plasma was thawed at room temperature before analysis. 200µL of thawed plasma was transferred to a clean 1.5mL centrifuge tube and 50µL of 0.2M o-phosphoric acid was added to it. This was followed by the addition of 1mL of 0.5µg/mL internal standard (1-naphthol) prepared in methanol. The mixture was vortexed for 10 seconds and then centrifuged at 5000 rpm for 5 minutes. The resultant clear supernatant solution was filtered through a 0.45µ nylon membrane and 50µL of this was injected directly into the HPLC for analysis.

3.3.1.3: Chromatographic Conditions

Analysis of silymarin was performed using a Phenomenex Luna C-18(2) column (5µ, 150 x 4mm). The binary gradient method used water (Solvent A) and methanol (Solvent B) in the following composition and corresponding time program as shown in Table 3.1.

Table 3.1: Gradient Solvent B Concentration (%) and Time Program

Time (minutes)	Composition of B (%)	Time (mins)	Total Flow (ml/min)
0	20	0	2
0-28	50	0-10	2
28-36	55	10-38	1
36-41	20	38-41	2

1-naphthol (0.5µg/mL) was used as an internal standard. The UV detector was set at 288nm and the volume of injection was 50µL. The total run time, including column equilibration was 41 minutes and carried out at ambient temperature.

3.3.2: METHOD VALIDATION

The HPLC method was validated for complete separation and quantification of all the six silymarin isomers using 1-naphthol as internal standard. The ratio of the peak area of the analyte to peak area of the internal standard was used to calculate the analyte response.

3.3.2.1: Linearity

The linearity for silymarin extract was tested in a concentration range from 2.5µg/mL to 250µg/mL comprising of 7 standard solutions. silymarin stock solutions were prepared in methanol. These concentrations of the silymarin extract yielded the corresponding isomer concentrations as per their proportion determined from the standardization of the extract shown in Table 3.11. Linearity samples were injected in triplicate and the peak area ratios of the isomers to the internal standard was calculated. A linear regression analysis was carried out on the peak area ratio versus concentration profiles to determine the slope, intercept and the correlation coefficient. Unknown concentrations of isomers in the plasma samples were calculated using the resultant regression equation for each isomer.

The plasma linearity solutions were prepared in a way such that, when 150µL of drug free rat plasma was spiked with 50µL of silymarin, yielded 200µL of plasma solution containing the respective concentrations of the isomers as described in Table 3.2.

Table 3.2: Linearity Concentrations for Silymarin Isomers.

Calibration Point	Silymarin (µg/ml)	Sc (µg/ml)	Sd (µg/ml)	SbA (µg/ml)	SbB (µg/ml)	ISbA (µg/ml)	ISbB (µg/ml)
1	2.5	0.61	0.09	0.47	0.86	0.20	0.06
2	5	1.22	0.18	0.94	1.72	0.41	0.128
3	12.5	3.06	0.45	2.36	4.32	1.02	0.32
4	25	6.13	0.90	4.72	8.64	2.05	0.64
5	62.5	15.32	2.26	11.81	21.61	5.13	1.60
6	125	30.65	4.56	23.63	43.23	10.27	3.20
7	250	61.30	9.04	47.27	86.46	20.55	6.40

3.3.2.2: Interday and Intraday Precision

Three concentrations of each silymarin isomer were tested for interday (3 days, n=3) and intraday (n=6) precision. Peak ratio of the respective isomer to internal standard was used in the subsequent calculations. The concentrations of isomers tested are shown in Table 3.3.

Table 3.3: Concentrations of Silymarin Isomers Tested for Intraday and Interday Precision.

Silycristin Concentration (µg/mL)	**Silydianin Concentration (µg/mL)**	**Silybin A Concentration (µg/mL)**
1.22	0.09	0.189
3.06	0.452	0.945
6.13	0.905	2.36
Silybin B Concentration (µg/mL)	**Isosilybin A Concentration (µg/mL)**	**Isosilybin B Concentration (µg/mL)**
0.345	0.082	0.064
1.73	0.411	0.320
4.32	1.027	0.640

3.3.2.3: Accuracy and Recovery

Method accuracy and extraction recovery for the six isomers was determined for one concentration (12.5µg/mL) at three levels, by spiking with silymarin. The spiked concentrations (L1, L2, & L3) of silymarin and its corresponding six isomers are shown in Table 3.4

Table 3.4: Spiked Concentrations of Silymarin Isomers for Accuracy and Recovery.

Silymarin (µg/mL)	Sc (µg/mL)	Sd (µg/mL)	SbA (µg/mL)	SbB (µg/mL)	ISbA (µg/mL)	ISbB (µg/mL)
Std.(12.5)	3.06	0.45	2.36	4.32	1.02	0.32
L1 (15)	0.61	0.09	0.47	0.86	0.21	0.06
L2 (20)	1.84	0.27	1.41	2.59	0.62	0.19
L3 (45)	7.97	1.17	6.14	11.24	2.67	0.83

* L1= Recovery Level; L2= Recovery Level; L3= Recovery Level 3

**Sc=silycristin, Sd=silydianin, SbA=silybin A, SbB=silybin B, ISbA=isosilybin A, ISbB=isosilybin B

3.3.2.4: Limit of Detection and Quantitation

Limit of detection (LOD) for the isomers was defined as the lowest analyte concentration having a signal to noise ratio of 3 that could be distinguished when compared to its blank. Limit of quantitation was the lowest silybin A&B concentration that could be measured with a signal to noise ratio of 10 and had a relative standard deviation of less than 20%.

3.3.2.5: Stability

Plasma samples spiked with silymarin were subjected to 3 freeze-thaw cycles and then assayed for their isomeric content. Rat plasma spiked with silymarin, post sample preparation, was tested for stability at room temperature for 36 hours. Stock solutions of silymarin and internal standard (1-naphthol) in methanol were also tested for stability at room temperature for 24 hours.

3.3.3: RESULTS

3.3.3.1: Linearity

The mean slopes and intercepts for the silymarin isomers, obtained from the various calibration curves are listed in Table 3.5.

Table 3.5: Mean Slopes and Intercepts of Silymarin Isomers.

	Sc (n=3)	Sd (n=3)	SbA (n=3)	SbB (n=3)	ISbA (n=3)	ISbB (n=3)
Mean Slope ±SD	0.1143 ±0.014	0.0526 ±0.019	0.2303 ±0.006	0.233 ±0.01	0.238 ±0.002	0.203 ±0.005
Mean Intercept ±SD	0.0266 ±0.001	0.003 ±0.001	0.0235 ±0.001	0.01 ±0.003	0.009 ±0.001	0.004 ±0.002
R^2 ±SD	0.996 ±0.001	0.989 ±0.012	0.998 ±0.002	0.996 ±0.004	0.998 ±0.001	0.996 ±0.001

3.3.3.2: Interday and Intraday Precision

The summary data for the intra-day and inter-day precision is shown in Table 3.6 and 3.7.

Table 3.6: Intra-day Precision

Silycristin (n=6)				Silydianin (n=6)			
Conc. (µg/ml)	1.22	3.06	6.13	**Conc. (µg/ml)**	0.090	0.452	0.904
***Mean Ratio**	0.112	0.339	0.725	***Mean Ratio**	0.005	0.033	0.049
SD	0.0045	0.023	0.0300	**SD**	0.0001	0.002	0.006
CV (%)	4.07	6.80	4.14	**CV (%)**	3.47	8.12	13.07
Silybin A (n=6)				**Silybin B (n=6)**			
Conc. (µg/ml)	0.189	0.945	2.36	**Conc. (µg/ml)**	0.345	1.73	4.32
***Mean Ratio**	0.024	0.223	0.540	***Mean Ratio**	0.062	0.418	0.989
SD	0.004	0.002	0.0145	**SD**	0.009	0.005	0.006
CV (%)	4.98	0.97	2.68	**CV (%)**	15.7	1.22	0.61
Isosilybin A (n=6)				**Isosilybin B (n=6)**			
Conc. (µg/ml)	0.082	0.411	1.027	**Conc. (µg/ml)**	0.064	0.320	0.640
***Mean Ratio**	0.013	0.093	0.235	***Mean Ratio**	0.008	0.055	0.125
SD	0.0010	0	0.0121	**SD**	0.001	0.0013	0.001
CV (%)	8.01	0.317	5.17	**CV (%)**	11.64	2.43	0.88

*Mean Ratio=Mean peak area ratio of analyte to internal standard
Conc.= Concentration; SD=Standard Deviation; CV=Coefficient of Variation

Table 3.7: Inter-day Precision

	Silycristin (n=3)				Silydianin (n=3)			
Day 1	**Conc. (µg/ml)**	***Mean Ratio**	**SD**	**CV (%)**	**Conc. (µg/ml)**	***Mean Ratio**	**SD**	**CV (%)**
	1.22	0.119	0.0045	4.07	0.09	0.0053	0.0001	3.47
	3.06	0.339	0.321	6.80	0.452	0.033	0.0027	8.12
	6.13	0.725	0.0300	4.14	0.905	0.049	0.006	13.07
Day 2	1.22	0.1088	0.0073	6.70	0.09	0.010	0.001	13.97
	3.06	0.283	0.0054	1.91	0.452	0.026	0.0001	0.58
	6.13	0.609	0.027	4.53	0.905	0.065	0.010	15.40
Day 3	1.22	0.134	0.013	10.35	0.09	0.0026	0.0001	6.54
	3.06	0.227	0.004	1.77	0.452	0.015	0.0012	7.78
	6.13	0.688	0.009	1.44	0.905	0.035	0.002	5.98
	Silybin A (n=3)				**Silybin B (n=3)**			
Day 1	**Conc. (µg/ml)**	***Mean Ratio**	**SD**	**CV (%)**	**Conc. (µg/ml)**	***Mean Ratio**	**SD**	**CV (%)**
	0.189	0.024	0.004	4.98	0.345	0.063	0.009	15.7
	0.945	0.223	0.0021	0.97	1.73	0.418	0.0051	1.82
	2.36	0.540	0.0145	2.68	4.32	0.989	0.0060	0.61
Day 2	0.189	0.024	0.001	4.98	0.345	0.056	0.005	8.89
	0.945	0.095	0.002	2.65	1.73	0.393	0.018	4.72
	2.36	0.249	0.003	1.36	4.32	0.898	0.008	0.895
Day 3	0.189	0.043	0.003	8.68	0.345	0.068	0.008	12.65
	0.945	0.221	0.012	5.57	1.73	0.411	0.001	0.268
	2.36	0.519	0.005	0.96	4.32	0.952	0.001	1.19

Mean Ratio=Mean peak area ratio of analyte to internal standard
Conc.= Concentration; SD=Standard Deviation; CV=Coefficient of Variation

Table 3.7: Intra-day Precision (Contd.)

	Isosilybin A (n=3)				Isosilybin B (n=3)			
	Conc. (µg/ml)	***Mean Ratio**	**SD**	**CV (%)**	**Conc. (µg/ml)**	***Mean Ratio**	**SD**	**CV (%)**
Day 1	0.082	0.013	0.001	8.01	0.064	0.008	0.001	11.64
	0.411	0.093	0.0002	0.317	0.320	0.055	0.0013	2.43
	1.027	0.235	0.0121	5.17	0.640	0.125	0.0011	0.88
Day 2	0.082	0.011	0.0005	4.58	0.064	--	--	--
	0.411	0.093	0.0002	0.397	0.320	0.055	0.001	2.32
	1.027	0.235	0.012	5.17	0.640	0.126	0.011	9.06
Day 3	0.082	0.009	0.001	11.67	0.064	0.008	0.0005	6.90
	0.411	0.089	0.001	1.67	0.320	0.047	0.001	3.01
	1.027	0.227	0.004	1.96	0.640	0.107	0.018	17.04

Mean Ratio=Mean peak area ratio of analyte to internal standard
Conc.= Concentration; SD=Standard Deviation; CV=Coefficient of Variation

3.3.3.3: Accuracy and Recovery

The method was found to be accurate according to the guidelines set by the FDA. The extraction efficiency of the isomers from rat plasma was also found to be within acceptable limits. Data for accuracy and extraction recovery is shown in Table 3.8.

Table 3.8: Accuracy and Recovery Data for Silymarin Isomers

	Std. Conc. (µg/mL)	Conc.Added (µg/mL)	Conc. Found, n=3 (µg/mL)	Accuracy(%)	%CV	Extraction Recovery(%)
Sc	3.06	0.61	0.609 ±0.008	-0.59	1.35	99.40
		1.84	2.07 ±0.14	12.89	6.78	112.88
		7.97	8.43 ±0.155	5.89	1.84	105.89
Sd	0.45	0.09	0.095 ±0.010	5.38	10.62	105.38
		1.17	1.26 ±0.09	8.00	7.84	107.99
SbA	2.36	0.47	0.47 ±0.023	0.10	4.91	100.10
		1.41	1.47 ±0.204	4.06	13.82	104.06
		6.14	6.28 ±0.338	2.31	5.28	102.31
SbB	4.32	0.86	0.79 ±0.093	-8.21	11.79	91.79
		2.59	2.78 ±0.230	7.21	8.27	107.21
		11.24	10.87 ±0.162	-3.31	1.49	96.69
ISbA	1.02	0.21	0.242 ±0.01	17.58	5.49	117.58
		0.62	0.61 ±0.063	-0.15	10.24	99.85
		2.67	2.65 ±0.24	-0.93	9.13	99.07
ISbB	0.32	0.06	0.088 ±0.004	7.00	5.18	107.00
		0.19	0.196 ±0.020	2.09	9.99	102.09
		0.83	0.857 ±0.018	2.88	2.17	102.88

3.3.3.4: Limit of Detection and Quantitation

The limits of detection (LOD) and limit of quantitation (LOQ) for the silymarin isomers are specified in Table 3.9. The LOQ %CV was ≤11% for all the isomers.

Table 3.9: LOD and LOQ for the silymarin isomers

	Sc	Sd	SbA	SbB	ISbA	ISbB
LOD (µg/mL)	0.03	N/A	0.02	0.02	0.03	0.03
LOQ (µg/mL)	0.06	0.08	0.05	0.05	0.05	0.06

The LOD for Silydianin was tested at 0.03, 0.04, 0.05µg/mL and the peak obtained at all the three concentrations was debatable and without any visual precision. On the contrary, at 0.08µg/mL the peak for Silydianin could be detected and quantified. Thus, only a LOQ for Silydianin was assigned at 0.08µg/mL.

3.3.3.5: Stability

Silymarin isomers in plasma when subjected to 3 freeze-thaw cycles, upon analysis did not show any significant degradation (<5%). Plasma samples spiked with silymarin, post sample preparation were stable for 24 hours. At the end of 36 hours, this aliquot showed light precipitation settling at the bottom of the sample vial, but analysis results did not show any degradation of silymarin isomers, suggesting precipitation of plasma proteins.

Stock solutions of silymarin in methanol and internal standard (1-naphthol) in methanol were also stable for 24 hours at room temperature.

3.4: STANDARDIZATION OF THE EXTRACT

Standardization of extract involved assaying two products obtained from Sigma Aldrich prior to their use in our experimental work. The first product was a mixture silymarin diastereomers, Silybin A&B only and the second product was the pure silymarin extract that contained all the six silymarin isomers. Silybin USP containing Silybin A&B was the only USP reference standard available prior to Caco-2 cell transport studies, while Silybin USP and Silydianin USP were available prior to *in vitro* dissolution and assay studies. Silymarin USP was available only prior to the start of *in vivo* studies in rats.

Assay of silybin (A&B) and silymarin obtained from Sigma Aldrich was determined by HPLC and calculated by the method of area normalization

The certificate of analysis for Silybin USP indicated an assay of Silybin equal to 94.00%, without any individual diastereomeric content. Assay of Silybin USP by area normalization indicated a total assay of Silybin (A&B) equal to 94.00% comprising of 44.91% Silybin A and 49.08% Silybin B.

Assay of Silybin obtained from Sigma Aldrich was confirmed by area normalization and was calculated as 99.46% comprising of 48.00% Silybin A and 51.46% Silybin B. Silybin USP was used as a reference to confirm the retention times of the diastereomers during method development.

Assay of silymarin obtained from Sigma Aldrich was determined by HPLC and calculated using the method of area normalization. Table 3.10 shows the individual assay of each isomer in silymarin.

Assay and Chromatographic Conditions: An accurately weighed quantity of silymarin extract was dissolved in methanol so as to obtain a concentration of 100µg/mL. This solution was filtered using a 0.45µm nylon membrane and 10µL of the clear solution was injected onto the HPLC was analysis. The assay used the following chromatographic conditions:

Table 3.10: Chromatographic Conditions for Determination of Isomer Proportions in Silymarin Extract

Method	Reverse Phase, Binary Gradient
Column Type & Dimensions	Phenomenex, Luna C-18 (2)
Mobile Phase	Water (A); Methanol (B) %B: 20-65 in 40 minutes
Total Flow Rate	1 mL/minute
Detection	Ultraviolet; 288nm
Injection Volume	10 µL
Run Time	Analysis: 40 minutes Equilibration: 10 minutes
Temperature	Ambient

The results of the assay of individual isomers in the silymarin extract are shown in Table 3.11.

Table 3.11: Assay of Silymarin Isomers by Area Normalization

Silymarin isomer	Average Assay % (n=6)	SD
Silycristin (Sc)	24.52	0.312
Silydianin (Sd)	3.61	0.278
Silybin A (SbA)	18.90	0.263
Silybin B (SbB)	34.58	0.264
Isosilybin A (ISbA)	8.22	0.037
Isosilybin B (ISbB)	2.56	0.026
TOTAL	92.42	0.312

SD= Standard Deviation

3.5: ASSAY OF SILYMARIN ISOMERS IN VARIOUS CHEMICAL MATRICES

(CaCo-2 Cell Transport Buffer, Tablets, Capsules *& in vitro* Dissolution Media, Equilibrium Solubility and Partition Coefficient Media)

The HPLC method used for the quantitation of silymarin isomers in rat plasma was used to analyze the isomers in different chemical matrices, with minor modifications in the gradient program, flow rate and the volume of injection. The type of HPLC column, mobile phase (methanol + water), the wavelength of detection (UV, 288nm) and temperature were kept constant. The sample preparation method and the chromatographic specifications for each chemical matrix are explained in the following sections. Due to the limited solubility of the silymarin extract, Caco-2 cell transport experiments involved were carried out using only the Silybin A&B mixture obtained from Sigma Aldrich.

3.5.1: Assay of Silybin A and B in Caco-2 Cell Transport Media

3.5.1.1: Sample Preparation

After the transport experiments, aliquots of transport buffer from the basal chamber were mixed with an appropriate quantity of internal standard (1-naphthol in methanol) and filtered using a 0.45µ nylon membrane before injecting on to a HPLC for analysis.

3.5.1.2: Chromatographic Conditions

Table 3.12: Chromatographic Conditions for the Quantitation of Silybin A&B in a CaCo-2 Cell Transport Media

Method	Reverse Phase, Binary Gradient
Column	Phenomenex, Luna C-18 (2)
Mobile Phase	Water (A); Methanol (B) %B: 20-65 in 40 minutes
Total Flow Rate	1 mL/minute
Detection	Ultraviolet; 288nm
Injection Volume	10 µL
Run Time	Analysis: 40 minutes Equilibration: 10 minutes
Temperature	Ambient

Figures 3.3-3.5 are representative chromatograms for the matrix blank, the internal standard alone, and the separation of silybin A, silybin B and internal standard.

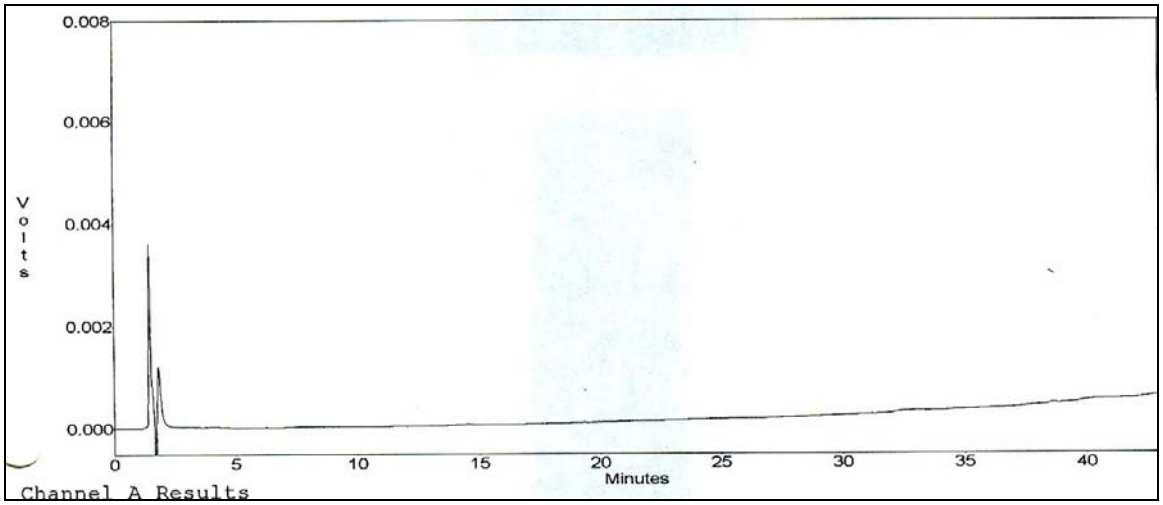


Figure 3.3: Sample Chromatogram- Blank CaCo-2 Cell Transport Media

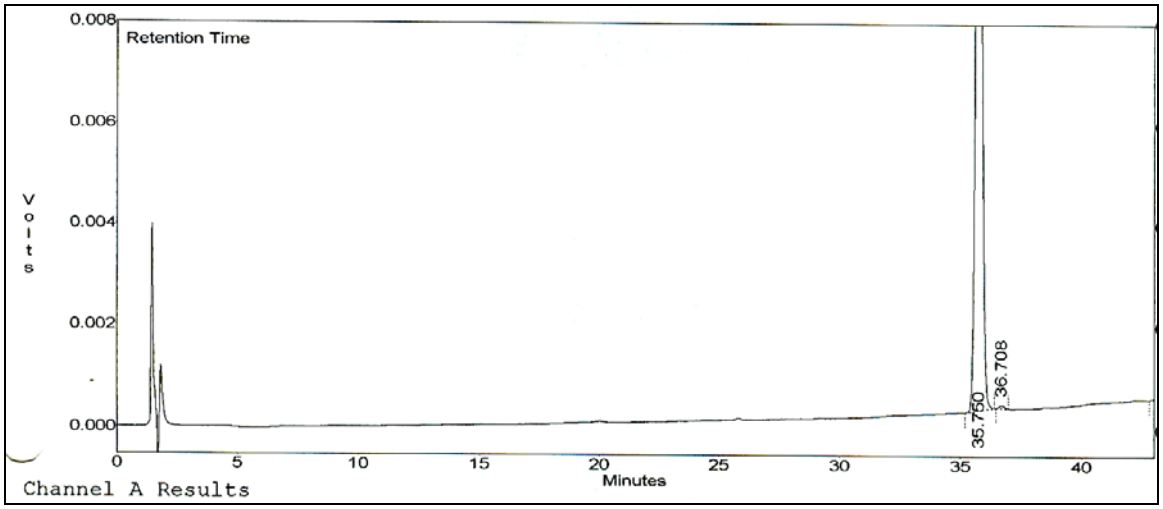


Figure 3.4: Sample Chromatogram- Internal Standard (1-Naphthol 10µg/mL; 35.7 mins) in CaCo-2 Cell Transport Media

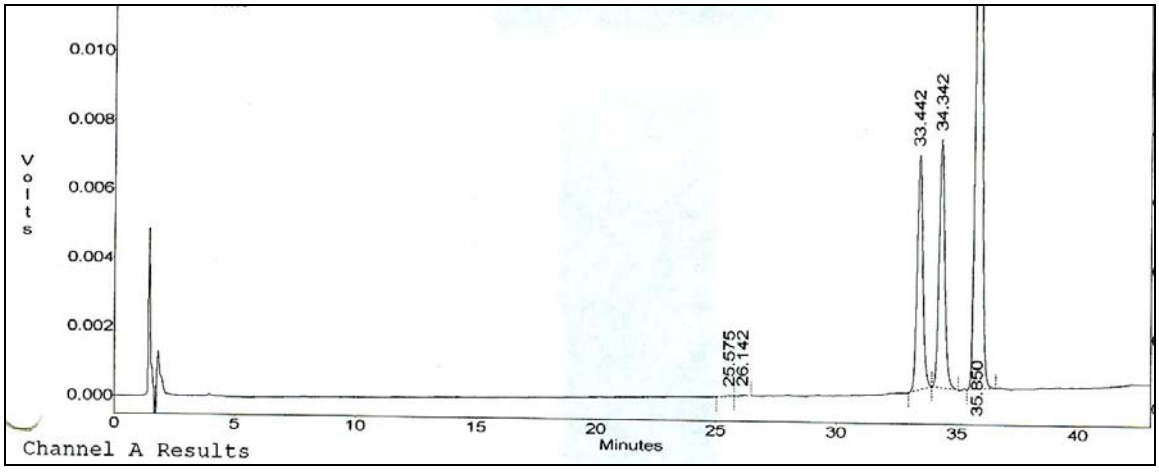


Figure 3.5. Sample Chromatogram-Separation of Silybin A and Silybin B in CaCo-2 Cell Transport Media. (Silybin A: 33.44 minutes; Silybin B: 34.34 minutes; Internal Standard: 35.85 minutes

3.5.2: Assay of Silymarin Isomers in Product Formulations, In Vitro Dissolution media and Equilibrium Solubility and Partition Coefficient Media

Assay and in vitro dissolution testing of silymarin isomers in commercial formulations was done only for Silybin A, Silybin B and Silydianin only. Equilibrium solubility and the experimental octanol-water partition coefficient values were determined for all the isomers in the silymarin extract.

3.5.2.1: Sample Preparation

(i) Assay of Silybin A, Silybin B and Silydianin in Product Formulations: Powdered sample approximately equal to 0.1g of silymarin was accurately weighed and transferred to a 100 ml volumetric flask. After the addition of 50 ml of methanol, the

solution was sonicated for around 20 minutes and then the volume made up to 100ml with methanol. This solution was then filtered through a 0.45µ nylon membrane, and 2 ml of clear aliquot was transferred to a 10 ml volumetric flask. 1 ml of internal standard (1-naphthol; 75µg/ml prepared in methanol) was added to this solution and the volume made up to 10ml with methanol. 10µL of this solution was injected directly into the HPLC.

(ii) Quantitation of Silybin A, Silybin B and Silydianin in In Vitro Dissolution Media: A sufficient volume of sampled aliquot was filtered through 0.45µ nylon membrane and to 1 ml of clear filtrate was added 0.5ml of internal standard (1-naphthol, 420µg/mL) and 0.5ml of 0.5% ortho phosphoric acid. This solution was then injected into the HPLC for analysis.

(iii) Partition Coefficient and Equilibrium Solubility Media: A sufficient volume of octanol aliquot was filtered through 0.45µ nylon membrane and to 0.1 ml of this clear aliquot was added 0.1 ml of internal standard prepared in methanol. 20µL of this solution was injected into the HPLC for analysis. Different dilutions of the aliquots were required, due to the high concentrations of silymarin isomers being distributed into the octanol phase. Sample preparation was similar for the water phase of the partition coefficient media and phosphate buffer pH 7.2 used in the determination of the equilibrium solubility of silymarin isomers.

The solutions of the internal standard were prepared in such a way that the final concentration of the internal standard in the sample solution was 75µg/mL.

3.2.1.2.2: Chromatographic Conditions

Chromatographic conditions for the quantitation of isomers during assay, *in vitro* dissolution, equilibrium solubility and partition coefficient experiments are shown in Table 3.13 and a sample chromatogram showing the separation of all the isomers along with the internal standard is shown in Figure 3.6.

Table 3.13: Chromatographic Conditions for the Quantitation of Silymarin Isomers in Assay, In Vitro Dissolution, Equilibrium Solubility and Partition Coefficient Experiments.

Method	Reverse Phase, Binary Gradient
Column Type & Dimensions	Phenomenex, Luna C-18 (2)
Mobile Phase	Water (A); Methanol (B) %B: 20-60 in 36 minutes 60-65 in 45 minutes
Total Flow Rate	1 mL/minute
Detection	Ultraviolet; 288nm
Injection Volume	10 µL/20µL
Run Time	Analysis: 45 minutes Equilibration: 10 minutes
Temperature	Ambient

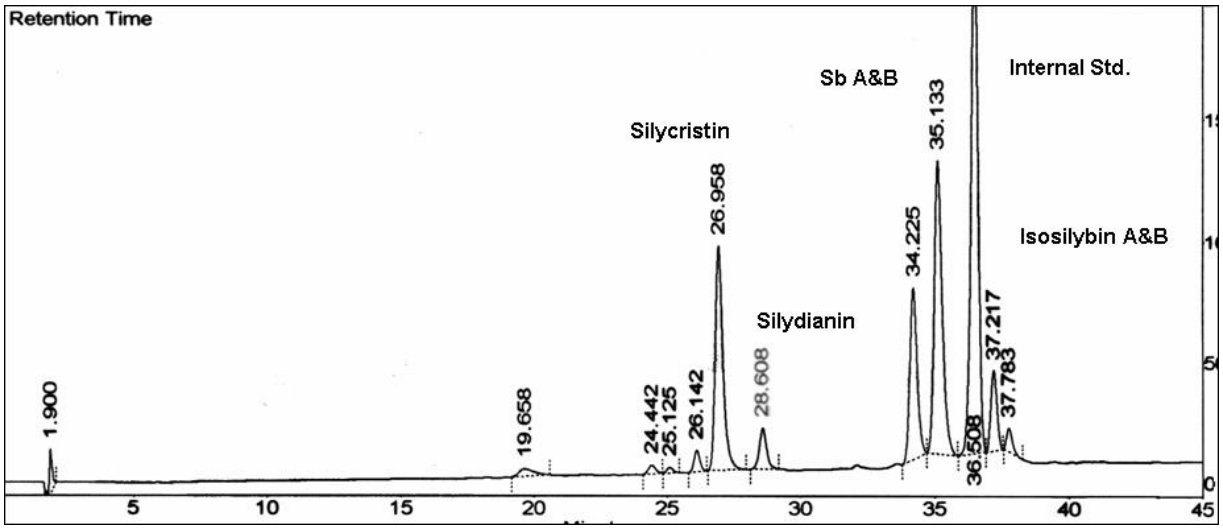


Figure 3.6. Sample Chromatogram for the Separation of Silymarin Isomers in Dissolution Media (Silycristin: 26.95 minutes; Silydianin: 28.60 minutes; Silybin A: 34.22 minutes; Silybin B: 35.13 minutes; Internal Standard: 36.50 minutes; Isosilybin A: 37.21 minutes; Isosilybin B: 37.78minutes

3.6: SUMMARY:

A specific, sensitive and reproducible reverse phase, binary gradient HPLC method was developed and validated for the quantitation of six silymarin isomers (silycristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B) in various chemical matrices and in rat plasma. The method used for the quantitation of the isomers in rat plasma was validated and applied for the determination of silymarin isomers in other chemical matrices. The method had an approximate analysis runtime of 45 minutes and the process of extraction of the isomers especially from rat plasma was very simple and accompanied with high extraction efficiency. This method can be confidently applied for the routine assay and in vitro dissolution of silymarin isomers in various milk thistle market formulations and also for the quantitation of isomers in rat plasma.

CHAPTER 4: EQUILIBRIUM SOLUBILITY, IN VITRO DISSOLUTION AND PARTITION COEFFICIENT OF SILYMARIN ISOMERS

This chapter describes the determination of equilibrium solubility, apparent partition coefficient and *in vitro* dissolution of silymarin isomers in extract and in market formulations, respectively. The apparent octanol water partition coefficients are compared to the predicted values (CLogP) for verification of prediction. Based on their equilibrium solubility and partition coefficient (calculated and experimental) values the silymarin isomers are classified according to the Biopharmaceutics Classification System. [143] The model drug metoprolol is used as a reference to classify the isomers into the high permeability and low permeability class.

The assay content of silymarin isomers (silybin A, silybin B and silydianin) was determined for three selected market formulations to check variation between dosage forms having the same label claim. *In vitro* dissolution experiments were performed to investigate the release of silybin A, silybin B and silydianin from a select formulation.

4.1: EQUILIBRIUM SOLUBILITY AND BIOPHARMACEUTICS CLASSIFICATION SYSTEM

The Biopharmaceutics Classification System (BCS) [143] is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability. When combined with the dissolution of the drug product, the BCS takes into account three major factors that govern the rate and extent of drug absorption from IR solid oral dosage forms: dissolution, solubility, and intestinal permeability.

According to the BCS, drug substances are classified as follows:

Class 1: High Solubility – High Permeability

Class 2: Low Solubility – High Permeability

Class 3: High Solubility - Low Permeability

Class 4: Low Solubility - Low Permeability

In addition, immediate release solid oral dosage forms are categorized as having rapid or slow dissolution. Within this framework, when certain criteria are met, the BCS can be used as a drug development tool to help justify waivers for bioequivalence studies.

Determination of Solubility Class: The solubility class boundary for the silymarin isomers was designated as shown in Table 4.1. [144] A drug substance was considered highly soluble when the highest dose strength is soluble in 250 ml or less of aqueous media over the pH range of 1-7.5. The volume estimate of 250 ml was derived from typical bioequivalence study protocols that prescribe administration of a drug product to fasting human volunteers with a glass (about 8 ounces) of water. The dose number (D_0) was calculated using the following equation:

$$D_0 = \frac{M_0 V_0}{C_s} \qquad \textbf{Equation 4.1}$$

where,

M_0=highest dose strength in milligrams

V_0=250mL

C_s=Solubility in mg/mL

Drugs with dose numbers of ≤1 were classified as high-solubility drugs. Conversely, drugs with dose numbers of >1 were classified as low solubility drugs.

Table 4.1: Solubility Definitions

Descriptive Solubility Term	Part of solvent required for 1 part of solute	Solubility Range (mg/mL)	Solubility Assigned
Very Soluble (vs)	<1	≥10000	1000
Freely Soluble (fs)	From 1 to 10	100-1000	100
Soluble	From 10 to 30	33-100	33
Sparingly Soluble (sps)	From 30 to 100	10-33	10
Slightly soluble (ss)	From 100 to 1000	1-10	1
Very Slightly Soluble (vss)	From 1000 to 10000	0.1-1	0.1
Practically Insoluble (pi)	≥10000	<0.1	0.01

Determination of Permeability Class: The permeability class of the isomers needs to be determined in order to classify them in to the low permeability and high permeability class. According to the BCS guidance, permeability class can be determined in humans by using the mass balance method, absolute bioavailability or intestinal perfusion method. Non human methods to determine intestinal permeability would be *in situ* or intestinal perfusion techniques in an animal model, or in vitro permeability methods involving monolayer of suitable epithelial cells. When a single *in vitro* method fails to conclusively demonstrate the permeability class, a second method such as the partition coefficient derived experimentally (using the right solvent systems)

or determined by calculations from the structure of the drug molecule (CLogP) can be used.

Silymarin isomers in the following work are classified in to high permeability and low permeability classes by comparing their experimental and calculated partition coefficient values to model drug metoprolol. Isomers which were more lipophilic than metoprolol will be classified as high permeability compounds and like wise.

4.2: ESTIMATION OF EQUILIBRIUM SOLUBILITY

The following section discusses the estimation of saturation solubility of six silymarin isomers silycristin, silydianin, silybin A & B and isosilybin A & B. There have been no published reports on the estimation of saturation solubility of each of the mentioned isomers of silymarin. Koch et al. studied the solubility of silybin (A+B) as a single isomer, silydianin and silycristin in water as a function of increasing temperature and pH. [145] They found that aqueous solubility of silybin, silydianin and silycristin increased with the increasing temperature, respectively, and the heats of solution were found to be as follows: silybin (2.607 kcal/mol), silydianin (7.154 kcal/mol), silycristin (2.005 kcal/mol).

4.2.1: Experimental Design

Saturation solubility for the silymarin isomers was estimated at 37°C in phosphate buffer (0.2M) at pH 7.2, representative of the human small intestine.

Preparation of pH 7.2 buffer: Accurately weighed, 6.9g of monobasic sodium phosphate and 1.51g of sodium hydroxide were completely dissolved in 1 liter of distilled

water. The pH of the solution was adjusted to 7.2 by adding drop wise, either 1N sodium hydroxide or 1N hydrochloric acid as required.

Determination of Equilibrium Solubility: The following procedure was used to estimate the equilibrium solubility of the silymarin isomers. An excess of compound was placed in a vial containing the pH 7.2 phosphate buffer (0.2M). The vial was then allowed to equilibrate in a shaking water bath maintained at 37°C for 16 hours. At the end of 16 hours aliquots of the supernatant solution were withdrawn and filtered using a pre-warmed syringe filter (37-40°C) and analyzed for drug content. The HPLC method used for the quantitation of silymarin isomers is described in detail in Chapter 3 of this dissertation. The equilibration process was continued until successive aliquots withdrawn were similar in drug content indicating equilibrium which allowed the estimation of equilibrium solubility.

Quantitation of Silymarin Isomers: Quantitative determination of the silymarin isomers was accomplished using a reverse phase HPLC method with UV detection at 288 nm. Concentration range of the calibration curves used to determine the amount of each dissolved isomer were as follows: Silycristin: 1.2- 17 µg/mL; silydianin: 0.16-2.49 µg/mL; silybin A: 0.96-13.8 µg/mL; silybin B: 1.76-25.2 µg/mL; isosilybin A: 0.41-6.0 µg/mL; isosilybin B: 0.13-1.87 µg/mL. Each standard curve concentration and sample was injected in triplicate into the HPLC and the relative standard deviation was found to be no more than 7% for all the injections.

4.2.2: Results and Discussion

Table 4.1 shows the equilibrium solubility for each silymarin isomer at 37°C and at a pH of 7.2.

Table 4.2: Equilibrium Solubility for Silymarin Isomers at 37°C and pH 7.2.

Silymarin Isomer	M_0 (mg)	Equilibrium Solubility (C_s) (mg/mL)	Dose Number* $D_0 = \frac{M_0 V_0}{C_s}$	BCS Solubility Class*
Silycristin (Sc)	34.52	0.0155	$5x10^5$	LS
Silydianin (Sd)	5.05	0.0191	$6.6x10^4$	LS
Silybin A (SbA)	26.46	0.0033	$2x10^6$	LS
Silybin B (SbB)	48.41	0.0077	$1.5x10^6$	LS
Isosilybin A (ISbA)	11.50	0.00094	$3x10^6$	LS
Isosilybin B (ISbB)	3.58	0.00045	$1.9x10^6$	LS
Metoprolol (Reference Drug)	**100**	**1000**	**0.0004**	**HS**

M_0 =Maximum dose strength in 140mg of extract; V_0= 250mL; HS=High Solubility; LS= Low Solubility

*D_0>1 = LS; $D_0 \leq 1$= HS

Results shown in Table 4.2 indicate that silydianin is the most soluble isomer and isosilybin B is the least soluble isomer. The solubility rank for the isomers decreases in the order Sd>Sc>SbB>SbA>ISbA>ISbB. Koch et al. [145] studied the solubility of silymarin isomers silybin, silydianin and silycristin and found that the solubility decreases in the order silycristin>silydianin>silybin. This trend is similar to the trend reported here, though the solubility experiments done here are only at 37°C. Also it

should be noted that Koch et al. found the solubility of silybin isomer as a single compound without separating them into their respective diastereomers.

Based on the calculation of dose number (D_0) it can be seen that all the silymarin isomers fall into the Low Solubility (LS) class. This is because D_0 for all the isomers is much greater than 1 which means that the silymarin isomers are practically insoluble. The maximum dose strength (M_0) was calculated by considering the amount of each isomer present in the extract as found from Table 3.11 in Chapter 3, where the standardization if the silymarin extract is explained.

4.2: DETERMINATION OF APPARENT OCTANOL-WATER PARTITION COEFFICIENT

The octanol-water partition coefficient provides a thermodynamic measure of the tendency of a substance to prefer a non aqueous or oily phase rather than water (the hydrophilic/hydrophobic balance). Thus, the partition coefficient is the ratio of the concentrations of unionized compounds between the oil phase and the aqueous phase. To measure the partition coefficient of ionizable solutes, the pH of the aqueous phase is adjusted such that the predominant form of the compound is unionized. The logarithm of the ratio of the concentrations of the unionized solute in the two phases is known as LogP (Equation 4.1).

$$LogP_{\text{Octanol/ Water}} = Log\left(\frac{[Solute]_{\text{Octanol}}}{[Solute]^{\text{Unionized}}_{\text{Water}}}\right) \qquad \textbf{Equation 4.1}$$

The 1-octanol-water system is an established system for simulating the partitioning between biological membranes and their natural environment, particularly

water saturated 1-octanolic phase which is highly structured. [146] Also, 1-octanol is an amphiprotic solvent having hydrogen bond donor and acceptor properties. Water saturated 1-octanol is a reasonably good solvent for many organic compounds while other organic solvents have a more limited range due to solubility problems. A further reason to 1-octanol-water partition coefficients is the fact that large compilations of experimental log P values are available, various theoretical approaches to estimate log P values are based on this solvent system and that theories referring to drug absorption as presently reviewed are all based on this system.

Octanol-water partition coefficient for the silymarin isomers was determined using the classical shake flask (tube) method. This was done by placing an excess of silymarin extract which contained the 6 isomer, in a vial containing equal amounts of 1-octanol and water, which in turn was placed in a shaking water bath maintained at 37°C. The water-1-octanol mixture used for the experiments was equilibrated by shaking for 24 hours at 37°C, before the addition of the extract for partition coefficient determination. The compound was allowed to partition between the 1-octanol and water phase for 16 hours by shaking at 37°C. At the end of 16 hours, samples were withdrawn from each layer, aqueous and organic and analyzed by HPLC for isomer content. The log ratio (LogP) of the concentrations of isomer content in octanol and water was calculated as per Equation 4.1.

4.2.1: Results and Correlation to Predicted Octanol-Water Partition Coefficient (CLogP)

The experimental results obtained for the six silymarin isomers were compared to their predicted 1-octanol-water partition coefficient (CLogP) values calculated, using the program CLogP in Chapter 2 of this dissertation. The values and the correlation results are shown here in Table 4.3.

Table 4.3: Apparent LogP and CLogP values for the Silymarin Isomers

Silymarin Isomer	Mol. Weight	CLogP (Calculated)	Apparent LogP (Experimental)	BCS Permeability Class	BCS Solubility Class	BCS Class**
Silycristin	482.44	1.38	1.44	CLogP=HP Expt. LogP=LP	LS	II/IV
Silydianin	482.44	-0.39	1.05	LP	LS	IV
Silybin A	482.44	1.95	1.97	HP	LS	II
Silybin B	482.44	1.94	1.87	HP	LS	II
Isosilybin A	482.44	1.94	2.19	HP	LS	II
Isosilybin B	482.44	1.94	1.95	HP	LS	II
$^{*}R^2$		0.835				
Metoprolol* (Reference Drug)		**1.35**	**1.72**	**Reference**	**HS**	

Metoprolol =Reference standard for Permeability;
*= R^2 for CLogP and Experimental LogP of silymarin isomers
HP=High Permeability; LP= Low Permeability
**I =High Permeability-High Solubility
II=High Permeability-Low Solubility
III=Low Permeability-High Solubility
IV=Low Permeability-Low Solubility

Table 4.3 also shows the BCS classification of silymarin isomers based on the solubility class obtained from the dose number (D0) and the permeability class obtained by using the partition coefficient of metoprolol as a reference standard. Isomers having a partition coefficient higher than metoprolol were classified as highly permeability and those having a partition coefficient lower than metoprolol were classified as low permeability. Based on its calculated partition coefficient (CLogP) silycristin is classified as a highly permeable compound and based on experimental LogP it is classified as a low permeability compound. Since experimental values are always considered more accurate than calculated values, we classify silycristin as a low permeability compound. Thus, silydianin and silycristin are the only BCS class IV compounds meaning that they belong to the Low-Permeability and Low-Solubility Class. All the other 4 isomers silybin A, silybin B, isosilybin A and isosilybin B belong to the BCS Class II i.e. High Permeability-Low Solubility.

Koch et al. [147] determined the true partition coefficients for silybin, silydianin and silycristin and got values similar to each other for the three isomers (LogP~ 1.08). They also found that the partition coefficient values increased with increasing pH. Though the values found by us are not identical to the values of LogP determined by Koch et al., the estimates make sense considering that all these are either isomers or diastereomers, and they also have a reasonable correlation (r^2=0.835) with the CLogP values calculated theoretically. Also Koch et al. determined the partition coefficient of silybin diastereomers as a single compound where as we have determined the partition coefficient of both the diastereomers (silybin A and silybin B) separately.

4.3: ASSAY AND IN VITRO DISSOLUTION STUDIES

This section discusses the assay and *in vitro* dissolution tests carried out for the silymarin isomers. Out of the six isomers, only silybin A, silybin B and silydianin were quantified due to the lack of pure reference extract of silymarin and lack of pure individual standards for silymarin isomers other than silybin A and silybin B. Pure reference standards for diastereomers silybin A&B and silydianin were obtained from the USP.

4.3.1: Previous Related Studies

A standardized extract of silymarin should contain not less than 80% of silymarin isomers (flavonolignans) in the extract. Silymarin formulations (tablets and capsules) found on the market have a label claim of 140mg of standardized silymarin. The United States Pharmacopoeia specifies that a standardized extract of Milk thistle (also known as Powdered Milk thistle extract in the USP) should contain not less than 90% and not more than 110% of the labeled amount of silymarin calculated as silybin, and consisting of not less than 20% and not more than 45% for the sum of silydianin and silycristin and not less than 40% and not more than 65% for the sum of silybin A and silybin B and not less than 10% and not more than 20% for the sum of Isosilybin A and Isosilybin B. USP limits for content of silymarin in dosage forms (tablets and capsules) are not less than 90 percent and not more than 110 percent of the labeled amount of silymarin as silybin calculated as the sum of the six isomers silycristin, silydianin, silybin A, silybin B,

isosilybin A and isosilybin B. USP limits for *in vitro* dissolution of dosage forms are not less than 75 percent of the labeled amount of silymarin as silybin in 45 minutes.

4.3.2: Assay and Dissolution Methodology

Three commercially available oral dosage forms of silymarin were analyzed for isomer content and *in vitro* release profile of silybin A, silybin B and silydianin. The criteria for selection of market products were:

(i) Milk thistle should be the only herbal extract present in the formulation and should not exist as a combination product with any other herbal extract or dietary supplement.

(ii) Should be in the form of an oral dosage unit such as a capsule, tablet or a caplet intended to be swallowed with water.

(iii) The formulation should not contain any solubility aid or ingredient such as surfactant nor should the dosage form or extract be altered with an aim to increase oral absorption after oral administration.

(iv) Each dosage unit was required to have a label claim of 140 mg of silymarin, which is the standardized dose of silymarin.

Based on these criteria, formulations manufactured by Company A, Company B and Company C were randomly selected from the market and the same lot of each product was used for the assay and dissolution studies as shown in Table 4.4.

The analytical method specifications for the assay of diastereomers silybin A, silybin B and silydianin are described in section 3.2.1.2 of this dissertation. Though the

analytical method could separate and quantify all the isomers, only silybin A, silybin B and silydianin could be assayed due to their availability as pure reference standards.

Table 4.4: Dosage Forms for Assay and Dissolution Studies

Manufacturer	Lot number	Dosage Form	Silymarin Label Claim (mg)
Company A	32070901	Capsule	140
Company B	429676	Capsule	140
Company C	235467	Capsule	140

Weight variation for each product was determined by individually weighing 20 filled capsules and their respective empty shells, thus giving the weight of the powder contained in each capsule. The filled weight, the standard deviation and the relative standard deviation (%) for each product is shown in Table 4.5

Table 4.5: Weight Variation for the Silymarin Dosage Forms

Manufacturer	Average Weight of powder±SD (g) (n=20)	CV (%)
Company A	0.510 ±0.012	2.37
Company B	0.459 ±0.009	2.01
Company C	0.396 ±0.006	1.72

CV= Coefficient of variation; SD= Standard deviation

As seen from Table 4.5, the % RSD for the capsule powder content for each of the three manufacturers was not more than 3% indicating that the capsules were uniformly filled and passed USP specification for weight variation.

The assay for the content of silybin A, silybin B and silydianin in capsules was done by extracting the isomers in methanol and analyzing them using HPLC with 1-naphthol as an internal standard.

4.3.2.1: Assay of Silybin A, Silybin Band Silydianin

Preparation of Standard Silybin A and Silybin B: Silybin USP contains silybin A (44.91%) and silybin B (49.09%). An accurately weighed quantity of silybin USP was dissolved in methanol (HPLC grade) to obtain a stock solution from which 3 concentrations of silybin A and silybin B could be prepared. The three concentrations of silybin A were: 1.18µg/mL, 11.85µg/mL, 79.04µg/mL and that of silybin B were: 1.29µg/mL, 12.9µg/mL, and 86.39µg/mL. 1-naphthol was used as the internal standard to calculate the principle peak to internal standard area ratio. The area ratio (drug/internal standard) versus concentration was plotted for each isomer. A linear relationship was obtained with an r^2 of 0.999 and the content of each isomer was calculated by considering the highest concentration on the calibration curve as a standard (79.04µg/mL for silybin A and 86.39µg/mL for silybin B).

Preparation of Standard Silydianin: Silydianin USP contains 100 percent silydianin. An accurately weighed quantity of silydianin USP was dissolved in methanol

(HPLC grade) to obtain a stock solution from which 4 concentrations of Silydianin (3.4, 5.6, 17 and 51µg/mL) could be prepared. 1-naphthol was used as the internal standard to calculate the principle peak to internal standard area ratio. The area ratio (drug/internal standard) versus concentration for the isomer was plotted to obtain a linear relationship with an r^2 of 0.999. The third highest concentration on the calibration curve (17µg/mL) was then considered as a standard for calculating the assay of silydianin in milk thistle formulations.

4.3.2.2: Dissolution Methodology for Silybin A, Silybin B and Silydianin

In vitro dissolution testing was performed as per the procedure described in USP XXVI, method 2 (paddle method). Nine hundred milliliters of pH 7.5 phosphate buffer with 2% sodium lauryl sulfate was used as the dissolution medium. The temperature of the medium was maintained at 37±0.1°C. The stirring rate of the paddle was set at 100 revolutions per minute. One capsule was placed in the medium of each of the dissolution vessels. In order to keep the capsule at the bottom of the vessel a sinker made of stainless steel metal wire was fixed uniformly to each of the capsules. Samples at fixed time intervals were withdrawn with a syringe and filtered through a 0.45µ nylon membrane. Samples were collected at 10, 20, 30, 40, 50 and 60 minutes. Equal volume of medium was replaced after each sample withdrawal and the volume changes were taken into consideration to calculate the actual amount dissolved. Samples were immediately analyzed using reverse phase HPLC with UV detection at 288 nm after the addition of an appropriate concentration of internal standard, 1-naphthol. The details of this method are explained in section 3.2.1.2 of chapter 3 of this dissertation.

Preparation of Dissolution Medium: 41.4g of monobasic sodium phosphate and 9.1g of sodium hydroxide were accurately weighed and completely dissolved in 6 liters of distilled water. The pH of the solution was adjusted to 7.5 by adding drop wise, either 1N sodium hydroxide or 1N hydrochloric acid as required. 120g of sodium lauryl sulfate was added to this solution and gently dissolved to obtain the required dissolution media.

4.3.3: Results and Summary

4.3.3.1: Assay of Silybin A, Silybin B and Silydianin

The results for the assay of silybin A, silybin B and silydianin are shown in Table 4.6. The assay was carried out on three market formulations each from a different manufacturer. All the three dosage forms tested were capsules.

Table 4.6: Assay Results for Silybin A, Silybin B & Silydianin in Market Formulations

Capsule	Company A	Company B	Company C
Lot No.	32070901	429676	235467
Weight Variation-CV (%)	2.37	2.01	1.72
Assay±SD (mg/capsule)			
Silybin A	17.20 ±0.05	17.20 ±0.01	47.20 ±0.82
Silybin B	29.9 ±0.11	30.19 ±0.21	69.36 ±1.02
Silydianin	11.08 ±0.07	12.81 ±0.09	5.13 ±0.08

CV= Coefficient of variation; SD=Standard deviation

Table 4.6 shows the assay of three market formulations for Milk thistle. The capsules have a label claim of 140 mg of silymarin, meaning that each capsule should contain not less than 140mg of silymarin extract standardized to contain at least 80% of flavonolignan isomers. The assay for the three market formulations indicated that Company A and Company B products contained similar amounts of silybin A (~17.3mg/capsule) and silybin B (~30mg/capsule), where as capsules from Company C contained a significantly high amount of both isomers (silybin A~47mg/capsule) and silybin B (~70mg/capsule). Company A and Company B capsules also had similar amounts of silydianin (~12 mg/capsule), and significantly less silydianin in Company C capsules (~5.13 mg/capsule). Silybin A and silybin B are considered as the main active ingredients and many researchers and manufacturers base their silymarin assays based on the content of silybin diastereomers.

According to USP specifications for assay of milk thistle capsules, the sum of all isomer content should be between 90%-110% of the label claim. Since we have reported the assay for silybin A, silybin B and silydianin content only, the total of these 3 isomers add up to 58.18% for Company A capsules, 43% for Company B capsules and 86.98% for Company C capsules. Though it cannot be concluded whether these products pass USP specifications for assay content, it is obvious that there is significant variation in the isomer content between formulations having the same label claim.

4.3.3.2: Dissolution of Silybin A, Silybin B and Silydianin

In vitro dissolution test was done for capsules manufactured by Company A. The release of silybin A, silybin B and silydianin was studied in 900ml of pH 7.5 phosphate buffer (0.2M) with 2% sodium lauryl phosphate.

The data for the release of silybin A, silybin B and silydianin is shown in Tables 4.7-4.9 along with the plot of percent release versus time in minutes (Figure 4.1-4.3).

Table 4.7: *In Vitro* dissolution profile of Silybin A in Company A Capsules

Company A Capsules (Silybin A: 17.20 mg/capsule)								
	Capsule Number (Percent Released)							
Time (minutes)	1	2	3	4	5	6	Mean	SD
10	7.29	4.84	6.57	9.16	14.21	12.99	9.18	3.39
20	8.72	16.33	19.57	20.60	24.19	25.41	19.13	5.53
30	30.16	25.40	26.30	24.26	28.09	26.89	26.85	1.90
40	31.39	25.11	29.12	31.26	27.76	30.42	29.17	2.21
50	33.45	25.35	32.14	30.47	32.46	33.36	31.20	2.79
60	34.37	32.42	32.08	30.94	32.02	30.74	32.09	1.18

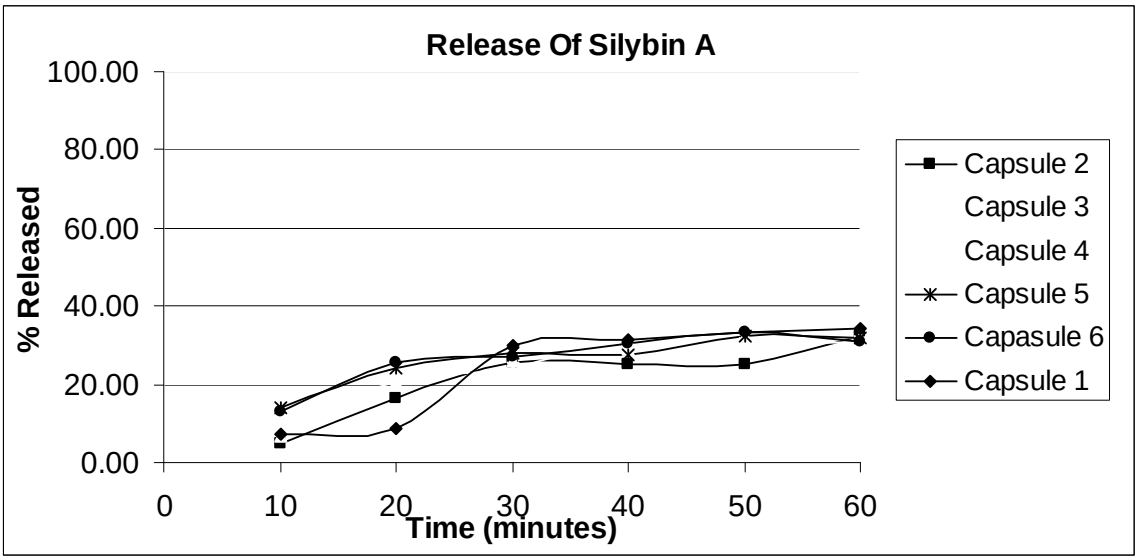


Figure 4.1. Plot of Percent release of Silybin A versus Time (Company A Capsules)

Table 4.8: *In Vitro* dissolution profile of Silybin B in Company A Capsules

Company A Capsules (Silybin B: 29.90 mg/capsule)								
	Capsule Number (Percent Released)							
Time (min)	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SD**
10	16.73	12.45	13.75	16.61	25.41	22.66	17.94	5.07
20	17.22	26.40	31.32	33.09	38.49	40.29	31.13	8.46
30	44.88	38.03	40.08	37.27	43.10	42.03	40.90	2.97
40	45.65	36.88	42.94	45.92	40.70	45.21	42.88	3.55
50	47.90	36.51	46.10	43.97	47.49	48.52	45.08	4.50
60	49.34	46.85	46.77	45.65	47.88	44.94	46.91	1.57

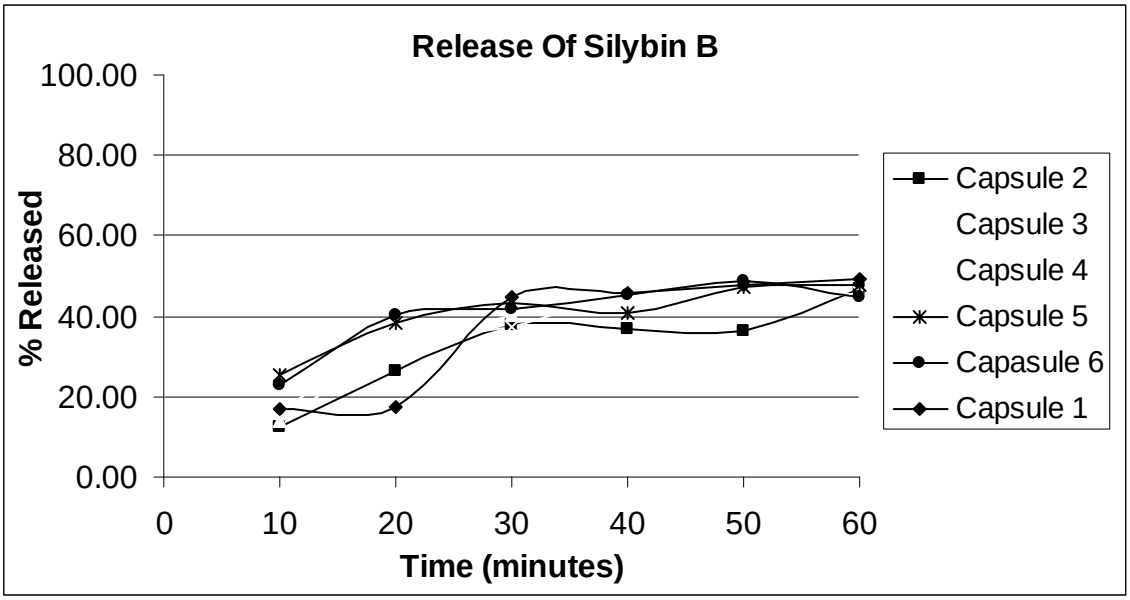


Figure 4.2. Plot of Percent release of Silybin B versus Time (Company A Capsules)

Table 4.9: *In Vitro* dissolution profile of Silydianin in Market Formulations

Company A Capsules (Silydianin: 11.08 mg/capsule)								
	Capsule Number (Percent Released)							
Time (mins)	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SD**
10	16.08	10.48	13.09	17.95	26.13	21.33	17.51	5.65
20	18.01	24.21	30.55	32.46	41.59	47.29	32.35	10.80
30	47.14	39.81	46.18	39.91	48.56	49.55	45.19	4.28
40	47.14	38.10	46.21	48.49	40.91	49.30	45.03	4.49
50	51.15	35.03	49.64	45.42	51.84	51.94	47.50	6.57
60	52.47	48.71	50.42	48.58	51.32	50.05	50.26	1.50

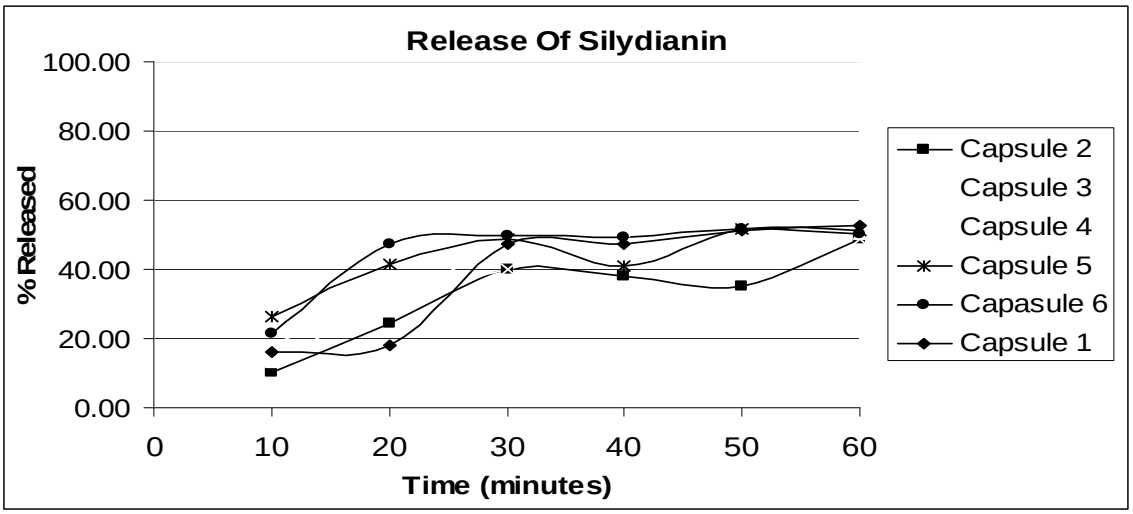


Figure 4.3. Plot of Percent release of Silydianin versus Time (Company A Capsules)

Results presented in Tables 4.7-4.9 show the *in vitro* dissolution of silybin A, silybin B and silydianin from Company A Milk thistle capsules. According to the dissolution studies and based on the assay of Company A capsules we see that at the end of 60 minutes, for silybin A:5.51mg dissolved from 17.2 mg/capsule (32.09%); for silybin B: 14.02mg dissolved from 29.9 mg/capsule (46.91%); for silydianin: 5.56 mg

dissolved from 11.08 mg/capsule (50.26%). Thus, the total dissolution from a label claim of 140 mg of isomers /capsule was 17.92 % which is extremely low after considering only 3 out of six isomers. According to USP 30 specifications, not less than 75% of the labeled amount of silymarin measured as silybin (calculated as the sum of silydianin, silycristin, silybin A, silybin B, isosilybin A and isosilybin B) should dissolve in 45 minutes. Considering the low solubility nature of the isomers, it is evident that the tested capsules manufactured by Company A will surely fail the *in vitro* dissolution specifications set by the USP 30 for milk thistle capsules indicating potential issues into the quality control of these products and the requirement more stringent regulatory protocols to maintain the quality and efficacy of these products and other herbal supplements.

The dissolution results also indicate that silybin A is the least soluble, followed by silybin B and silydianin is the most soluble among the three isomers tested for dissolution. These results are in correlation with the equilibrium solubility results obtained for the isomers where in silybin A (0.0033 mg/mL) is less soluble than silybin B (0.0077 mg/mL) which is less soluble than silydianin (0.019 mg/mL).

4.3.3.3: Summary

Silymarin capsules manufactured by Company A, Company B, and Company C were analyzed for content of isomers silybin A, silybin B and silydianin. Company A capsules were further studied for the *in vitro* dissolution profile of silybin A, silybin B and silydianin. Assay results indicated that capsules manufactured by Company C contained the highest amount of silybin A (47.2 mg/ capsule) and silybin B (67.3mg/capsule) and the lowest amount of silydianin (5.13mg/capsule). Capsules

manufactured by Company B and Company A contained similar amounts of silybin A (~17mg/capsule), silybin B (~30mg/capsule) and silydianin (~12 mg/capsule). Capsules manufactured by Company A were selected for studying the *in vitro* dissolution profile of silybin A, silybin B and silydianin. Based on the content of the three isomers assayed, we expect milk thistle capsules tested from all the manufacturers to pass USP specifications. The dissolution results indicate that for Company A capsules, at the end of 60 minutes, silybin A was the least dissolved (~32.09%) followed by silybin B (~46.91%) and then silydianin (~50.26%) which was the most soluble in the medium tested. This is agreement with the solubility experiments done for the silymarin isomers where in silydianin is the most soluble (0.019 mg/mL) and silybin A (0.0033 mg/mL) and silybin B (0.0077 mg/mL) have a lesser solubility, but not less than Isosilybin A and Isosilybin B. The dissolution profile of the diastereomers silybin A and silybin B is also in agreement with the solubility profile where in silybin A is less soluble than silybin B. The dissolution results for the Company A capsules are not expected to pass the specifications set by the USP 30 leading to stricter regulatory protocols and better GMP.

CHAPTER 5:PHARMACOKINETICS OF SILYMARIN ISOMERS

5.1: INTRODUCTION

Silymarin, the extract of milk thistle has been in traditional use for upper gastrointestinal disturbances, diseases of the liver and the biliary tract. It has been reported to be effective in improving the clinical courses of acute and chronic, viral, drug and alcohol-induced hepatitis. silymarin consists of six flavonolignan isomers known to be therapeutically effective, mainly against liver intoxication and poisoning. Accurate pharmacokinetic and metabolism studies with silymarin isomers are extremely rare or non existent. Pharmacokinetic studies are either inaccurate or not precise mainly due to the inability to quantify all the silymarin isomers in a biological matrix. Researchers tend to quantify only Silybin- a single component as an active marker for silymarin [148], or quantify four components: Silycristin, Silydianin, Silybin and Isosilybin, where in Silybin and Isosilybin each are quantified as one component rather than as individual diastereomers [77, 136]. Thus, pharmacokinetic parameters of silymarin and the active principle of any silymarin containing product is usually referred to and standardized as Silybin. *In vivo* experiments studying the pharmacokinetics of all the six silymarin isomers do not exist till date. It has been reported that after intravenous administration of 100mg/kg of Silybin N-methylglucamine, significant amounts of unconjugated Silybin were found in feces during the following 24 hours and in the intestine 2 hours after administration. Further Mennicke et al. studied the biliary excretion of silymarin as Silybin after rectal administration in rats, and found 3-5% of the administered dose after

6 hours. This data together with the presence of unmodified Silybin in the intestinal content and feces is strong evidence of the entero hepatic circulation of Silybin.

5.1.1: Absorption

Absorption of silymarin isomers, in human and animal models, after oral administration of the plain extract, is very low mainly due to the low solubility of the isomers. Other factors that govern the extent of absorption of the isomers are content of accompanying substances in the formulation such as solubilizers, the concentration of isomers in the extract and the dose administered. The bioavailability of silybin (A&B) can be greatly enhanced by its complexations with phosphatidylcholine (Lecithin) or β-cyclodextrin, choice of the capsule material or by using modified drug delivery systems such as self-micro-emulsifying drug delivery systems and liposomes. [77, 78, 136, 149-155] Complexation of Silybin with phosphatidylcholine is the most common method for the enhancing the bioavailability of silybin. It should be noted that in all these studies, complexation is done with silybin (A&B) alone and not with the extract. When studying different products, the amount of silymarin isomers dissolved during *in vitro* dissolution tests or the extent of absorption of silymarin or Silybin during *in vivo* studies should be compared with caution keeping in mind the method of analysis and the type of isomer analyzed. Significant variation has been observed when comparing different market products claiming to have the same label claim. [76, 156] Thus, when silybin is being reported as the isomer of interest in a certain study, it should be studied whether the amount of Silybin measured is the sum of all silymarin isomers reported as Silybin or the sum of diastereomers Silybin A and Silybin B. If Silybin alone is being reported as the

isomer of interest, is should be observed whether the method of analysis measures Silybin content as a single entity or the combination of diastereomers silybin A and silybin B. Schulz et al. performed assay and *in vitro* dissolution test in seven market formulations of silymarin and based on these results selected three formulations for bioequivalence testing. Their results showed a two fold higher bioavailability for silybin from one of the market products (Legalon™ by Madaus Pharma®) when compared to the other two products. Since the liver is the site of action of silymarin isomers, many researchers measure the conjugated and unconjugated forms of silymarin in bile and the systemic circulation, respectively. Thus, systemic plasma concentrations of silymarin isomers should be measured, as they provide an estimate on the quantity of isomers being absorbed from the gastrointestinal tract. *In vivo* studies reporting the absorption of silymarin or Silybin after oral administration indicate a very high variability in the values of C_{max} and T_{max}. As mentioned earlier, when comparing *in vivo* studies for silymarin, it should be made clear what isomer of interest was being measured. As for the earlier reports it can be safely assumed that since the analytical separation of all the silymarin isomers was not evident, the isomer reported was silybin, which might have been a measure of all the isomers or particularly silybin. Regardless of the isomer measured, all studies indicate a very low absorption extent of silymarin and a highly variable plasma peak concentration (C_{max}) and time to plasma peak concentration (T_{max}).

One of the earliest reports on the experimental pharmacokinetics of silymarin is by Meyer-Burg [58, 125] who reported that after oral administration of 500-1000 mg/kg of silymarin in rats, 75% of the dose was found in the feces, where as after intraperitoneal administration of 100mg/kg, no silymarin was found in the serum, urine or feces. Janiak

et al. reported peak plasma concentrations of 500µg/mL of silymarin as silybin, 90 minutes after oral administration of 200mg/kg of silymarin as silybin in mice. [58, 157]

Unpublished work done by Chasseaud and cited by Mennicke [58, 126] described the blood-time concentration profile of radio labeled ^{3}H-Silybin after oral administration in rats, where silybin showed peak concentrations at 30 minutes and an absorption rate of 30% of the administered dose. Morazzoni et al. compared the bioavailability of silymarin with silybin-phosphatidylcholine (Silipide®) complex. [136] The low bioavailability of silymarin was confirmed when after oral administration of a 200 mg/kg dose to rats, plasma levels of Silybin were under the analytical limit of detection. In another study, Morazzoni studied the pharmacokinetic profile of separate isomers, silybin, silycristin, silydianin, and isosilybin in comparison to Silipide®. [77] Unconjugated Silipide® had a significantly higher bioavailability ($AUC_{0\text{-}24h}$ = 8.31µg.hr/mL) as compared to unconjugated silymarin isomers. The sum of the $AUC_{0\text{-}24h}$ values for all the silymarin isomers was less than the $AUC_{0\text{-}24h}$ reported for Silipide® alone. Unconjugated silycristin could not be detected after oral administration and unconjugated Isosilybin had the highest $AUC_{0\text{-}24h}$ (1.65) among the silymarin isomers. For both Silipide® and silymarin, a major proportion of the isomers were found in the form of metabolites in bile as glucuronides and sulfates.

Quineng and Yanyu [154] compared the bioavailability of silybin-N-methylglucamine and silybin phospholipids complex in rats. They found that the silybin phospholipids complex had significant enhanced bioavailability when compared to silybin-N-methylglucamine.

Table 5.1 gives an idea of the variability in absorption parameters (Cmax, Tmax) for the various silymarin isomers in the plain extract or in the form of a phospholipids complex for studies done in rats by various researchers.

Table 5.1: Comparison of Absorption Parameters Following Oral Administration of Unconjugated Silybin or Silymarin (Plain or Complex) as Silybin in Rats and Rabbit. *

Silymarin Form/Isomer	**Dose (mg/Kg)**	**T_{max} (Hour)**	**C_{max} (µg/mL)**	**AUC (µg.hr/mL)**	**Reference**
Silybin (200mg/kg)	200	NA	Below LOD	--	[136]
§Silipide® (200mg/kg)	200	0.36	2-21∏	9.78±3.29	[136]
Silybin (200mg/kg)	200	0.13±0.09	0.06 ±0.04	0.02±0.01	[77]
Silycristin		ND	ND	0	
Silydianin		6 ±0	0.1 ±0.07	0.2±0.15	
Isosilybin		1.65±1.09	0.44±0.04	1.14±0.31	
§Silipide® (200mg/kg)	200	0.63±0.13	9.02±2.96	8.31±0.35	
Silybin-N-methylglucamine	9.1	0.08	0.104	0.235	[154]
Silybin-phospholipid Complex	9.1	0.17	0.126	1.220	[154]
Silymarin in PEG 400 (300mg/kg) *	300	2.39±1.54	0.70 ±0.18	3.17±1.63	[153]
Silymarin-SMEDDS as silybin (300mg/kg)*	300	4.33±0.82	1.01 ±0.21	6.23±1.75	[153]

§=Silipide® is a complex of silybin (A&B) with phosphatidylcholine. Doses of Silipide are measured as silybin (A&B) equivalents.

*=Study in Rabbit; ∏=Range of C_{max} observed

Thus, it can be seen from Table 5.1 that T_{max} for silybin (A&B) after oral administration in an animal model varies significantly from 5 minutes (Cmax=0.104µg/mL) for silybin-N-methylglucamine to 4.33 hours (Cmax= 1.01µg/mL) for silybin in silymarin-SMEDDS formulation. The T_{max} and C_{max} reported here are only for the unconjugated silybin isomer levels found in plasma. Variation can also be seen after administration of plain silymarin as silybin (Tmax range: 0.13 hours-2.39 hours).

Kim and coworkers [148] conducted a comparative bioavailability study in humans with three silymarin market formulations: Liverman Capsule (Dong A Pharmaceutical Company, Korea, silymarin tablet (Kunhil Pharmaceutical Company, Korea) and Legalon capsule (Madaus Pharma, Germany). All subjects received an equal dose strength containing silymarin as 120 mg of silybin. Significant variability in the area under the plasma concentration-time curve (AUC_{inf}) was observed between the three formulations in spite of the same dose administered to subjects. AUC_{inf} for Liverman capsules was found to be highest (15.1±3.68µg.hr/mL) than Legalon (6.0±2.2µg.hr/mL) and silymarin tablet (4.63±1.96µg.hr/mL). The Cmax for Liverman was 6.04±1.9 µg/mL where as that for Legalon and silymarin tablet was ~1.2µg/mL which was significantly lower. The publication does not give any explanation for the observed variability in the extent of absorption, but this could possibly be due to the varying contents of silybin in the extract used in the formulation. Further silymarin is known to be insoluble in aqueous media suggesting that the variability in the AUC might be due to dissolution limited absorption. Also, it has not been specified whether any of the formulations tested had any solubility enhancers present in the formulations which might lead to varying amounts of isomers being dissolved in the gastrointestinal contents.

Lorenz et al. studied the serum concentration levels of silybin in healthy human volunteers after the administration of a dose of 560mg of silymarin corresponding to 240 mg of silybin. The maximum plasma concentration was found to be 0.34±0.16µg/mL, time to peak was 1.32 hours, and AUC was 1.14µg.hr/mL. [158] Human pharmacokinetics of silymarin in healthy as well as cirrhotic patients was studied by Barzaghi et al. and Orlando et al. [58, 158] They reported that after a single dose of 360 mg of silybin as silymarin, silybin was rapidly absorbed with a Tmax of 1.4 hours, a Cmax ranging from 0.024µg/mL - 0.2µg/mL, and mean AUC observed was 0.252µg.hr/mL . In cirrhotic patients these parameters did not show a significant change but the Tmax was observed to be 2.6 hours, indicating slower absorption as compared to healthy patients.

Table 5.2 shows the comparative bioavailability parameters for free Silybin after oral administration in humans.

Table 5.2: Comparison of Pharmacokinetic Parameters for Free Silybin After Oral Administration to Humans

Silybin Formulation	**Dose (mg)**	**Cmax (µg/mL)**	**Tmax (hour)**	**$AUC_{0\text{-}inf}$ (µg.hr/mL)**	**Reference**
Silipide®	360	0.298	1.6±0.3	0.881±207	[150]
Silymarin Extract	360	0.102	1.4±0.3	257±66	[150]
Silipide (Hard gel Capsule)	80	0.193±0.182	2.9±1.3	0.387±0.340*	[78]
Silipide® (Soft Gel Capsule)	80	0.710±0.799	1.25±0.62	1.068±1.040*	[78]
Silymarin Extract	101.7	0.116	--	0.254	[79]
	152.6	0.250	--	0.423	[79]
	203.4	0.239	--	0.670	[79]
	245.3	0.317	--	0.751	[79]
Legalon Capsule	120	1.33±0.54	1.83±0.94	6.0±0.22**	[148]
Liverman Capsule	120	6.04±1.9	0.875±0.36	15.1±3.68**	[148]
Silymarin Tablet	120	1.13±0.51	2.10±1.07	4.63±1.96**	[148]

* = $AUC_{0\text{-}24h}$

** = $AUC_{0\text{-}26h}$

5.1.2: Distribution, Metabolism and Excretion

In vitro studies on the plasma protein binding of silycristin, silydianin and silybin indicate that the isomers are extensively but reversibly bound to human albumin. [159] In a later study, the volumes of distribution for silycristin, silydianin and silybin were calculated from the partition coefficient between octanol and pH 7.4 buffer and binding to human albumin. Based on the data, a one-compartmental model was fit for silybin, and silycristin and silydianin fit a two-compartment model. [160]

As seen from the data in Tables 5.1 and 5.2, most of the early *in vivo* experiments concerning silymarin, were performed with silybin as a standard. [126, 136, 161, 162] Though results were measured as silybin, later experiments have shown that major silymarin isomers other than silybin, such as silycristin, silydianin and isosilybin show a similar behavior as silybin in terms of excretion and metabolism, the only difference being their individual proportion in the extract. [163] Sonnenbichler et al. studied the subcellular distribution and liver uptake of silybin and its metabolites following intravenous, intraperitoneal, and oral administration of radioactive ^{3}H-Silybin in rats. [162] After 8 hours of intraperitoneal administration, the following percentages of radioactivity were found in various organs and tissues: 5.1% in liver; 2.1% in blood, 0.3% in spleen and 0.1% in brain. A similar trend in the percent distribution was found following other forms of administration. Further qualitative analysis indicated that 80% of the silybin found in the liver homogenate and cytosol of the hepatic cell was in the unconjugated form while the remaining 20% was in the form of glucuronide and sulfate metabolites.

One of the earliest reports on the metabolism and elimination of silymarin was by Bulles et al. in 1975. They published a study on the urinary and biliary excretion of silybin in rats after intravenous (20mg/kg) and oral (2-20mg/kg) administration of silybin-N-methylglucamine. [58, 126] The results indicated that independent of the route of administration, silybin was excreted in the urine unmodified and in the bile as metabolites, such as glucuronides and sulfates of silybin and dehydrosilybin. The amount of silybin excreted in urine during 48 hours post administration, was minimum (~2-5% post oral) and ~8% after intravenous administration. Biliary excretion of silybin, as glucuronide and sulfate metabolites, after 48 hours was about 40-45% for the highest oral dose (20mg/kg) and 80% after intravenous administration. The ratio between the dose and the quantity excreted in the bile was found to be linear and the minimum bioavailability based on the excretion data was found to be 45%. Kinetic studies of biliary excretion indicated that, maximum excretion of metabolites in bile occurred within 1 hour after oral after oral administration, indicating rapid absorption of silybin.

After intravenous administration of silybin-N-methylglucamine (100mg/kg), Bulles et al. reported considerable quantities of unconjugated silybin, in feces collected during 24 hours and intestinal contents 2 hours after administration. Mennicke [161] evaluating the biliary excretion of silybin after rectal administration, reported detecting 3-5% of the administered dose. This along with the presence of silybin in the intestinal content as reported by Bulles, may indicate possible entero hepatic circulation. Thus, silybin or silymarin isomers exhibit entero hepatic circulation in the sequence: intestinal absorption, conjugation in the liver, excretion in the bile, hydrolysis by the intestinal flora and finally reuptake in the intestine. [56, 161]

Elimination half lives for silybin or silymarin after intravenous administration have not been reported. Elimination half lives for Silybin after oral administration have been reported to be in the range from less than 1 hour up to six hours. Weyhenmeyer et al. reported concentrations of unchanged silybin diastereomers in plasma from 4 hours to 13 hours, with concentration of one isomer being three times higher than the second isomer. A one compartment model was fit to the obtained plasma data which resulted in elimination half lives of less than 1 hour. The actual half lives were not reported based on the one compartment model as it was suspected that another additional elimination phase existed which was at that time, hidden below the sensitivity of the analytical assay. The assay detected the beginning of a second elimination phase which was below the limit of detection. They also found an irregular absorption pattern resulting in multiple concentration peaks. [79] Lorenz et al. reported an elimination half of 6 hours for silybin post oral administration. [158]

Silymarin extract consists of six flavonolignans: Silycristin, Silydianin, Silybin A, Silybin B, Isosilybin A and Isosilybin B. Silybin (the mixture of diastereomers Silybin A and B) has long been considered to be the most active component of the extract and hence early research has been focused on Silybin as a measure of silymarin. Quantitative separation of all silymarin isomers or diastereomers was not possible during the earlier experiments-*in vitro* or *in vivo*, and thus silybin measured would be a measure of all the silymarin isomers collectively, unless specified. This resulted in the incomplete pharmacokinetic profiling or evaluation of bioavailability of each isomer. The overall bioavailability of silybin or silymarin from the extract is low mainly due to the low solubility of the extract. Significant variation has been found in content, dissolution and

bioavailability of different formulations having the same label claim. This indicates formulation differences resulting in lack of bioequivalence or variation in isomer content at source such as geographical location of the plant, time of harvest and method of processing to obtain the standardized extract. Comparison of pharmacokinetic parameters determined from different *in vivo* studies should be done with caution, considering the differences in the analytical methods used, the types of isomers quantified and whether the level of free, conjugated or the total isomer is being reported. Systemic plasma concentrations of all detectable isomers should be measured even if the liver is the site of action as this provides an estimate on the quantity of the particular isomer being absorbed from the gastro intestinal tract.

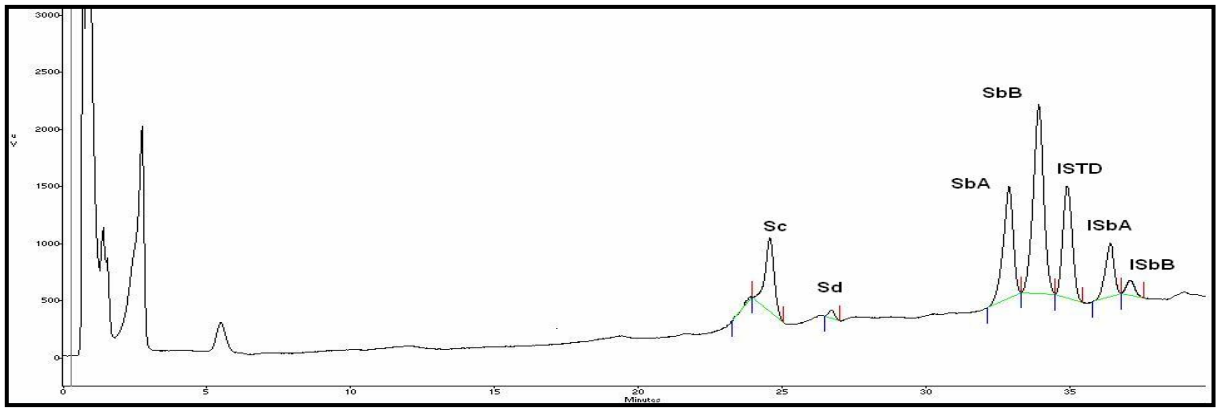
5.2: EXPERIMENTAL METHODS AND DESIGN

5.2.1: Method of Analysis

A reverse phase HPLC method was developed to detect the silymarin isomers in rat plasma. The development and complete validation of the method has been discussed in the Analytical Methodology Chapter of this dissertation. A skeletal description of the method and a sample chromatogram are shown in Table 5.3 and Figure 5.1.

Table 5.3: HPLC Specifications for Detection of Silymarin Isomers in Rat Plasma

Method	HPLC, Reverse Phase, Binary Gradient
Column Type	Phenomenex, Luna C-18(2)
Column Dimensions	150 x 4.6mm, particle size: 5µ
Mobile Phase Conditions	Water (A): Methanol (B) %B: 20-50 in 28 minutes 50-55 in 36 minutes Total Flow: 2ml/min for10 minutes 1ml/min for 10-38 minutes 2ml/min for 38-41 minutes
Internal Standard	1-naphthol (0.5µg/mL)
Detection	UV, 288nm
Injection Volume	50µL
Temperature	Ambient



Time (Minutes)

Figure 5.1. Sample Chromatogram for Detection of Silymarin Isomers in Rat Plasma

The following limit of quantitation was set for the following isomers: silycristin: 0.06µg/mL; silydianin: 0.08µg/mL; silybin A: 0.05µg/mL; silybin B: 0.05µg/mL; Isosilybin A: 0.05µg/mL; Isosilybin B: 0.06µg/mL.

The concentration range for each silymarin isomer for the development of respective standard curves in rat plasma is illustrated in Table 5.4.

Table 5.4: Standard Curve Concentration Range for Each Silymarin Isomer in Rat Plasma

Silymarin Isomer	**Concentration Range (µg/mL)**
Silycristin	0.24 - 15.32
Silydianin	0.08-2.26
Silybin A	0.47-11.81
Silybin B	0.86-21.61
Isosilybin A	0.20-5.13
Isosilybin B	0.06-1.6

5.2.2: Animal Model Used

Male Sprague Dawley rats weighing between 280-320g were purchased from Charles River (Wilmington, MA) and the Animal Resource Center (University of Texas at Austin, Austin, Tx). The rats were housed individually in wired bottom cages, at 22°C, 50% humidity and on a 12 hour light-dark cycle. The rats were allowed free access to commercial rodent diet (Harlan, IN) and water was allowed *ad libitum.* All amimal

research was carried out according to protocols pre-approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin

5.2.3: Study Design and Dosing Regimen

Thirty six rats were randomly divided into two groups, each group containing eighteen rats. One group was subjected to intravenous bolus dosing and the second group was subjected to oral dosing of silymarin extract.

Silymarin extract obtained from Sigma Aldrich was used to prepare the dosing solutions. The assay of the extract for isomer content is described in the Analytical Methodology section of this dissertation. Dosing solutions were prepared by accurately weighing and dissolving the appropriate quantity of silymarin extract in a mixture of water: PEG 400: Ethanol in the ratio 50:40:10 respectively.

5.2.3.1: Intravenous Bolus Dosing

Eighteen rats were divided into three groups of six rats each. Three silymarin concentrations were selected for the intravenous bolus dose: (i) **Treatment A:** 25mg/Kg; (ii) **Treatment B:** 50mg/Kg; (iii) **Treatment C:** 100mg/Kg.

These silymarin concentrations yielded the corresponding isomer concentrations as per their proportion in the extract as shown in Table 5.5.

Table 5.5: Individual Isomer Assay and Concentrations in Silymarin I.V Bolus Doses

Silymarin Isomer	Assay (%)	Treatment A (25mg/Kg)	Treatment B (50mg/Kg)	Treatment C (100mg/Kg)
Silycristin (Sc)	24.50	6.13	12.26	24.52
Silydianin (Sd)	3.62	0.90	1.80	3.61
Silybin A (SbA)	18.9	4.72	9.45	18.90
Silybin B (SbB)	34.6	8.6	17.29	34.58
Isosilybin A (ISbA)	8.22	2.05	4.11	8.22
Isosilybin B (ISbB)	2.56	0.64	1.28	2.56
Total	**92.40**			

Rats were dosed according to a cross over design as shown in Table 5.6 such that each rat received all the three treatments A, B & C during the three periods tested. A wash out time of 10 days was maintained between any two periods. Rats were fasted 12 hours prior to dosing. However, water was allowed *ad libitum*.

Table 5.6: Intravenous Bolus Dosing Sequence

Rat #	Sequence	Treatment (Period 1)	Treatment (Period 2)	Treatment (Period 3)
1	ABC	A	B	C
2	BCA	B	C	A
3	CAB	C	A	B
4	ABC	A	B	C
5	BCA	B	C	A
6	CAB	C	A	B

Rats were fasted 12 hours prior to dosing and water was restricted from one hour before dosing up to four hours after dosing. Food was restricted up to 12 hours after dose administration after which they had free access to food and water.

5.2.3.2: Oral Dosing

Eighteen rats were divided into three groups of six rats each. Three silymarin concentrations selected for oral administration were: (i) Treatment A1: 125 mg/Kg; (ii) Treatment B1: 250 mg/Kg; (iii) Treatment C1: 500 mg/Kg. The silymarin concentrations yielded the corresponding isomer concentration in the dosing solutions as shown in Table 5.6.

Table 5.7: Individual Isomer Assay and Concentrations in Silymarin Oral Doses

Silymarin Isomer	Assay (%)	Treatment A1 (125mg/Kg)	Treatment B1 (250mg/Kg)	Treatment C1 (500mg/Kg)
Silycristin (Sc)	24.50	30.65	61.30	122.61
Silydianin (Sd)	3.62	4.52	9.04	18.08
Silybin A (SbA)	18.9	23.63	47.27	94.54
Silybin B (SbB)	34.6	43.23	86.46	172.93
Isosilybin A (ISbA)	8.22	10.27	20.55	41.11
Isosilybin B (ISbB)	2.56	3.20	6.40	12.81
Total	**92.40**			

Oral administration of silymarin was done using a cross over design over three periods such that each rat received all the treatments A1, B1 & C1 as shown in Table 5.7.

Table 5.8: Oral Dosing Sequence

Rat #	Sequence	Treatment (Period 1)	Treatment (Period 2)	Treatment (Period 3)
1	A1B1C1	A1	B1	C1
2	B1C1A1	B1	C1	A1
3	C1A1B1	C1	A1	B1
4	A1B1C1	A1	B1	C1
5	B1C1A1	B1	C1	A1
6	C1A1B1	C1	A1	B1

A wash out time of 10 days was maintained between any two periods. Rats were fasted 12 hours prior to dosing and water was restricted from one hour before dosing up to four hours after dosing. Food was restricted up to 12 hours after dose administration after which they had free access to food and water.

5.2.4: Catheterization of the Jugular Vein

All rats were anesthetized and a cannula inserted into their jugular vein to enable the injection of drug solution during the intravenous study and sampling of blood post intravenous and oral drug administration.

5.2.4.1: Anesthesia

Anesthesia was induced prior to surgery by an intraperitoneal injection of rodent anesthesia cocktail containing Ketamine HCl, Xylazine HCl and Acepromazine. The anesthetic cocktail was prepared as follows:

i. Ketamine HCl: 150mg, 100mg/mL, 1.5ml

ii. Xylazine HCl: 30mg, 20mg/mL, 1.5mL

iii. Acepromazine: 5mg, 10mg/mL, 0.5mL

An initial dose of 0.6 mL/Kg of the cocktail was administered to induce anesthesia. In the event that the animal regained consciousness during the surgical procedure, a 0.3 mL/Kg dose was administered again. The anesthetic cocktail does not cause deep central respiratory depression. Ketamine prevents the body temperature from dropping and Xylazine and Acepromazine provide muscle relaxation.

5.2.4.2: Surgical Procedure

Once an appropriate and stable plain of surgical anesthesia had been induced, the rat was moved to the surgical area. The collar bone area near the chest was shaved in order to make the jugular vein roughly visible. The head was also shaved to enable the mounting of the cannula on the head after the catheterization was complete. Rimadyl (NSAID), 1% body weight was administered and the time of administration noted. Initially a one inch long incision was made over the head, between the eyes in order to expose the lambda/bregma.

Catheterization of the jugular vein: The animal was laid on its back its legs taped using a peach tape. Then a 2-3 cm long incision was made in the collar bone area where the jugular vein could be roughly visible. Precaution was observed not to damage the jugular vein during the making of the incision. Once the jugular vein was exposed and isolated from the surrounding tissue, a tiny incision was made using a ball scissors. A catheter (6-7 cm polyethylene tubing, PE-50, trimmed to give a smooth beveled end) was attached to a syringe and its lumen filled with saline. A bent blunt needle (used as a place holder) was inserted into the vein incision to make way for the catheter, and the catheter inserted in to the vein. Once the catheter was inserted into the vein, the operation of the catheter was checked for free flow of blood by administering a small quantity of saline, from the syringe attached to it. The catheter was then secured in place by 2 sutures, one at the farther end from the incision and one near the incision. The catheter was checked for blood flow after each suture and then ensured that its lumen was filled with saline. The syringe was detached, and the catheter was immediately capped to

prevent blood coagulation. Gentamicin was applied to the incision cavity to prevent infection during recovery.

Using a large forcep a subcutaneous pathway was created between the head and the chest. The capped end of the catheter was then pulled gently through the subcutaneous pathway, such that the capped end of the catheter showed up on the head.

The chest incision was then closed with sutures, and wound powder and Noesporin was applied to the sutures to prevent infection and aid recovery.

Mounting of Catheter on the Head: The skull was cleaned with saline, and 4 tiny shallow holes were drilled into the skull to form four vertices of a square. The holes were drilled such that they were not more than 0.25 cm apart from each other and roughly 0.15 cm deep. Care was taken to ensure that the holes were shallow enough so as not to expose the brain to the screws. Tiny screws were inserted into these holes and slightly tightened half way through. The catheter was positioned between these screws and put in place with the help of cyanoacrylate (SuperGlu®) glue. The catheter was then further reinforced into place by filling the space between the screws with dental cement. The cement was allowed to dry and the catheter was again finally checked for free blood flow. Care was taken to prevent the cement from sticking to the exposed skin around the head, by filling the gap between the skin and the cement by wound powder. The rat was then placed on a heating pad to recover and gain consciousness.

5.2.4.2: Recovery Period

The rats were allowed to recover from surgery and regain the lost weight for approximately two weeks prior to the start of the pharmacokinetic study. As the weight of each rat approached 300 g only then were they assumed fit for drug administration.

5.2.4.3: Anticoagulant and Catheter Flushing

Heparin, 20units/mL was used as an anti coagulant throughout the experiments. Catheter flushing solution was composed of 0.9% NaCl injectable solution containing 20units/mL of Heparin. Catheters were flushed daily, during intravenous drug administration and during blood sampling with 0.3 mL of flushing solution in order to prevent clotting of blood inside the catheter.

5.2.5: Dose Administration

Dosing solutions were prepared fresh prior to dosing. Preparation of dosing solutions has already been described in the previous sections of this chapter. The volume of the administered dose was adjusted according to the weight of the animal such that the required concentration of drug was administered. The injection volume ranged from 0.1 to 0.4 ml.

5.2.5.1: Intravenous Dosing

During intravenous dosing, the appropriate quantity of drug solution was filled in a syringe and kept ready for administration. Prior to dosing, the catheter was flushed with Heparin/Saline solution (Flushing solution) to ensure that it had a free flow. With

the lumen of the catheter filled with the flushing solution, the syringe filled with drug solution was attached to the catheter and the drug solution pushed through the catheter. The empty drug syringe was then detached and 0.15 ml of flushing solution pushed through the catheter to ensure that there was no drug solution remaining in the catheter after administration.

5.2.5.2: Oral Administration

Silymarin was administered to the rats orally by using a rodent feeding needle. The feeding needle was pre-filled with the respective drug solution to ensure accurate administration of the drug concentration.

5.2.6: Blood Sampling Plasma Collection and Storage

Blood samples were drawn at 0, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 16, 18 and 24 hours time points. 400 µL of blood was drawn using a pre heparinised syringe and collected into a pre heparinised micro centrifuge tube. The tubes were centrifuged immediately at 4000 rpm for 10 minutes. The plasma which was transferred to a centrifuge tube and stored at -4°C until assayed.

5.2.8: Analysis of Plasma

Rat plasma was analyzed for the quantitation of silymarin isomers using the HPLC method described in the Analytical Methodology section of this dissertation. Calibration curve concentrations were injected thrice and a new calibration curve was prepared every 48 hours during the analysis of the plasma samples. The ratio of the isomer peak area to the internal standard peak area was used in the calculation of the

signal response and quantitative determination of each isomer in plasma. A linear relationship was obtained when the area ratio was plotted against the concentration of each of the isomers.

5.2.9: Pharmacokinetics Following Intravenous Bolus Dose

The plasma concentration-time data for each quantifiable isomer per rat was tabulated and their mean and standard deviation calculated.

5.2.9.1: Non Compartmental Analysis

Non compartmental pharmacokinetic parameters were calculated from the plasma concentration time profile, by using WinNonlin Professional Edition Version 2.1. The means and the standard deviations of the pharmacokinetic parameters were calculated. The AUC from time of dosing to the last measurable concentration (AUC0-t) was calculated using the Linear Trapezoidal Rule and extrapolated to infinity by the addition of the term C_{Last}/λ_Z where C_{Last} is the last observable and λ_Z is the terminal rate constant determined by log linear regression analysis.

The pharmacokinetic parameter and the respective equations used to calculate them are listed below:

$AUC_{0\text{-}t}$: Area under the curve from time of dosing to time of last measurable concentration, determined by the linear trapezoidal rule.

AUC0-∞: Area under the curve from time of dose extrapolated to infinity.

$$AUC_{0-\infty} = AUC_{0-t} + \frac{C_{Last}}{\lambda z} \qquad \textbf{Equation 5.1}$$

Total Body Clearance (CL): $$CL = \frac{Dose}{AUC_{0-\infty}} \qquad \textbf{Equation 5.2}$$

Volume of Distribution (V_d) $= \frac{Dose}{\lambda z * AUC_{0-\infty}}$ **Equation 5.3**

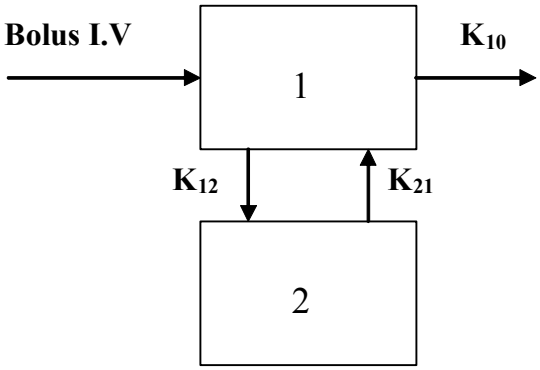
Volume of Distribution at Steady State (V_{ss}): = MRT_{inf} * CL **Equation 5.4**

where, MRT_{inf} is the Mean Residence Time extrapolated to infinity

5.2.9.2: Two-Compartmental Analysis

The plasma concentration-time profile was fitted to a two compartment model following an intravenous bolus dose with no lag time and a 1st order elimination rate to yield macro constants (A, B, α, β). The model is illustrated in figure 5.2 and can be described by Equation 5.2.

Figure 5.2: Schematic of a 2-Compartmental Model following an I.V Bolus Dose



$$Cp = A * e^{-\alpha t} + B * e^{-\beta t} \qquad \textbf{Equation 5.5}$$

where,

Cp is the plasma concentration at time a particular time 't' on the plasma concentration- time curve.

A and B are intercepts on the Y-axis for each exponential segment of the curve described by equation 5.5.

α and β are the hybrid rate constants for the distribution and elimination phase respectively.

A, B, α and β were obtained using the method of residuals.

Pharmacokinetic parameters were calculated using the same equations described in the non compartmental analysis Section 5.2.9.1, except for the calculation of volume of distribution at steady state (Vss) and Volume of distribution (Vd) which were calculated using the following equations:

$$Vss = Vp + \frac{K_{12}}{K_{21}} * Vp \qquad \textbf{Equation 5.6}$$

where, Vp = Dose/(A+B) = Volume of distribution of the central compartment

K_{12} = First order intercompartmental rate constant of the drug going into the tissue compartment

K_{21}= First order intercompartmental rate constant of the drug leaving the tissue compartment

$$V_D = \frac{Dose}{AUC_{0-\infty} * \beta} \qquad \textbf{Equation 5.7}$$

5.2.10: Determination of Bioavailability

Determination of pharmacokinetic parameters for the silymarin isomers post oral administration was done using WinNonlin Professional Version 2.1. Absolute bioavailability for the detected isomers was calculated using Equation 5.8

$$F = \frac{AUC_{0-\inf}^{Oral}}{AUC_{0-\inf}^{I.V}} \times \frac{Dose_{I.V}}{Dose_{Oral}} \qquad \textbf{Equation 5.8}$$

where,

F = Absolute Bioavailability

$AUC_{0-\inf}^{Oral}$ = Area under the plasma concentration-time curve from time zero to time infinity for a particular isomer X, after oral administration (µg-hr/mL)

$AUC_{0-\inf}^{I.V}$ = Area under the plasma concentration-time curve from time zero to time infinity for a particular isomer X, after an intravenous bolus administration (µg-hr/mL)

$Dose_{Oral}$ = Oral administered Dose of Isomer X (µg/Kg)

$Dose_{I.V}$ = Intravenous Bolus Dose administered of Isomer X (µg/Kg)

$AUC_{0-\inf}^{I.V}$ **for the particular isomer, in the above equation was the mean of the individual AUC's obtained by non compartmental analysis after intravenous bolus treatment C for the particular isomer.** The dose used was the corresponding concentration of the isomer in the intravenous treatment C dose, in micrograms.

Calculation of Hepatic Extraction Ratio and Maximum Oral Bioavailability:

The most direct quantitative measure of the liver's ability to eliminate a drug is hepatic clearance which includes biliary excretory clearance. Hepatic extraction ratio can be estimated if the hepatic clearance and the hepatic blood flow are approximated. Maximum oral bioavailability ($1-E_H$) dependent on physiologic factors, implies that no amount of pharmaceutical manipulation can improve this bioavailability value for an oral dosage formulation. Thus, any drug that has a high hepatic extraction ratio (EH) will have low bioavailability and vice versa. But even if the drug has a low hepatic extraction ratio (i.e. a high maximum oral bioavailability) there can be other factors such as low dissolution, that limit the drug from reaching the portal vein further decreasing the bioavailability.

Previous pharmacokinetic studies measuring silybin after the administration of silymarin have indicated that silybin is excreted in urine in minimum quantities, following oral (2-5%) and intravenous (~8%) administration. There have been contradictory reports on the biliary excretion of silybin or silymarin as silybin following oral/rectal administration, with values ranging between 5%-80%. [58] This high degree of variation can be attributed to the limited solubility of silymarin isomers.

Based on these observations, the value of total clearance (CL_T, mean calculated from individual PK parameters) reported here following intravenous administration, was used to calculate the hepatic extraction ratio (E_H) in the following equation:

$$E_H = CL_H / Q_H \qquad \textbf{Equation 5.9}$$

where,

E_H =hepatic extraction ratio

CL_H=Hepatic Clearance= CL_T

Q_H=Hepatic Blood Flow in Rat =25.6 mL/min ~5100 mL/Kg-hr for a 0.3 Kg Rat. The value for Q_H was obtained from experiments done by Sato et. al. [164]

5.3: RESULTS AND DISCUSSION

Individual rat plasma concentration-time profiles and individual pharmacokinetic parameters obtained using non-compartmental (NCA) and two-compartmental analysis, for each silymarin isomer following intravenous bolus and oral administration (NCA only) are presented in the Appendix (A1-A5) at the end of this dissertation.

In this section, the mean plasma concentrations after intravenous bolus administration (Treatments A, B&C) and oral administration (Treatments A1, B1&C1) for a particular silymarin isomer are presented. A Comparison of the mean of the individual pharmacokinetic parameters obtained after intravenous administration, using non-compartmental analysis (NCA) and two-compartmental analysis is presented followed by the non compartmental analysis and the calculation of absolute bioavailability for each silymarin isomer. The data for each isomer is followed by a discussion on the pharmacokinetics and the bioavailability of that isomer.

Plasma concentrations for Silydianin (Sd) following treatment A, B, C were either not detected or below the limit of quantitation. Plasma levels for Silybin A (SbA) after Treatment A were also not detected. Isosilybin B was detected in only two rats for each of the Treatments A, B&C.

Post oral administration, quantifiable levels could only be detected for silycristin, silybin A and silybin B in plasma.

The final number of rats ‘n’ per treatment per isomer following intravenous administration was as follows:

- Silycristin: Treatments A, C (n=6) B (n=7)
- Silydianin: Treatments A, B&C (n=0)
- Silybin A: Treatment A (n=0), Treatment B (n=5), Treatment C (n=6)
- Silybin B: Treatments A (n=6), B (n=8) & C (n=6)
- Isosilybin A: Treatments A&C (n=5), Treatment B (n=7)
- Isosilybin B: Treatments A, B&C (n=2)

The final number of rats ‘n’ per treatment per isomer following oral administration was as follows:

- Silycristin: Treatments A1 (n=5), B1 (n=3) C1 (n=4)
- Silydianin, Isosilybin A, Isosilybin B: Treatments A1, B1&C1 (n=0)
- Silybin A: Treatment A1 (n=3), Treatment B1 (n=2), Treatment C1 (n=4)
- Silybin B: Treatments A1 (n=2), B1 (n=5) & C1 (n=4)

5.3.1: Results for Silycristin

Table 5.9: Mean Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg (A), 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 6.13 mg/Kg, 12.26 mg/Kg, 24.52 mg/Kg of Silycristin respectively, to Male Sprague Dawley Rats-Treatment A, B, C

	Mean Silycristin Plasma Concentration (µg/mL)					
	Treatment A (6.13 mg/Kg)		**Treatment B (12.26 mg/Kg)**		**Treatment C (24.52 mg/Kg)**	
Time (Hrs)	Mean (n=6)	SD	Mean (n=7)	SD	Mean (n=6)	SD
0	0	0	0	0	0	0
0.25	5.12	2.05	15.85	7.24	33.96	13.32
0.5	1.25	0.64	6.07	5.03	13.52	7.65
0.75	0.47	0.17	2.00	1.47	7.03	3.47
1	0.40	0.16	0.89	0.43	3.95	1.83
2	0.14	0.09	0.54	0.63	0.95	0.32
4	0.07	0.02	0.13	0.04	0.28	0.16
6	--	--	0.07	0	0.08	0
Weight (Kg)	**0.29**	**0.017**	**0.30**	**0.01**	**0.30**	**0.01**

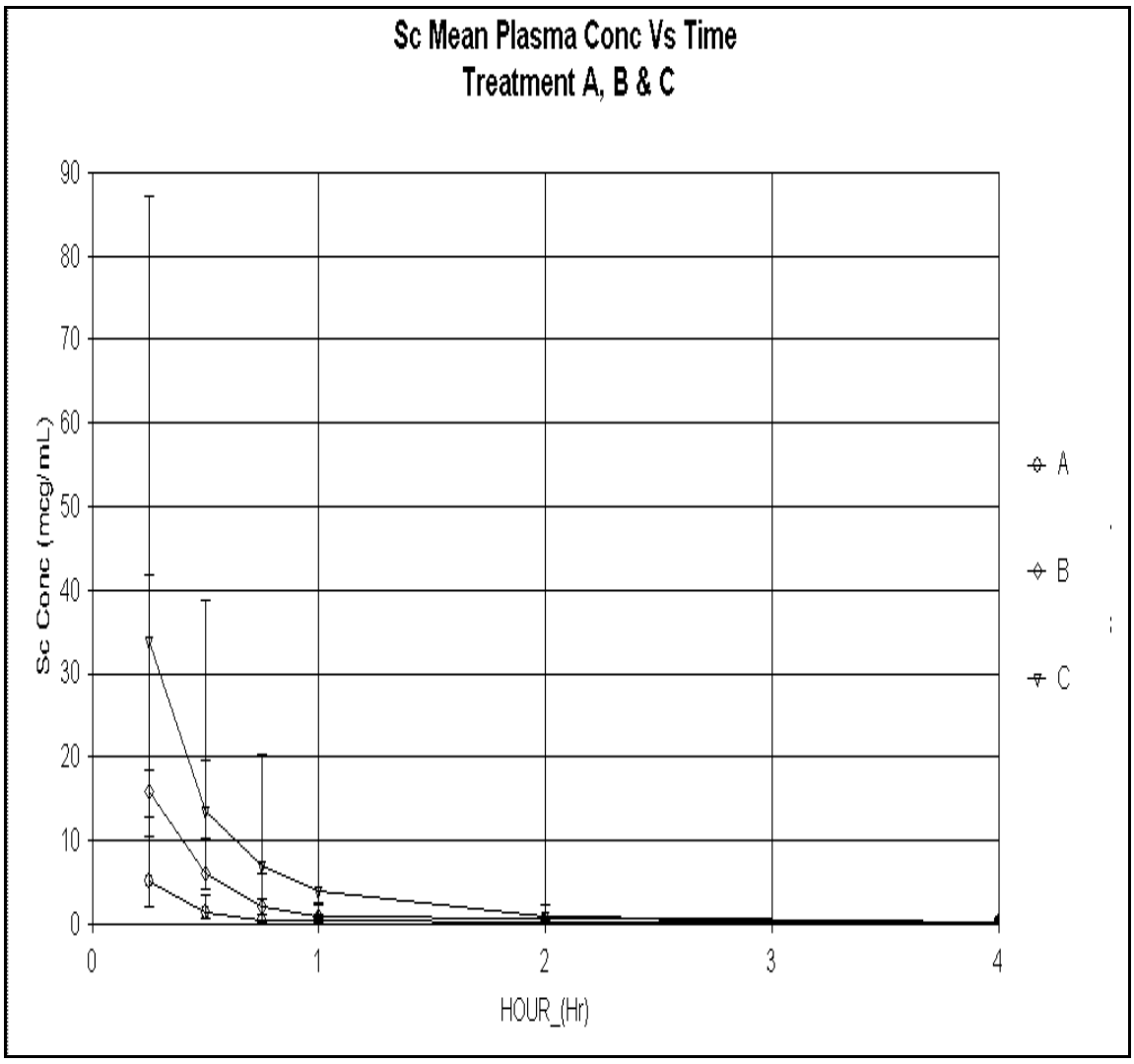


Figure 5.3. Mean Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg (A), 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 6.13 mg/Kg, 12.26 mg/Kg, 24.52 mg/Kg of Silycristin respectively, to Male Sprague Dawley Rats-Treatment A, B, C

Table 5.10:Mean Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg (A1), 250 mg/Kg (B1), 500 mg/Kg (C1) Silymarin equivalent to 30.65 mg/Kg, 61.30 mg/Kg, 122.61 mg/Kg of Silycristin respectively, to Male Sprague Dawley Rats-Treatment A1, B1, C1

	Mean Silycristin Plasma Concentration (µg/mL)					
	Treatment A1 (30.65 mg/Kg)		**Treatment B1 (61.30 mg/Kg)**		**Treatment C1 (122.61 mg/Kg)**	
Time (Hours)	Mean (n=5)	SD	Mean (n=3)	SD	Mean (n=4)	SD
0	0	0	0	0	0	0
0.25	0.52	0	0.35	0.32	0.51	0.11
0.5	0.29	0.06	0.21	0.15	0.38	0.22
0.75	0.24	0.17	0.28	0.05	0.37	0.10
1	0.31	0.33	0.14	0.08	0.45	0.17
2	0.37	0.28	0.28	0.36	0.40	0.23
4	0.54	0.55	0.43	0.48	0.43	0.28
6	0.52	0.11	0.53	0.12	0.92	0.83
8	0.42	0.23	0.30	0.19	0.93	0.29
12	0.29	0.16	0.22	0.11	0.38	0.05
16	0.27	0.04				
Weight (Kg)	**0.27**	**0.03**	**0.27**	**0.03**	**0.27**	**0.03**

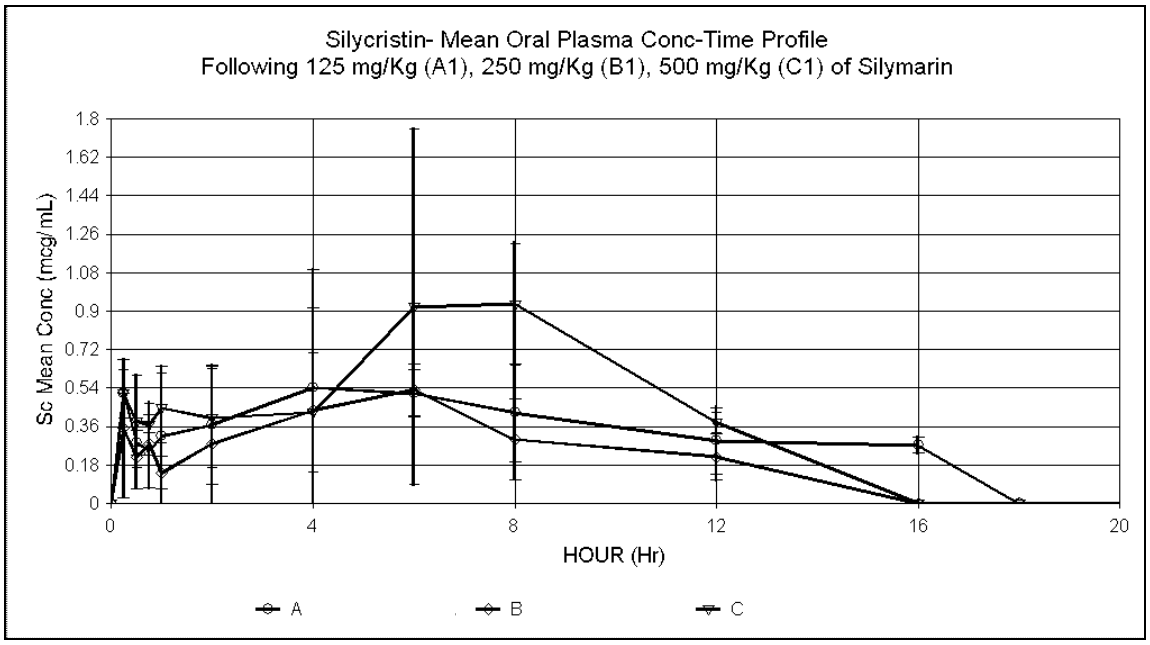


Figure 5.4:Mean Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg (A1), 250 mg/Kg (B1), 500 mg/Kg (C1) Silymarin equivalent to 30.65 mg/Kg, 61.30 mg/Kg, 122.61 mg/Kg of Silycristin respectively, to Male Sprague Dawley Rats-Treatment A1, B1, C1

Table 5.11: Comparison of Pharmacokinetic Parameters for Silycristin- (I.V Bolus Administration, Treatments A, B, C) from Mean Plasma Concentration-Time Data and Mean of Individual Pharmacokinetic Parameters

Non Compartmental Analysis (Cp= Plasma Conc.)						
Parameter	Treatment A Parameters n=6		Treatment B Parameters n=7		Treatment C Parameters n=6	
	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏
AUC_{0-t} (mcg-hr/ml)	4.65	5.45	12.84	13.66	28.82	29.04
$AUC_{0-\infty}$ (mcg-hr/ml)	4.79	5.58	12.99	13.77	28.95	29.65
Clearance (ml/Kg-hr)	1281.09	1351.81	944.02	1062.14	847.07	908.86
Vd (ml/kg)	1262.79	1726.07	1883.06	1007.75	1370.80	1134.76
Vss (ml/kg)	718.19	566.53	502.42	464.85	413.84	422.51
$t_{1/2}$ (hr)	0.68	0.86	1.38	0.65	1.12	0.90
E_H		0.26		0.18		0.18
2-Compartmental Analysis						
AUCo-inf (mcg-hr/ml)	4.87	6.49	12.57	13.98	26.05	28.71
Clearance (ml/Kg-hr)	1258.88	1192.22	975.69	1110.58	941.24	1007.84
Vd (ml/kg)	1234.23	1500.78	1840.94	1058.22	1518.58	1308.42
Vss (ml/kg)	378.18	474.34	568.19	470.68	514.46	559.25
$t_{1/2}$ (hr)	0.68	0.86	1.31	0.65	1.12	0.93
A (mcg/mL)	30.15	55.36	42.70	69.92	76.00	106.25
Alfa (hr^{-1})	7.75	8.93	4.31	6.32	3.67	4.75
B (mcg/mL)	1.00	1.11	1.41	5.36	3.30	5.48
Beta (hr^{-1})	1.02	0.97	0.53	1.33	0.62	0.81

***** Mean of plasma concentration time profile; **§** Mean of individual pharmacokinetic parameters
∏ Standard deviations for the means are listed in the Addendum at the end of this chapter.

Table 5.12:Mean of the Individual Non-Compartmental Pharmacokinetic Parameters obtained for Silycristin following Oral Administration of Silymarin (Treatments A1, B1, C1)

	Mean Non Compartmental Pharmacokinetic Parameters					
	Treatment A1 (30.65 mg/Kg)		**Treatment B1 (61.30 mg/Kg)**		**Treatment C1 (122.61 mg/Kg)**	
Parameter	Mean (n=5)	SD	Mean (n=3)	SD	Mean (n=4)	SD
AUC_{0-t} (mcg-hr/ml)	4.17	0.03	3.55	3.34	4.82	4.61
$AUC_{0-\infty}$ (mcg-hr/ml)	5.55	2.70	4.69	4.41	6.09	5.54
C_{max} (µg/mL)	0.73	3.62	0.61	0.47	0.94	0.58
T_{max} (Hr)	4.85	0.35	3.58	2.65	3.38	3.04
F	**0.15**	**0.10**	**0.06**	**0.06**	**0.04**	**0.04**

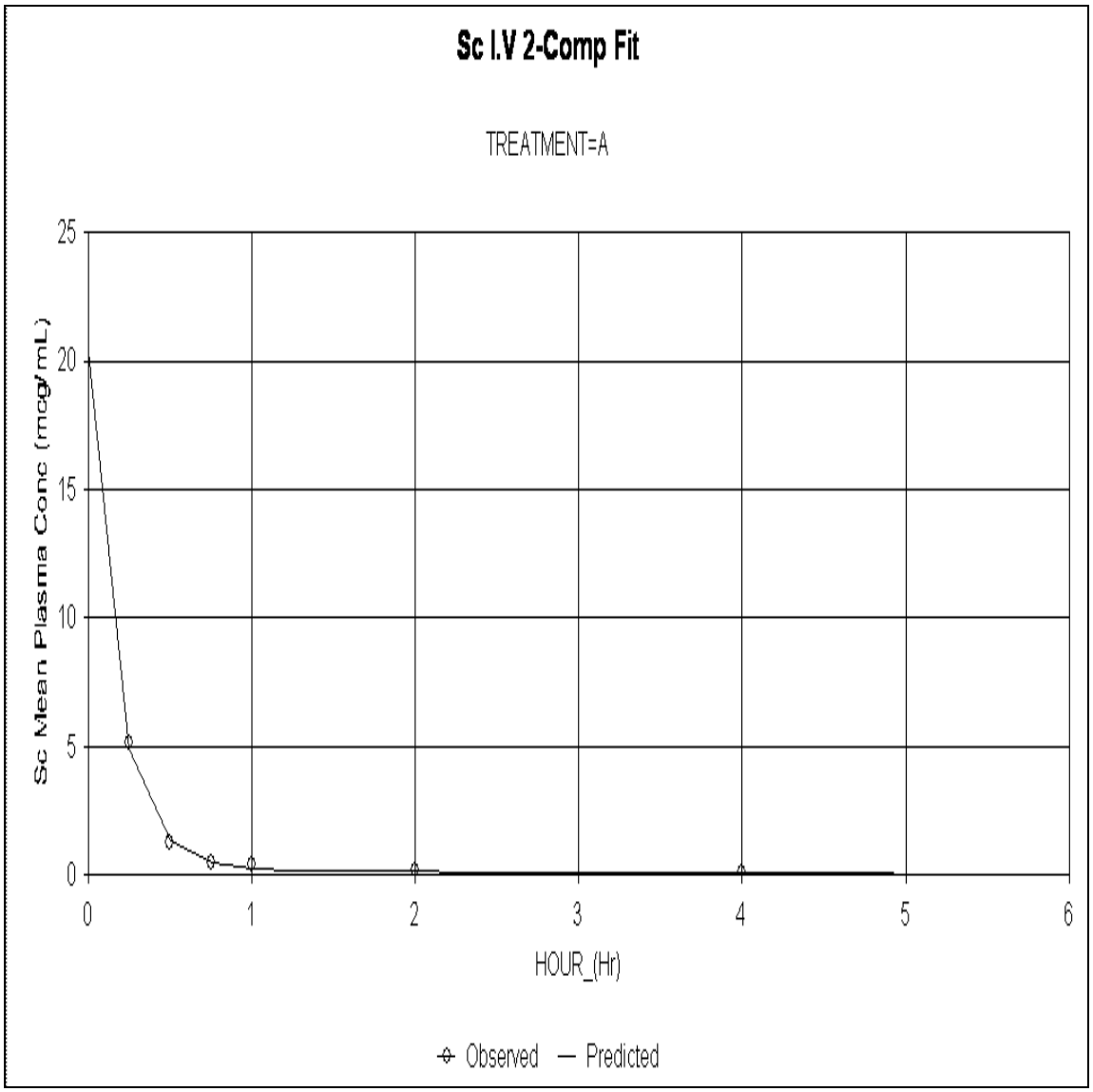


Figure 5.5: Representative Two-Compartmental Fit for Silycristin Following Intravenous Bolus Treatment A (Sc= Silycristin)

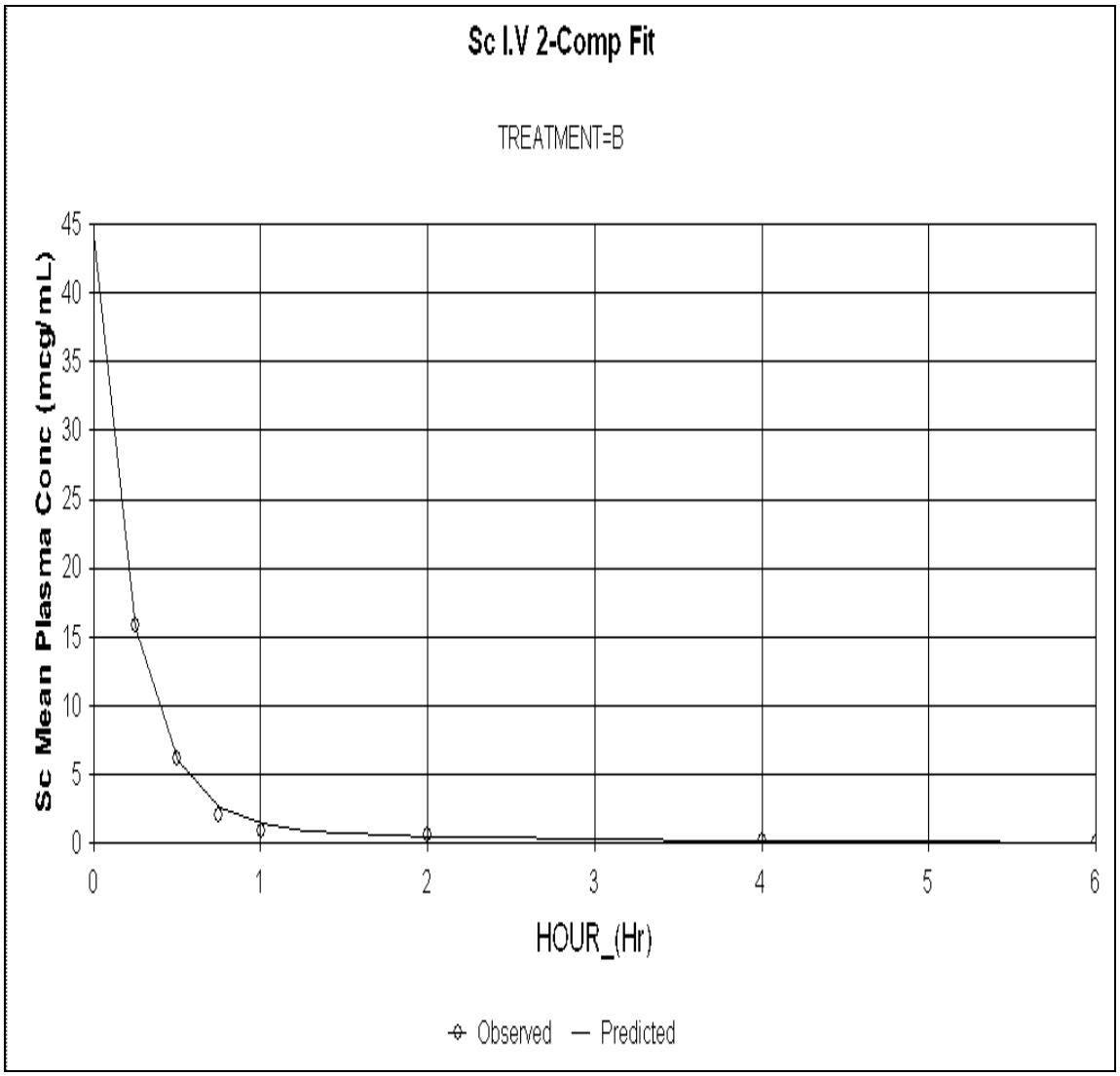


Figure 5.6: Representative Two-Compartmental Fit for Silycristin Following Intravenous Bolus Treatment B (Sc= Silycristin)

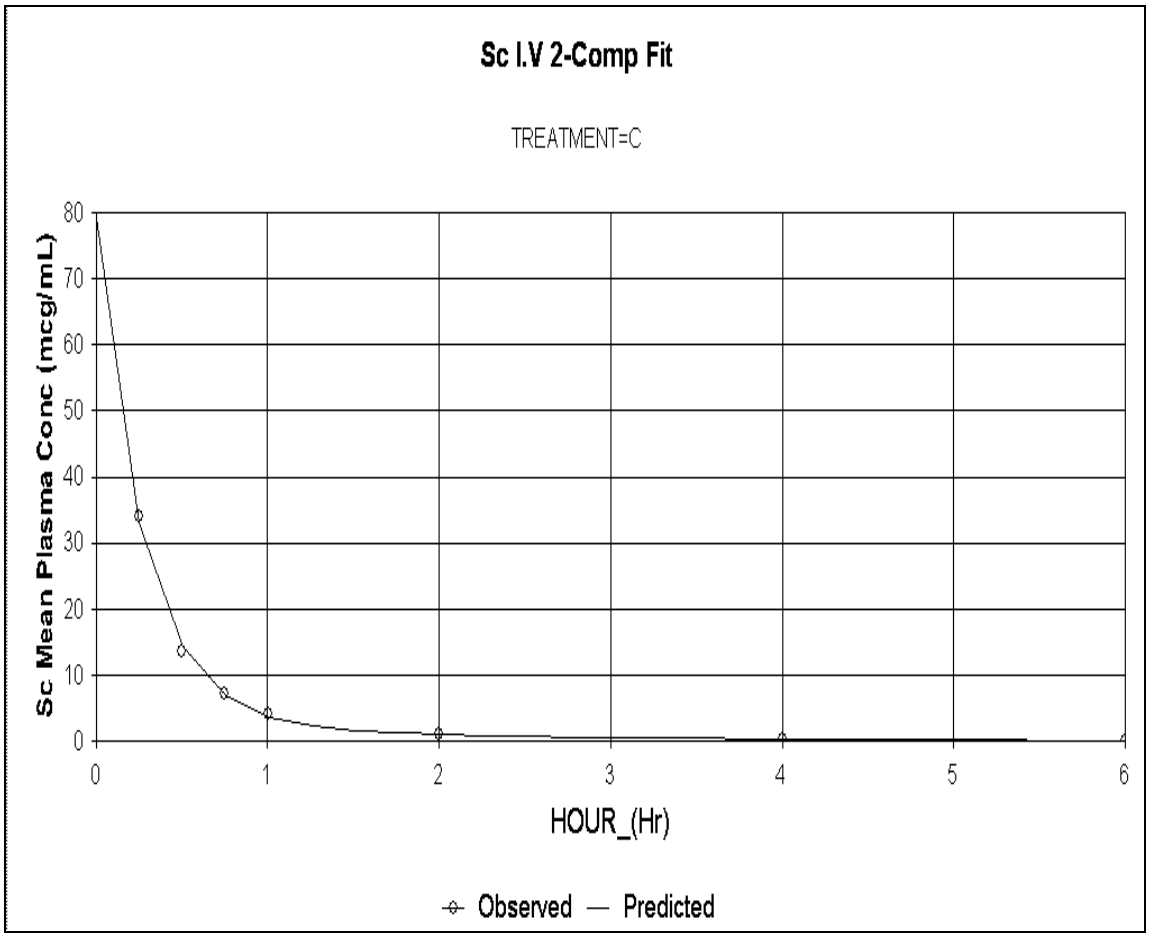


Figure 5.7: Representative Two-Compartmental Fit for Silycristin Following Intravenous Bolus Treatment C (Sc= Silycristin)

Table 5.13: Silycristin I.V Bolus Dose and Mean $AUC_{0\text{-}inf}$

(*Mean of the individual AUC's calculated using NCA)

Treatment (I.V Bolus)	Dose (µg/Kg)	*$AUC_{0\text{-}inf}$ (µg.hr/mL)
A	6130.63	5.58
B	12261.25	13.77
C	24522.51	29.65

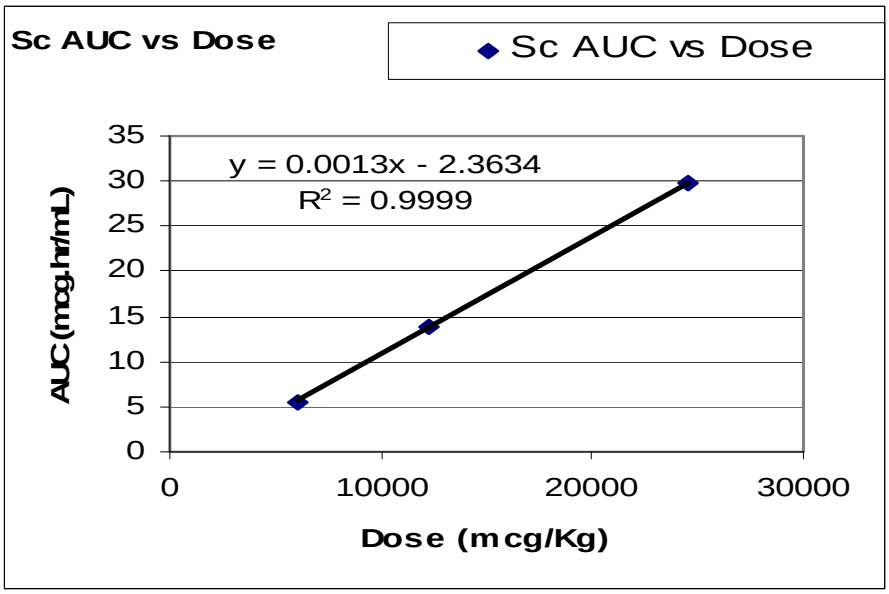


Figure 5.8: Dose Proportionality Plot for Silycristin $AUC_{0\text{-}inf}$ (µg.hr/mL) vs. Dose (µg/Kg) (Sc= Silycristin)

5.3.2: Pharmacokinetics of Silycristin

A comparison of the mean pharmacokinetic parameters obtained for silycristin following intravenous administration, using non compartmental analysis and 2-compartment modeling, are presented in the following Table 5.11. The plasma concentration-time data post intravenous administration fit a two compartment model, representative graphs of which are shown in Figures 5.5-5.7. A plot of the mean of the individual $AUC_{0\text{-}inf}$ obtained using non compartmental analysis for each I.V treatment (A,B&C) plotted against the silycristin dose equivalent of silymarin, indicated dose proportionality (Figure 5.8).

The values of $AUC_{0\text{-}inf}$, CL_T, V_d, and half life calculated from the mean plasma concentration-time profile and the means obtained from the individual pharmacokinetic parameters, using NCA do not differ significantly from those calculated using a two-compartment model confirming the assumption that the silycristin plasma data fits a two compartmental model. The mean half life for the three treatments (A,B,C) following intravenous administration ranges between 0.65-0.9 hour indicating no significant change in elimination with increasing dose.

Table 5.12 shows the absolute bioavailability for silycristin after oral administration of treatments (A1, B1 and C1). It is clearly seen that the bioavailability (F) decreases with the increasing dose (F_{A1}= 0.15±0.1, F_{B1}=0.06±0.06, F_{C1}=0.04±0.04) indicating dose dependency. This is possible mainly due to the overall low aqueous solubility of the silymarin extract. Low dissolution can cause dose dependency in bioavailability due to the fixed transit time through the gastrointestinal tract and thus

amount of silycristin absorbed is unlikely to increase in proportion with the dose administered.

Figure 5.4 shows the plot of silycristin mean plasma concentration versus time after oral administration of three treatments A1, B1 and C1. Though the highest plasma levels are observed for the lowest dose A1, all the three treatments show slight indications of an entero-hepatic cycle for silycristin. The silycristin mean plasma levels for the three doses, show a distinct rise between 0.25-0.5 hours followed by a decrease and then a Tmax between 3-6 hours before dropping to a minimum or below LOQ.

The hepatic extraction ratio (E_H) calculated from the CL_T for silycristin ranges between 0.18-0.26 indicating low clearance by the liver (<0.3). [165] This further supports the assumption that the low value of F calculated for silycristin is mainly due to dissolution limited absorption. The value of CL_T is used in the calculation of E_H instead of the CL_H based on previous reports about minimal amounts of silybin/silymarin being excreted from the urine. [58]

The mean V_d values (Table 5.11:Mean of Ind PK.Param), obtained either by NCA or 2-compartmental analysis for silycristin shows a distinct decrease when going from treatment A to B as compared to from B to C. This drop in the V_d from treatment A to B signifies possible saturability in binding to tissues.

5.3.3: Results for Silydianin

No detectable levels of silydianin were obtained following intravenous or oral administration. This could probably be due to the very low proportion of silydianin in the extract (~3.6%). After intravenous administration, traces of silydianin could be detected for the 0.25 hour and 0.5 hour time points respectively, after which the levels dropped below the limit of quantitation (0.08µg/mL). Thus, due to lack of sufficient data points for the determination of an elimination phase, pharmacokinetic parameters for silydianin could not be determined. Following oral administration, absolutely no traces of silydianin were detected in plasma. Along the low proportion of silydianin in the extract the low intestinal permeability of silydianin, as predicted by the high minimal cross sectional area (157.10 $Å^2$) and a low lipophilicity as predicted by the CLogP (-0.39), could be the most probable reasons for the absence of silydianin in plasma.

5.3.4: Results for Silybin A

Table 5.14: Mean Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg (A), 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 4.72 mg/Kg, 9.45 mg/Kg, 18.90 mg/Kg of Silybin A respectively, to Male Sprague Dawley Rats-Treatment A, B, C

	Mean Silybin A Plasma Concentration (µg/mL)					
	Treatment A (4.72 mg/Kg)		**Treatment B (9.45 mg/Kg)**		**Treatment C (18.90 mg/Kg)**	
Time (Hrs)	Mean (n=0)	SD	Mean (n=5)	SD	Mean (n=6)	SD
0	ND	--	0	0	0	0
0.25	ND	--	9.29	3.30	17.19	5.15
0.5	ND	--	3.19	1.92	5.08	1.83
0.75	ND	--	0.88	0.73	2.31	1.45
1	ND	--	0.63	0.43	1.35	0.63
2	ND	--	0.28	0.33	0.40	0.26
4	ND	--	--	--	0.16	0.13
6	ND	--	--	--	0.07	0
Weight (Kg)			0.299	0.006	0.305	0.013

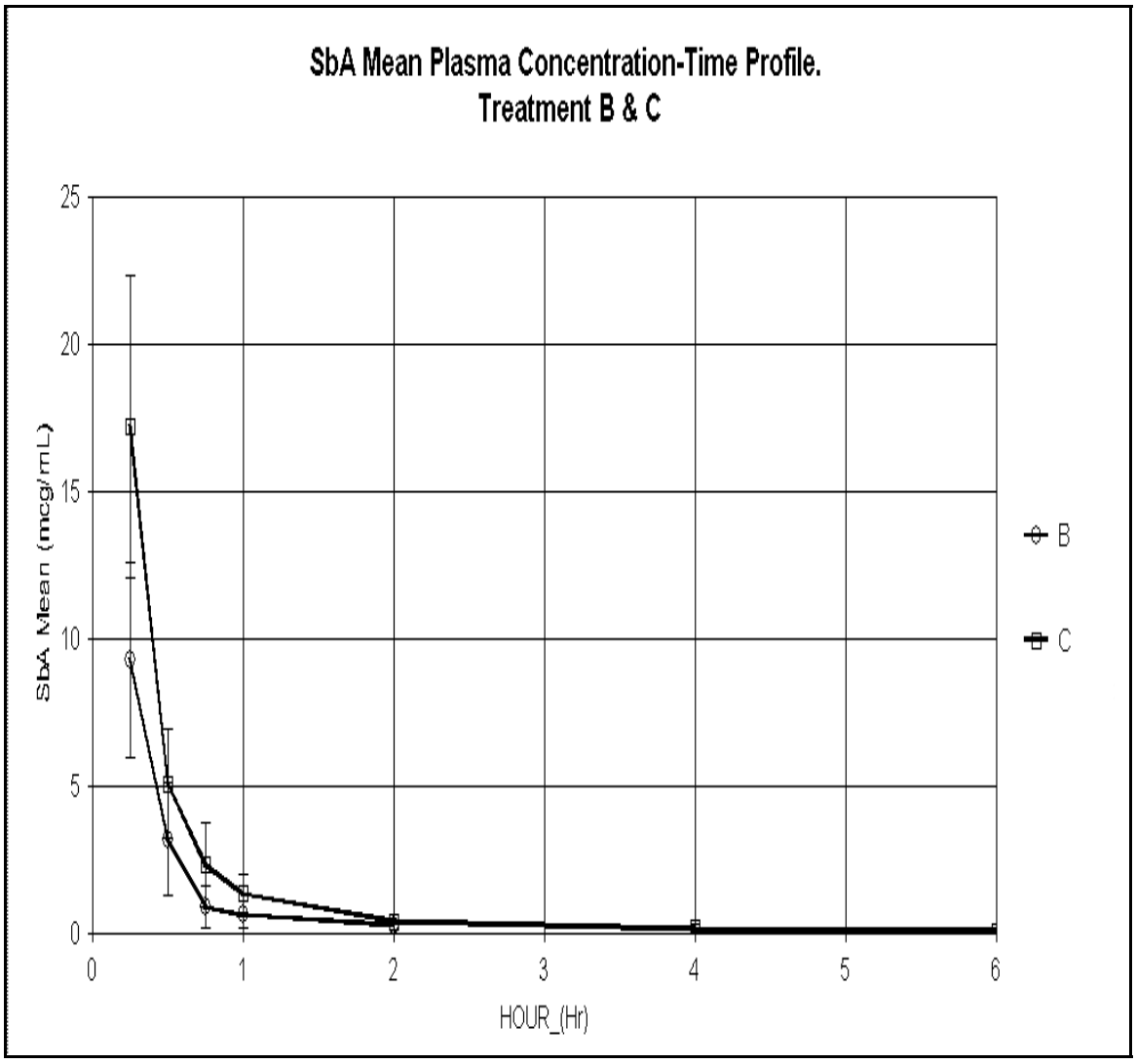


Figure 5.9: Mean Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 9.45 mg/Kg, 18.90 mg/Kg of Silybin A respectively, to Male Sprague Dawley Rats-Treatment B, C

Table 5.15:Mean Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg (A1), 250 mg/Kg (B1), 500 mg/Kg (C1) Silymarin equivalent to 23.63 mg/Kg, 47.27 mg/Kg, 94.54 mg/Kg of Silybin A respectively, to Male Sprague Dawley Rats-Treatment A1, B1, C1

	Mean Silybin A Plasma Concentration (µg/mL)					
	Treatment A1 (23.63 mg/Kg)		**Treatment B1 (47.27 mg/Kg)**		**Treatment C1 (94.54 mg/Kg)**	
Time (Hrs)	Mean (n=3)	SD	Mean (n=2)	SD	Mean (n=4)	SD
0	0	0	0	0	0	0
0.25	0.50	0.26	0.31	0	0.50	0.20
0.5	0.48	0	0.18	0.06	0.39	0.19
0.75	0.17	0.12	0.24	0	0.40	0.18
1	0.19	0.07	0.22	0.08	0.25	0.09
2	0.25	0.06	0.22	0.03	0.19	0.04
4	0.21	0.08	0.34	0.20	0.27	0.24
6	0.27	0.14	0.16	0.01	0.24	0.08
8	0.16	0.02	0.13	0	0.17	0.05
12	0.13	0	--	--	0.12	0
Weight (Kg)	**0.26**	**0.02**	**0.29**	**0.03**	**0.27**	**0.03**

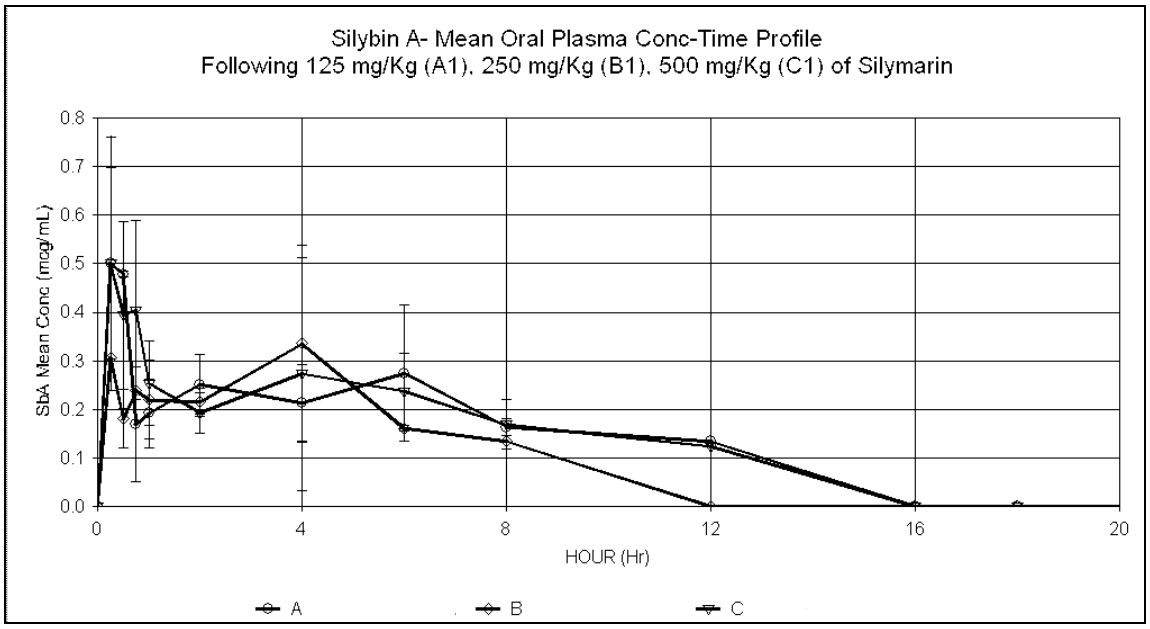


Figure 5.10: Mean Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg (A1), 250 mg/Kg (B1), 500 mg/Kg (C1) Silymarin equivalent to 23.63 mg/Kg, 47.27 mg/Kg, 94.54 mg/Kg of Silybin A respectively, to Male Sprague Dawley Rats-Treatment A1, B1, C1

Table 5.16: Comparison of Pharmacokinetic Parameters for Silybin A-(I.V Bolus Administration, Treatments A, B, C) from Mean Plasma Concentration-Time Data and Mean of Individual Pharmacokinetic Parameters

Non Compartmental Analysis (Cp= Plasma Conc.)						
Parameter	Treatment A Parameters n=0		Treatment B Parameters n=4		Treatment C Parameters n=6	
	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏
AUC_{0-t} (mcg-hr/ml)	ND	N/A	7.26	7.39	15.24	15.14
$AUC_{0-\infty}$ (mcg-hr/ml)	N/A	N/A	7.59	7.93	15.34	15.36
Clearance (ml/Kg-hr)	N/A	N/A	1245.58	1279.19	1232.95	1316.52
Vd (ml/kg)	N/A	N/A	1509.41	1561.42	1804.01	1768.92
Vss (ml/kg)	N/A	N/A	474.11	602.03	526.22	484.38
$t_{1/2}$ (hr)	N/A	N/A	0.84	0.87	1.01	0.95
E_H	N/A	N/A	--	0.25	--	0.26
2-Compartmental Analysis						
AUCo-inf (mcg-hr/ml)	N/A	N/A	7.73	8.33	13.88	14.71
Clearance (ml/Kg-hr)	N/A	N/A	1223.68	1192.37	1361.97	1429.35
Vd (ml/kg)	N/A	N/A	1474.31	1473.66	1974.12	1849.47
Vss (ml/kg)	N/A	N/A	509.43	569.56	641.95	634.39
$t_{1/2}$ (hr)	N/A	N/A	0.84	0.90	1.00	0.90
A (mcg/mL)	N/A	N/A	30.80	36.90	58.00	73.13
Alfa (hr^{-1})	N/A	N/A	1.40	5.84	2.17	5.63
B (mcg/mL)	N/A	N/A	5.10	1.31	5.40	2.47
Beta (hr^{-1})	N/A	N/A	0.83	0.88	0.69	0.94

* Mean of plasma concentration time profile; § Mean of individual pharmacokinetic parameters
∏ Standard deviations for the means are listed in the Addendum at the end of this chapter.

Table 5.17: Mean of the Individual Non-Compartmental Pharmacokinetic Parameters obtained for Silybin A following Oral Administration of Silymarin (Treatments A1, B1, C1)

	Mean Non Compartmental Pharmacokinetic Parameters					
	Treatment A1 (23.63 mg/Kg)		**Treatment B1 (47.27 mg/Kg)**		**Treatment C1 (94.54 mg/Kg)**	
Parameter	Mean (n=3)	SD	Mean (n=2)	SD	Mean (n=4)	SD
AUC_{0-t} (mcg-hr/ml)	1.98	0.26	1.55	0.63	1.66	0.51
$AUC_{0-\infty}$ (mcg-hr/ml)	3.92	0.77	2.53	0.16	2.54	0.47
C_{max} (µg/mL)	0.46	0.23	0.36	0.17	0.57	0.16
T_{max} (Hr)	2.25	3.25	2.38	2.30	1.31	1.81
F	**0.20**	**0.04**	**0.12**	**0.01**	**0.03**	**0.01**

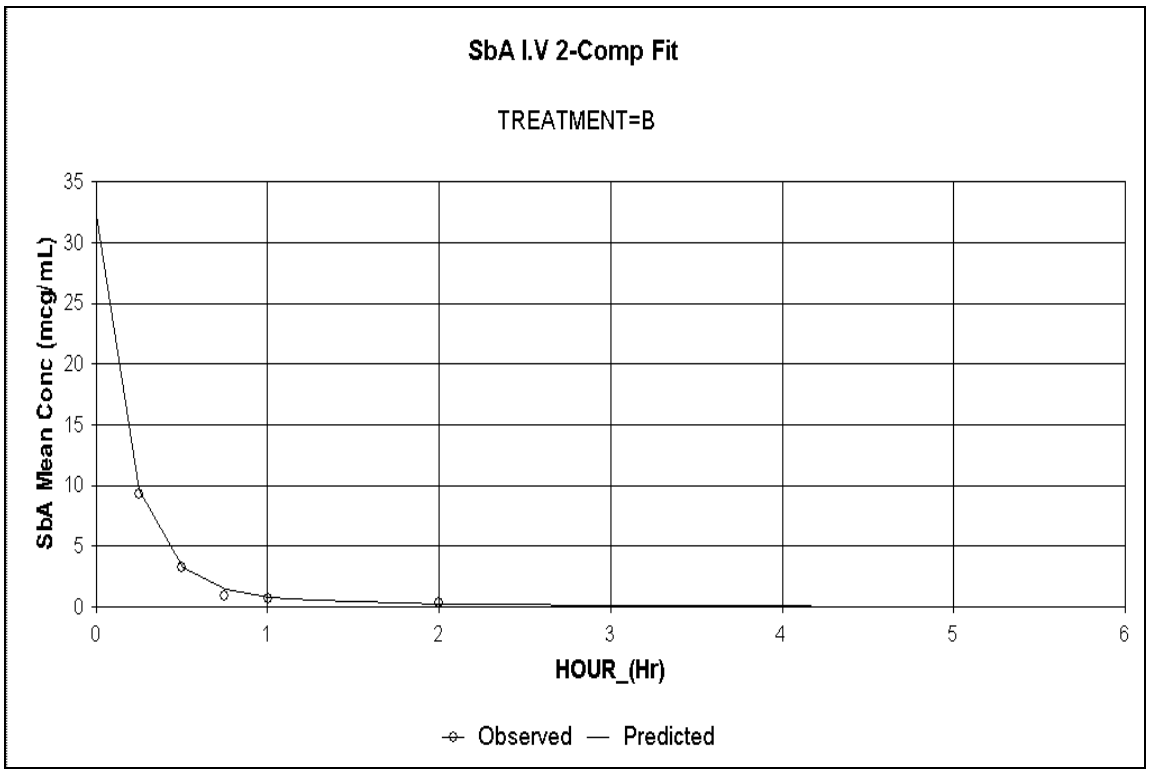


Figure 5.11: Representative Two-Compartmental Fit for Silybin A (Treatment B: 9.45mg/Kg) Following Intravenous Bolus Administration

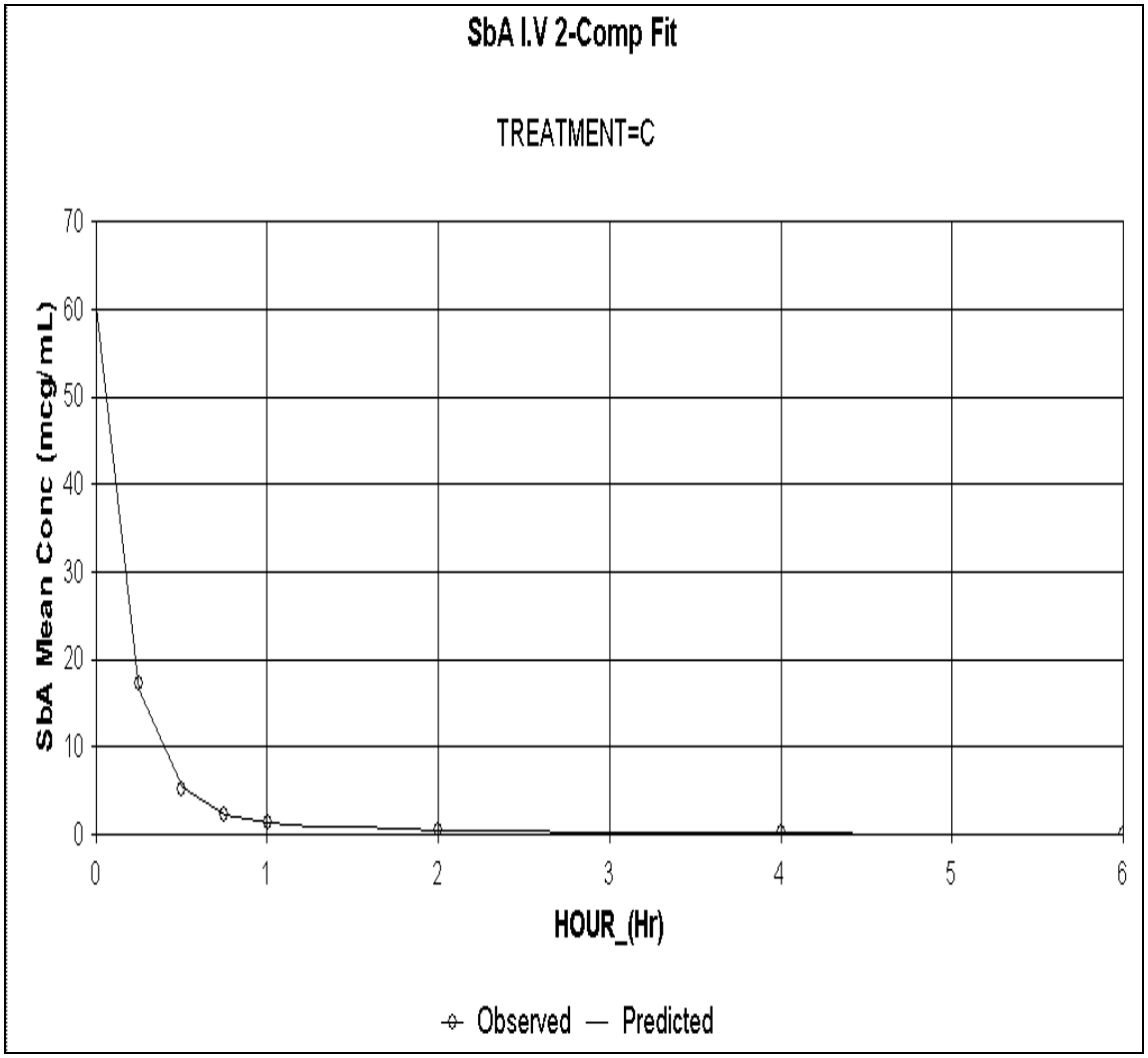


Figure 5.12: Representative Two-Compartmental Fit for Silybin A (Treatment C: 18.90mg/Kg) Following Intravenous Bolus Administration

5.3.5: Pharmacokinetics of Silybin A

Plasma levels for Silybin A, after I.V bolus administration of silymarin (Treatment A: 25 mg/Kg~4.72 mg/Kg silybin A) were not detected or were below LOQ (0.05µg/mL). Pharmacokinetic parameters were determined for silybin A: Treatment B (9.45 mg/Kg~50 mg/Kg silymarin) and Treatment C (18.90mg/Kg~100 mg/Kg silymarin), post I.V bolus administration using non-compartmental and two-compartmental analysis.

Dose proportionality for Silybin A could not be determined due to absence of plasma levels for Treatment A (4.72 mg/Kg). $AUC_{0\text{-}inf}$ after Treatment B (9.45 mg/Kg~50 mg/Kg silymarin) was calculated to be 7.93µg.hr/mL and after Treatment C (18.90mg/Kg~100 mg/Kg silymarin) was calculated as 15.36µg.hr/mL. Thus, the $AUC_{0\text{-}inf}$ increased approximately two fold with the doubling of dose, indicating a trend in dose proportionality but a regression analysis could not be done due to the presence of only 2 data points.

A comparison of the mean pharmacokinetic parameters obtained for silybin A following intravenous administration, using non compartmental analysis and 2-compartment modeling, are presented in the following Table 5.16. The plasma concentration-time data post intravenous administration fit a two compartment model, representative graphs of which are shown in Figures 5.11-5.12.

The values of $AUC_{0\text{-}inf}$, CL_T, V_d, and half life calculated from the mean plasma concentration-time profile and the means obtained from the individual pharmacokinetic parameters, using non compartmental analysis do not differ significantly from those

calculated using a two-compartment model confirming the assumption that the silybin A plasma data confidently fits a two compartmental model. The mean half life for each of the three treatments calculated from the individual plasma data ranges between 0.84-1.01 hour indicating no significant change in elimination of the drug with increasing dose.

Table 5.17 shows the absolute bioavailability for silybin A after oral administration of Treatments (B1=47.27 mg/Kg SbA~250 mg/Kg silymarin and C1=94.54 mg/Kg SbA~500 mg/Kg silymarin). The (F) values indicate that the bioavailability decreases with the increasing dose (F_{A1}=0.20±0.4, F_{B1}=0.12±0.01, F_{C1}=0.03±0.01) indicating dose dependency. The reason for the low bioavailability is probably the same as that for silycristin which is low aqueous solubility. Low dissolution can cause dose dependency in bioavailability due to the fixed transit time through the gastrointestinal tract thus making it unlikely for the increase in the absorption of silybin A with the increasing dose.

Figure 5.10 shows the plot of silybin A mean plasma concentration versus time after oral administration of three treatments A1, B1 and C1. The silybin A plasma concentration time profile shows signs of an entero-hepatic cycle with the plasma levels showing an initial increase between 0.25-0.5 hours and then another increase between 4-6 hours before finally dropping below LOQ. This trend is more prominent in silybin A as compared to the plot of silycristin (Figure 5.4).

The hepatic extraction ratio (E_H) calculated from the CL_T for silybin A ranges between 0.25-0.26 indicating low clearance by the liver (E_H<0.3). [165] This further supports the assumption that the decreasing value of F calculated for silybin A is mainly due to dissolution limited absorption with the increase in dose. The value of CL_T is used

in the calculation of E_H instead of the CL_H based on previous reports about minimal amounts of silybin/silymarin being excreted from the urine. [58]

The Vd values as calculated by two compartmental analysis, shows an approximate 33% increase between intravenous dose B and C. This increase in Vd with the increasing dose indicates saturable binding to plasma proteins. It should be noted that, Vd of silycristin as discussed earlier indicated saturable binding to tissues.

5.3.6: Results for Silybin B

Table 5.18: Mean Plasma Concentrations (μg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg (A), 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 8.64 mg/Kg, 17.29 mg/Kg, 34.58 mg/Kg of Silybin B respectively, to Male Sprague Dawley Rats-Treatment A, B, C

	Mean Silybin B Plasma Concentration (μg/mL)					
	Treatment A (8.64 mg/Kg)		**Treatment B (17.29 mg/Kg)**		**Treatment C (34.58 mg/Kg)**	
Time (Hrs)	Mean (n=6)	SD	Mean (n=8)	SD	Mean (n=6)	SD
0	0	0	0	0	0	0
0.25	4.62	1.53	12.06	6.23	25.37	9.46
0.5	2.18	1.97	4.34	2.44	7.93	4.14
0.75	0.87	0.34	1.79	0.88	4.34	3.18
1	0.76	0.39	1.22	0.60	2.44	1.01
2	0.54	0.38	0.80	0.45	1.04	0.61
4	0.20	0	0.65	0.45	0.82	0.35
6	--	--	--	--	0.63	0.56
8	--	--	--	--	0.13	0
Weight (Kg)	0.303	0.026	0.298	0.008	0.30	0.01

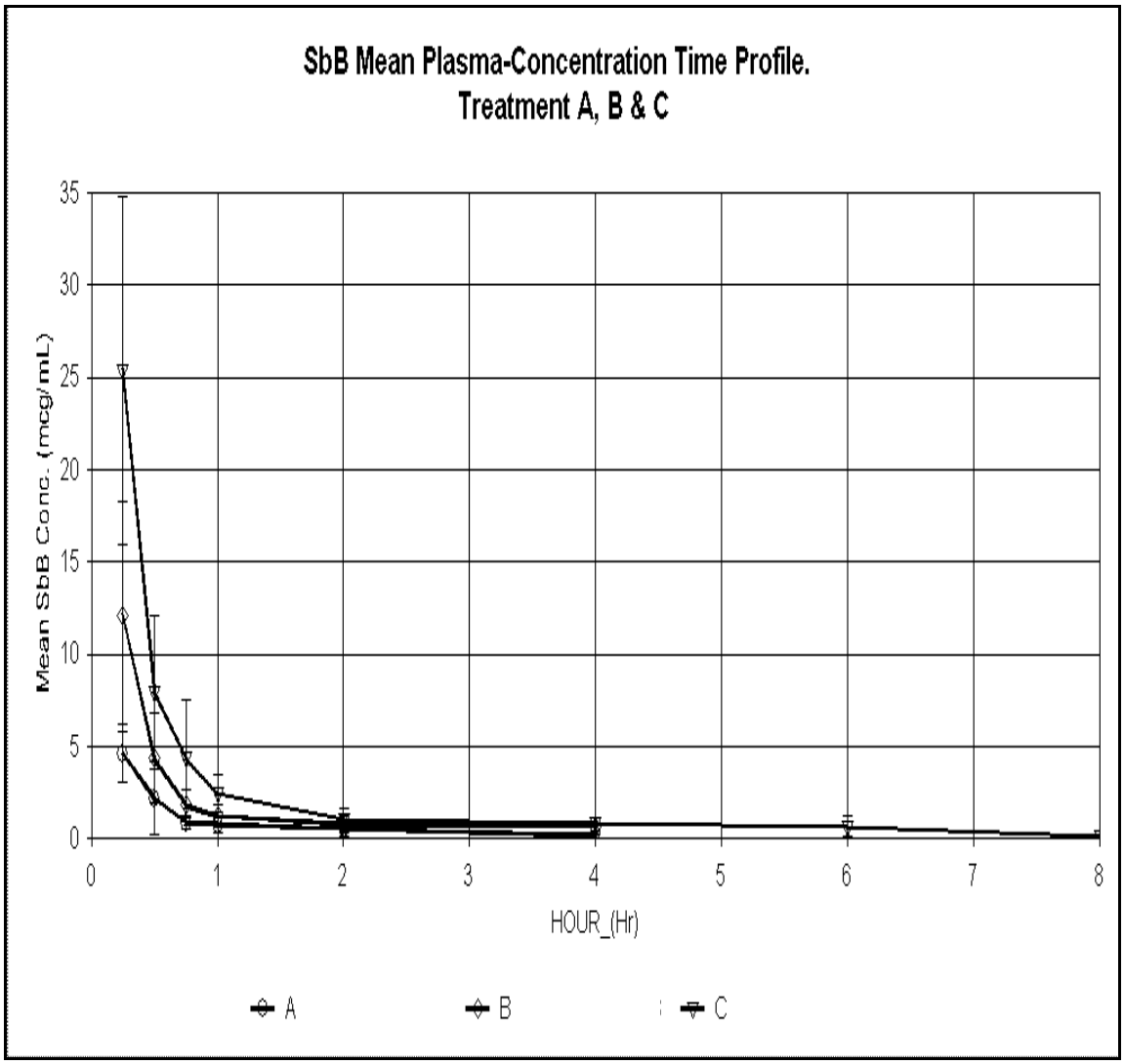


Figure 5.13: Mean Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg (A), 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 8.64 mg/Kg, 17.29 mg/Kg, 34.58 mg/Kg of Silybin B (SbB) respectively, to Male Sprague Dawley Rats-Treatment A, B, C

Table 5.19:Mean Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg (A1), 250 mg/Kg (B1), 500 mg/Kg (C1) Silymarin equivalent to 43.23 mg/Kg, 86.46 mg/Kg, 172.93 mg/Kg of Silybin B respectively, to Male Sprague Dawley Rats-Treatment A1, B1, C1

	Mean Silybin B Plasma Concentration (µg/mL)					
	Treatment A1 (43.23 mg/Kg)		**Treatment B1 (86.46 mg/Kg)**		**Treatment C1 (172.93 mg/Kg)**	
Time (Hrs)	Mean (n=2)	SD	Mean (n=5)	SD	Mean (n=4)	SD
0	0	0	0	0	0	0
0.25	0.19	0	0.25	0.12	0.63	0.14
0.5	0.11	0	0.19	0.13	0.49	0.30
0.75	0.17	0	0.24	0.09	0.43	0.19
1	0.19	0	0.18	0.12	0.21	0.07
2	0.30	0.23	0.22	0.15	0.34	0.26
4	0.81	0.81	0.51	0.47	0.80	0.84
6	0.22	0.04	0.23	0.17	0.80	0.74
8	0.14	0.02	0.15	0.04	0.43	0.19
12	0.12	0.02	0.24	0.19	0.12	0.01
16	--	--	0.16	0	--	--
Weight (Kg)	0.29	0.03	0.27	0.03	0.28	0.03

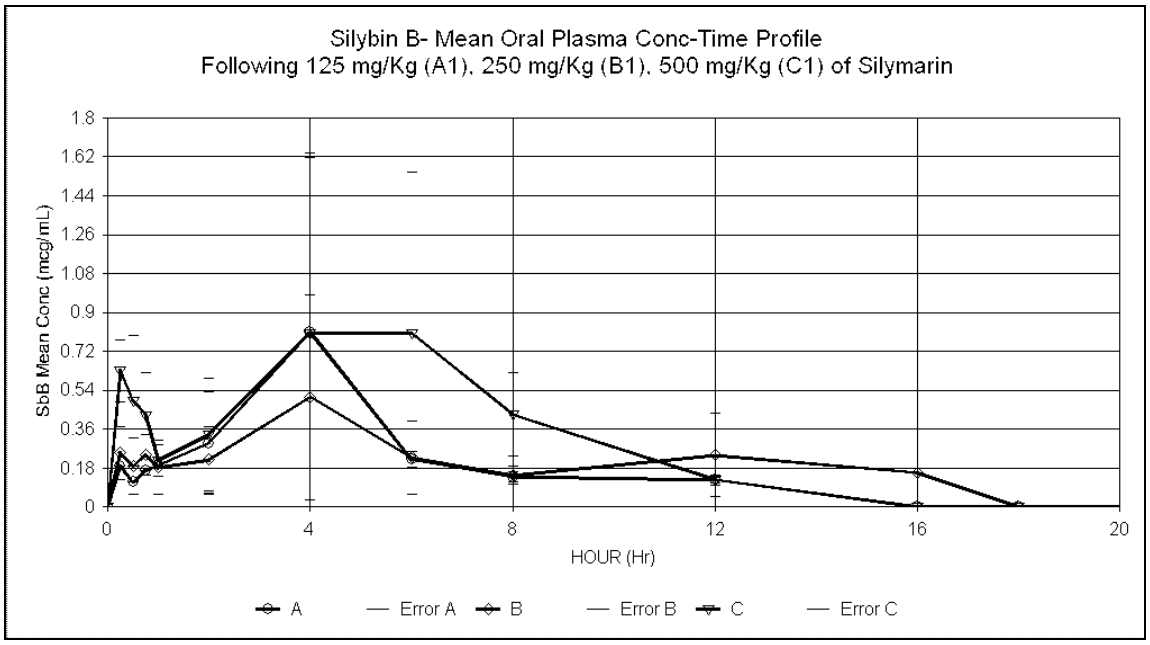


Figure 5.14: Mean Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg (A1), 250 mg/Kg (B1), 500 mg/Kg (C1) Silymarin equivalent to 43.23 mg/Kg, 86.46 mg/Kg, 172.93 mg/Kg of Silybin B respectively, to Male Sprague Dawley Rats-Treatment A1, B1, C1

Table 5.20: Comparison of Pharmacokinetic Parameters for Silybin B-(I.V Bolus Administration, Treatment A, B, C) from Mean Plasma Concentration-Time Data and Mean of Individual Pharmacokinetic Parameters

Non Compartmental Analysis (Cp= Plasma Conc.)						
Parameter	Treatment A Parameters n=5		Treatment B Parameters n=8		Treatment C Parameters n=6	
	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏
AUC_{0-t} (µg-hr/ml)	4.62	4.57	11.35	11.56	25.68	24.07
$AUC_{0-\infty}$ (µg-hr/ml)	5.16	7.17	13.77	14.08	26.07	26.48
Clearance (ml/Kg-hr)	1674.71	1314.30	1255.64	1272.04	1326.60	1381.00
Vd (ml/kg)	4577.14	3837.18	4645.06	3565.72	3837.02	4538.23
Vss (ml/kg)	2347.63	2469.51	2372.96	2459.83	1400.49	1818.23
$t_{1/2}$ (hr)	1.89	2.30	2.56	2.27	2.00	2.24
E_H	--	0.26	--	0.25	--	0.27
2-Compartmental Analysis						
AUCo-inf (mcg-hr/ml)	5.42	7.71	13.67	13.94	23.40	24.39
Clearance (ml/Kg-hr)	1595.77	1217.57	1265.33	1275.31	1478.08	1553.70
Vd (ml/kg)	4312.89	3545.23	4601.73	3997.97	4223.12	5169.78
Vss (ml/kg)	2553.48	2161.34	2259.48	2365.02	1765.69	2212.85
$t_{1/2}$ (hr)	1.87	2.27	2.52	2.25	1.98	2.22
A (mcg/mL)	10.40	25.70	38.70	48.59	70.50	89.12
Alfa (hr^{-1})	4.30	7.38	5.27	6.02	4.80	5.14
B (mcg/mL)	1.11	1.20	1.74	2.06	3.05	3.63
Beta (hr^{-1})	0.37	0.42	0.27	0.55	0.35	0.59

***** Mean of plasma concentration time profile; **§** Mean of individual pharmacokinetic parameters
∏ Standard deviations for the means are listed in the Addendum at the end of this chapter.

Table 5.21: Mean of the Individual Non-Compartmental Pharmacokinetic Parameters obtained for Silybin B following Oral Administration of Silymarin (Treatments A1, B1, C1)

	Mean Non Compartmental Pharmacokinetic Parameters					
	Treatment A1 (43.23 mg/Kg)		**Treatment B1 (86.46 mg/Kg)**		**Treatment C1 (172.93 mg/Kg)**	
Parameter	Mean (n=2)	SD	Mean (n=5)	SD	Mean (n=4)	SD
AUC_{0-t} (mcg-hr/ml)	3.29	2.09	2.62	1.12	4.07	2.98
$AUC_{0-\infty}$ (mcg-hr/ml)	5.17	0.68	3.13	1.25	4.56	3.09
C_{max} (µg/mL)	0.81	0.81	0.62	0.38	1.27	0.65
T_{max} (Hr)	4.00	0.00	5.40	4.10	2.69	2.79
F	**0.62**	**0.08**	**0.09**	**0.04**	**0.03**	**0.02**

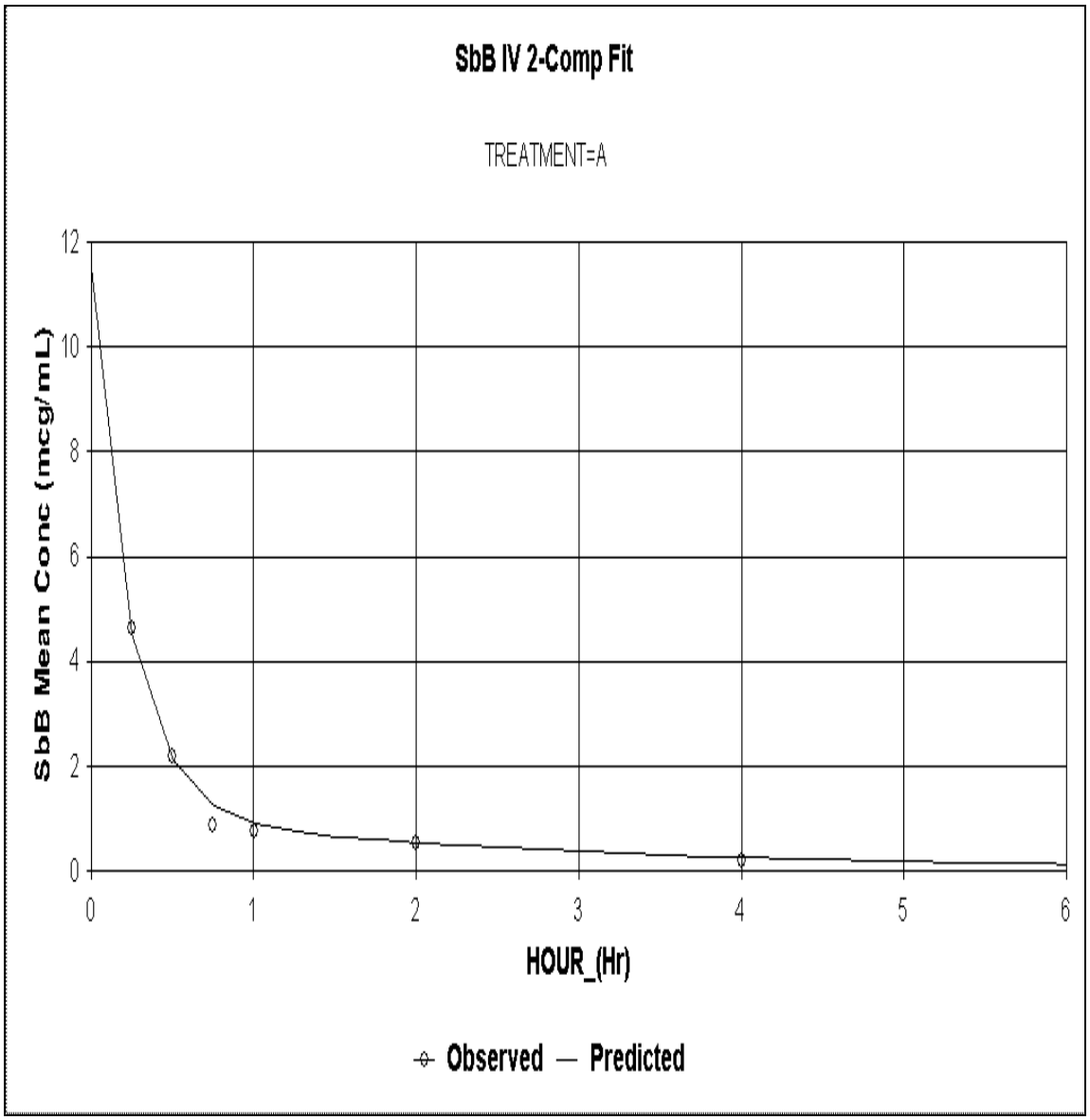


Figure 5.15: Representative Two-Compartmental Fit for Silybin B (SbB; Treatment A: 8.64 mg/Kg) Following Intravenous Bolus Administration

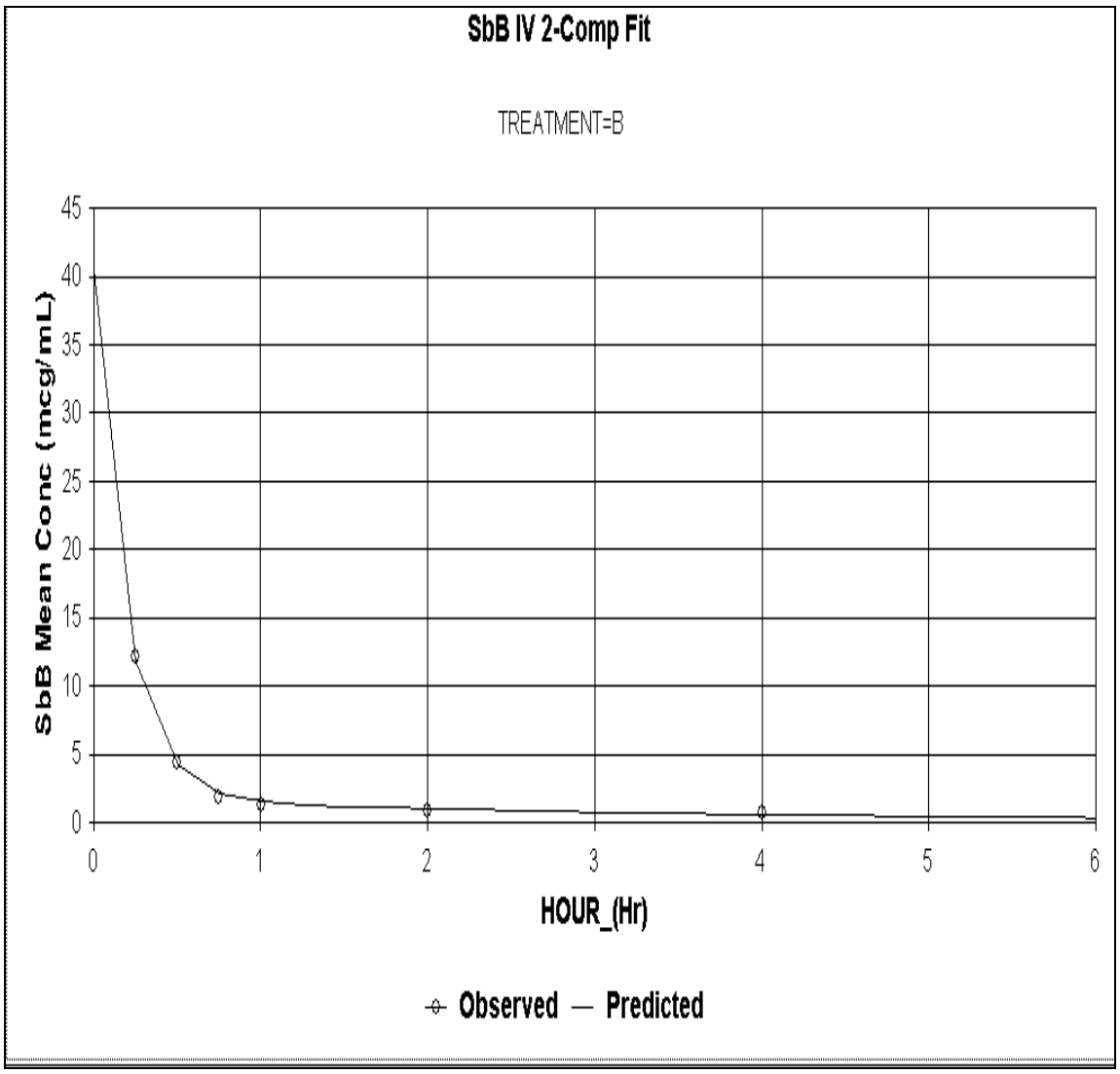


Figure 5.16: Representative Two-Compartmental Fit for Silybin B (SbB; Treatment B: 17.29 mg/Kg) Following Intravenous Bolus Administration

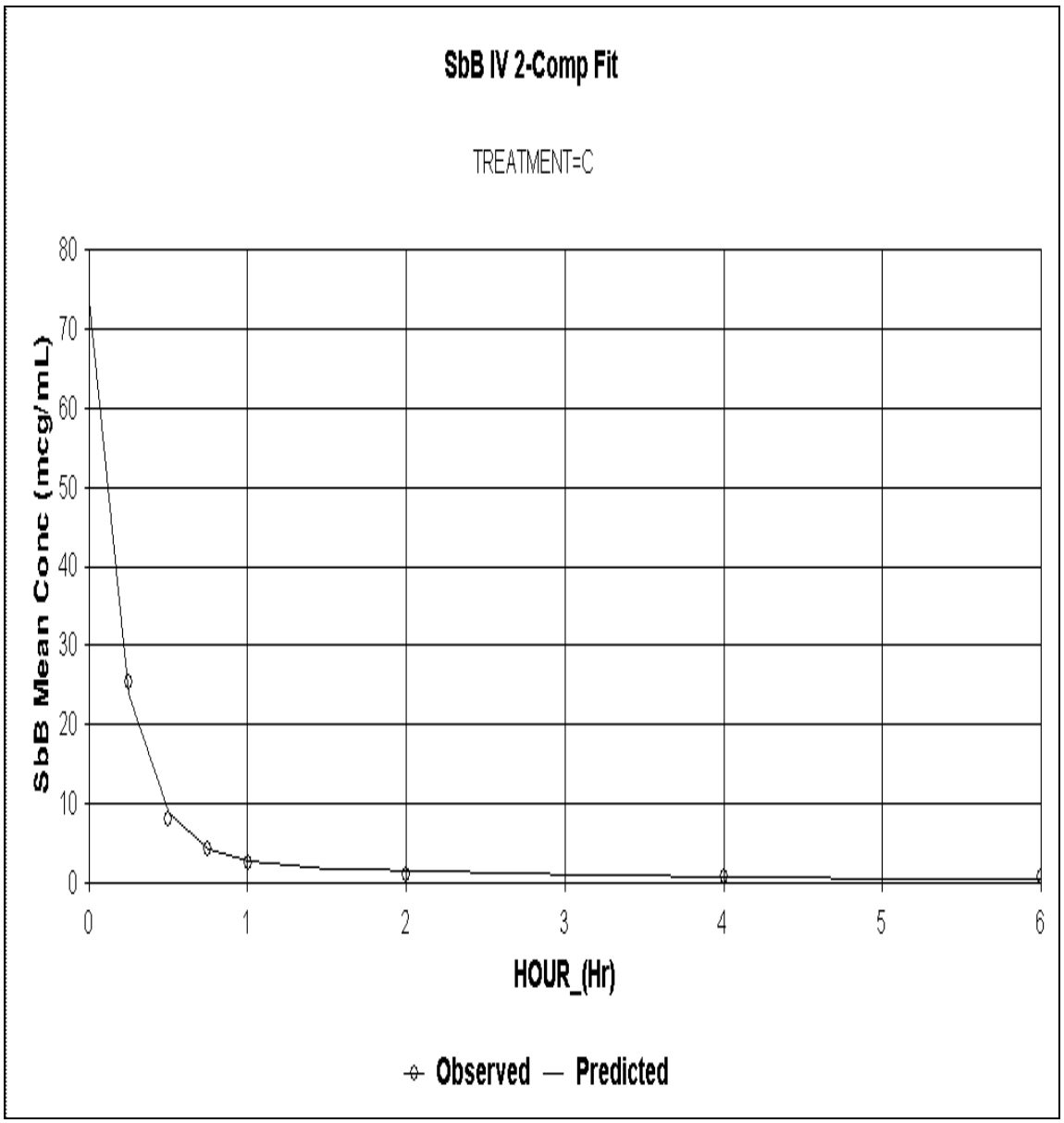


Figure 5.17: Representative Two-Compartmental Fit for Silybin B (SbB; Treatment C: 34.58 mg/Kg) Following Intravenous Bolus Administration

Table 5.22: Silybin B I.V Bolus Dose and Mean $AUC_{0\text{-}inf}$

(*Mean of the individual AUC's calculated using NCA)

Treatment (I.V Bolus)	Dose (µg/Kg)	*$AUC_{0\text{-}inf}$ (µg.hr/mL)
A	8646.82	7.17
B	17293.63	14.08
C	34587.27	26.48

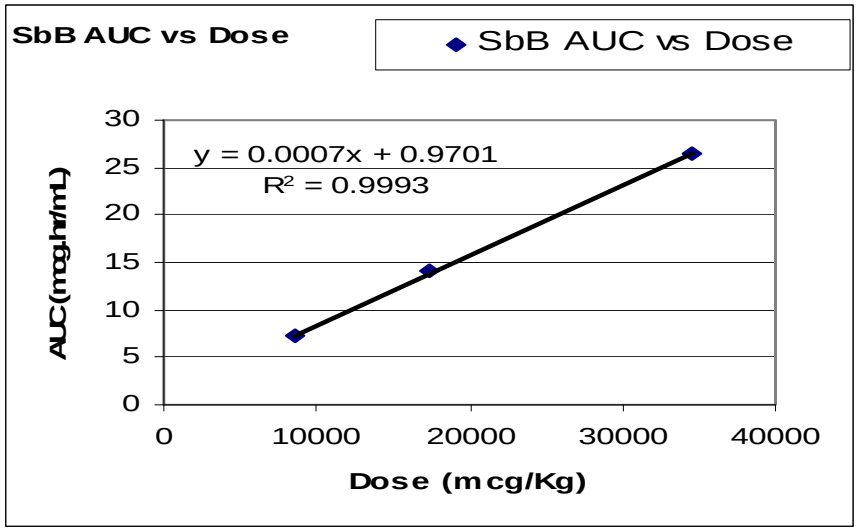


Figure 5.18: Dose Proportionality Plot for Silybin B $AUC_{0\text{-}inf}$ (µg.hr/mL) vs. Dose (µg/Kg) (SbB= Silybin B)

5.3.7: Pharmacokinetics of Silybin B

A comparison of the mean pharmacokinetic parameters obtained for silybin B following intravenous administration, using non compartmental analysis and 2-compartment modeling, are presented in Table 5.20. The plasma concentration-time data post intravenous administration fits a two compartment model, representative graphs of which are shown in Figures 5.15-5.17. A plot of the mean of the individual $AUC_{0\text{-}inf}$ obtained using non compartmental analysis for each I.V treatment (A,B&C) plotted against the silybin B dose equivalent of silymarin, indicated dose proportionality (Figure 5.18).

The respective values of $AUC_{0\text{-}inf}$, CL_T, V_d, and half life calculated from the mean plasma concentration-time profile and the means obtained from the individual pharmacokinetic parameters, using NCA are in close approximation with those calculated using two-compartmental analysis confirming the assumption that the silybin B plasma data confidently fits a two compartmental model. The mean half life for each of the three treatments calculated from the individual plasma data ranges between 2.2-2.3 hours indicating no significant change in elimination with increasing dose.

Table 5.21 shows the absolute bioavailability for silybin B after oral administration of treatments (A1=43.23 mg/Kg SbB~125 mg/Kg silymarin, B1=86.46 mg/Kg SbB~250 mg/Kg silymarin, and C1=172.93 mg/Kg SbB~500 mg/Kg silymarin). It is observed that the bioavailability (F) decreases with the increasing dose (F_{A1}= 0.62±0.08, F_{B1}=0.09±0.04, F_{C1}=0.03±0.02) indicating dose dependency. This is due to the overall low aqueous solubility of the silymarin extract. Low dissolution can cause

dose dependency in bioavailability due to the fixed transit time through the gastrointestinal tract and thus amount of silybin B absorbed is unlikely to increase in proportion with the dose administered.

It should be noted that even if the bioavailability decreased with the increasing dose for silycristin, silybin A and silybin B, F_{A1} for silycristin (0.15±0.1) was the lowest followed by silybin A (0.20±0.04) and then silybin B (0.62±0.08) in the increasing order.

Figure 5.14 shows the plot of silybin B mean plasma concentration versus time after oral administration of three treatments A1, B1 and C1. The oral plasma concentration-time profile weakly indicates an eneterohepatic cycle, but not as prominent as that for silybin A.

The hepatic extraction ratio (E_H) for silybin B ranges between 0.25-0.27 indicating low clearance by the liver (<0.3). [165] The value of F decreases with the increase in dose, but the value of EH stays constant over the three treatments, indicating that maximum oral bioavailability ($1-E_H$) for silybin B could be in the range of 0.73-0.75 indicating that the decrease in the value of bioavailability is mainly due to absorption factors such as limited dissolution rather than hepatic metabolism.

The mean V_d values (Table 5.15:Mean of Ind PK.Param), obtained by NCA remain constant with the increasing intravenous doses. But when the intravenous data is treated as a two-compartment model, the Vd between Treatment A (3.5 L/Kg) and Treatment C (5.16 L/Kg) increases by 62% indicating extensive plasma protein binding.

5.3.8: Results for Isosilybin A

Table 5.23: Mean Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg (A), 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 2.05 mg/Kg, 4.11 mg/Kg, 8.22 mg/Kg of Isosilybin A respectively, to Male Sprague Dawley Rats-Treatment A, B, C

	Mean Isosilybin A Plasma Concentration (µg/mL)					
	Treatment A (2.05 mg/Kg)		**Treatment B (4.11 mg/Kg)**		**Treatment C (8.22 mg/Kg)**	
Time (Hrs)	Mean (n=5)	SD	Mean (n=7)	SD	Mean (n=5)	SD
0	0	0	0	0	0	0
0.25	1.66	1.24	2.60	0.81	5.71	1.73
0.5	0.45	0.26	0.89	0.44	1.67	0.65
0.75	0.23	0.07	0.37	0.10	0.97	0.28
1	0.18	0.06	0.26	0.06	0.59	0.19
2	0.07	0.03	0.10	0.03	0.22	0.09
4	0.08	0	0.10	0.03	0.14	0.04
6	--	--	--	--	0.11	0.00
Weight (Kg)	0.30	0.02	0.30	0.008	0.31	0.01

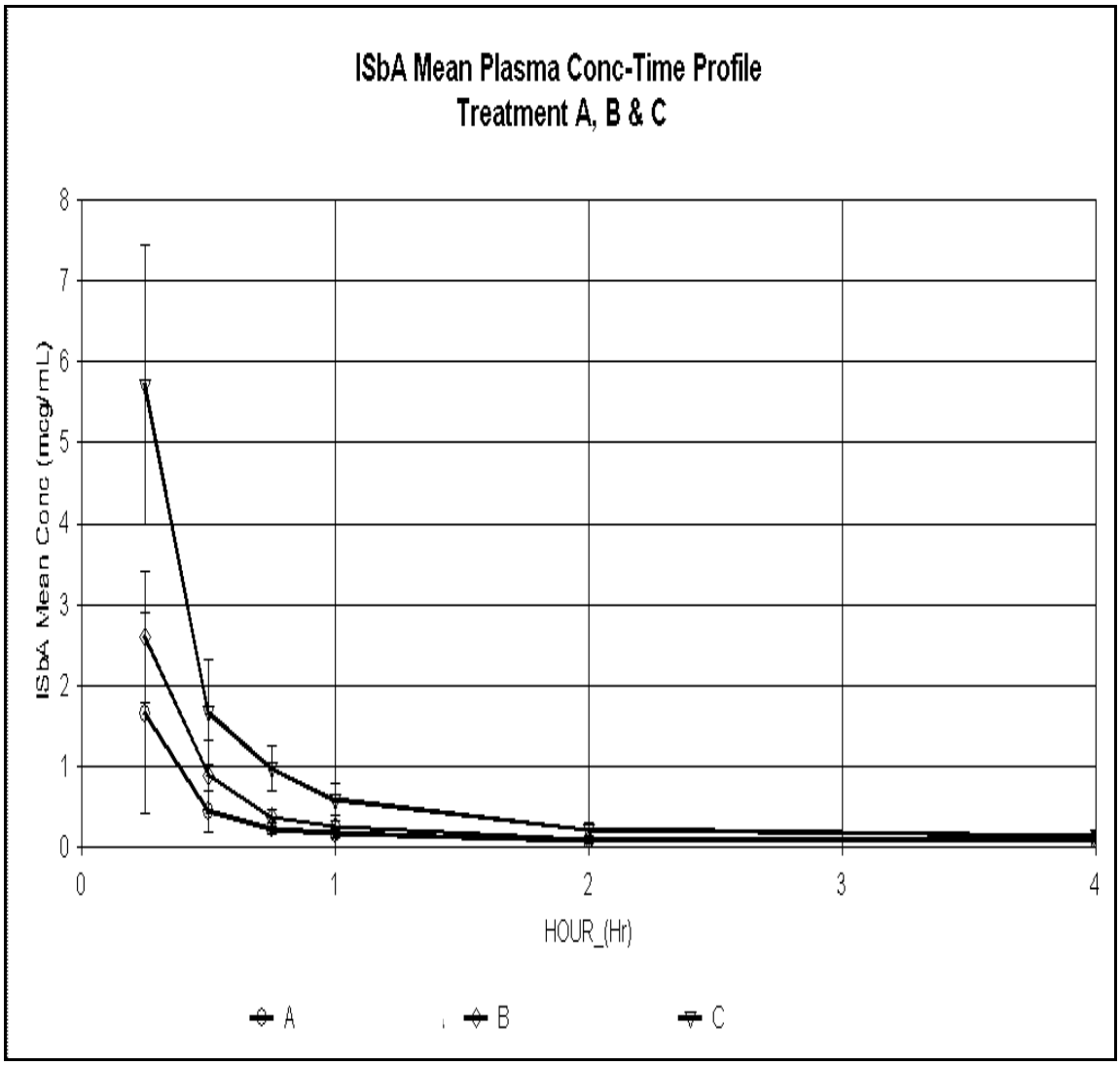


Figure 5.19: Mean Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg (A), 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 2.05 mg/Kg, 4.11 mg/Kg, 8.22 mg/Kg of Isosilybin A respectively, to Male Sprague Dawley Rats-Treatment A, B, C

Table 5.24: Comparison of Pharmacokinetic Parameters for Isosilybin A-(I.V Bolus Administration, Treatment A, B, C) from Mean Plasma Concentration-Time Data and Mean of Individual Pharmacokinetic

Non Compartmental Analysis (Cp= Plasma Conc.)						
Parameter	Treatment A Parameters n=4		Treatment B Parameters n= 7		Treatment C Parameters n= 5	
	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏
AUC_{0-t} (mcg-hr/ml)	1.65	1.04	2.32	2.33	5.36	5.47
$AUC_{0-\infty}$ (mcg-hr/ml)	1.93	1.17	2.56	2.51	5.70	5.77
Clearance (ml/Kg-hr)	1067.07	1918.90	1603.56	1780.37	1443.66	1518.61
Vd (ml/kg)	3643.66	3375.78	4128.04	3011.19	3344.82	2780.47
Vss (ml/kg)	1611.72	1824.19	1750.12	1294.44	1125.93	1100.04
$t_{1/2}$ (hr)	2.37	1.32	1.78	1.28	1.61	1.27
E_H	--	0.37	--	0.35	--	0.30
2-Compartmental Analysis						
AUCo-inf (mcg-hr/ml)	2.06	1.38	2.54	2.40	4.82	5.54
Clearance (ml/Kg-hr)	997.50	1671.09	1618.76	1797.01	1705.87	1578.40
Vd (ml/kg)	3381.39	3253.49	4150.67	3112.39	3876.98	2900.06
Vss (ml/kg)	1470.86	1775.52	1700.02	1368.77	1565.56	1298.48
$t_{1/2}$ (hr)	2.35	1.33	1.78	1.27	1.58	1.26
A (mcg/mL)	8.87	6.32	8.70	8.87	15.30	22.95
Alfa (hr^{-1})	7.31	6.40	5.38	5.83	4.84	5.70
B (mcg/mL)	0.25	0.34	0.36	0.51	0.73	1.33
Beta (hr^{-1})	0.37	0.42	0.27	0.55	0.35	0.59

***** Mean of plasma concentration time profile; **§** Mean of individual pharmacokinetic parameters
∏ Standard deviations for the means are listed in the Addendum at the end of this chapter.

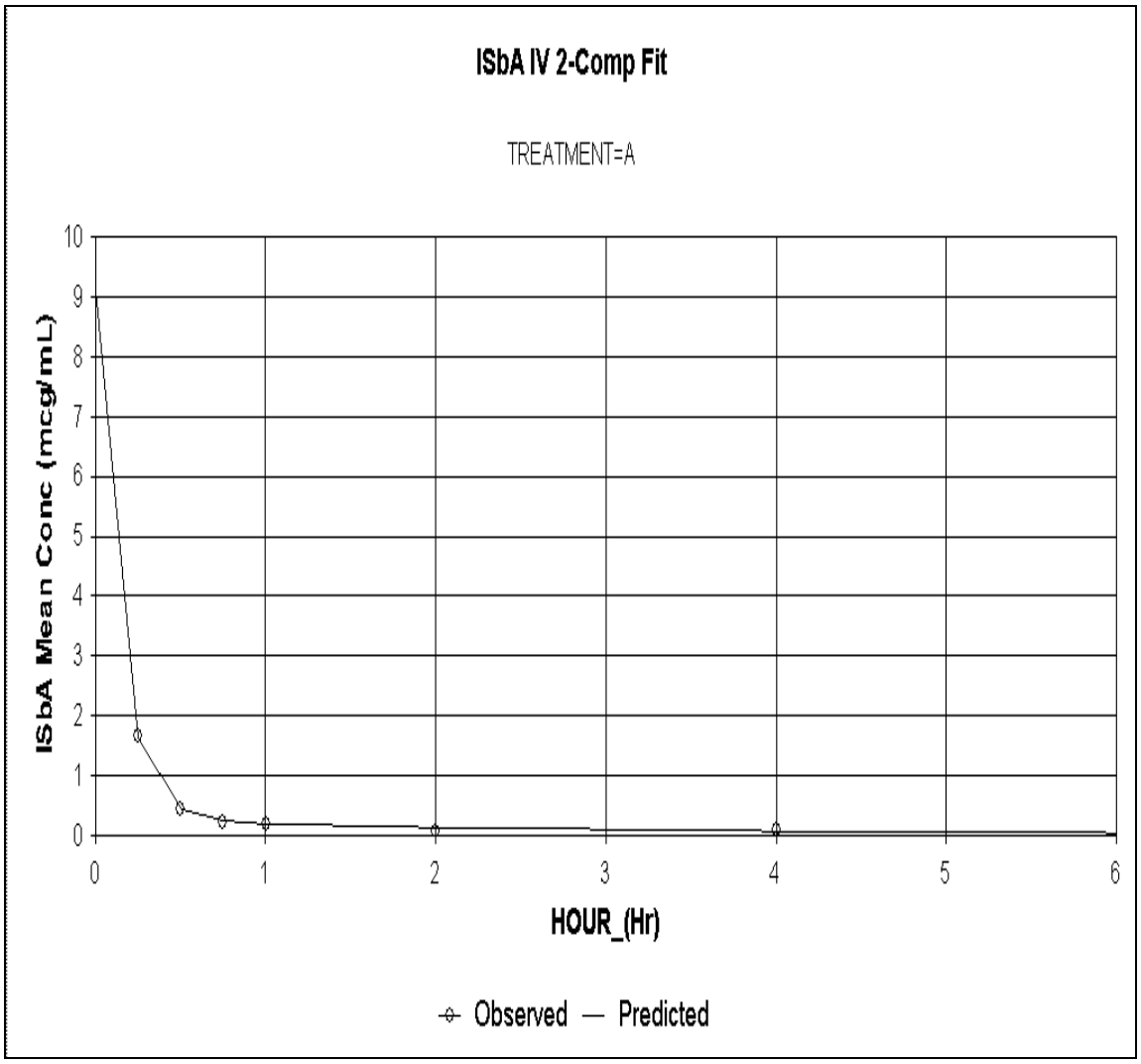


Figure 5.20: Representative Two-Compartmental Fit for Isosilybin A (ISbA; Treatment A: 2.05 mg/Kg) Following Intravenous Bolus Administration

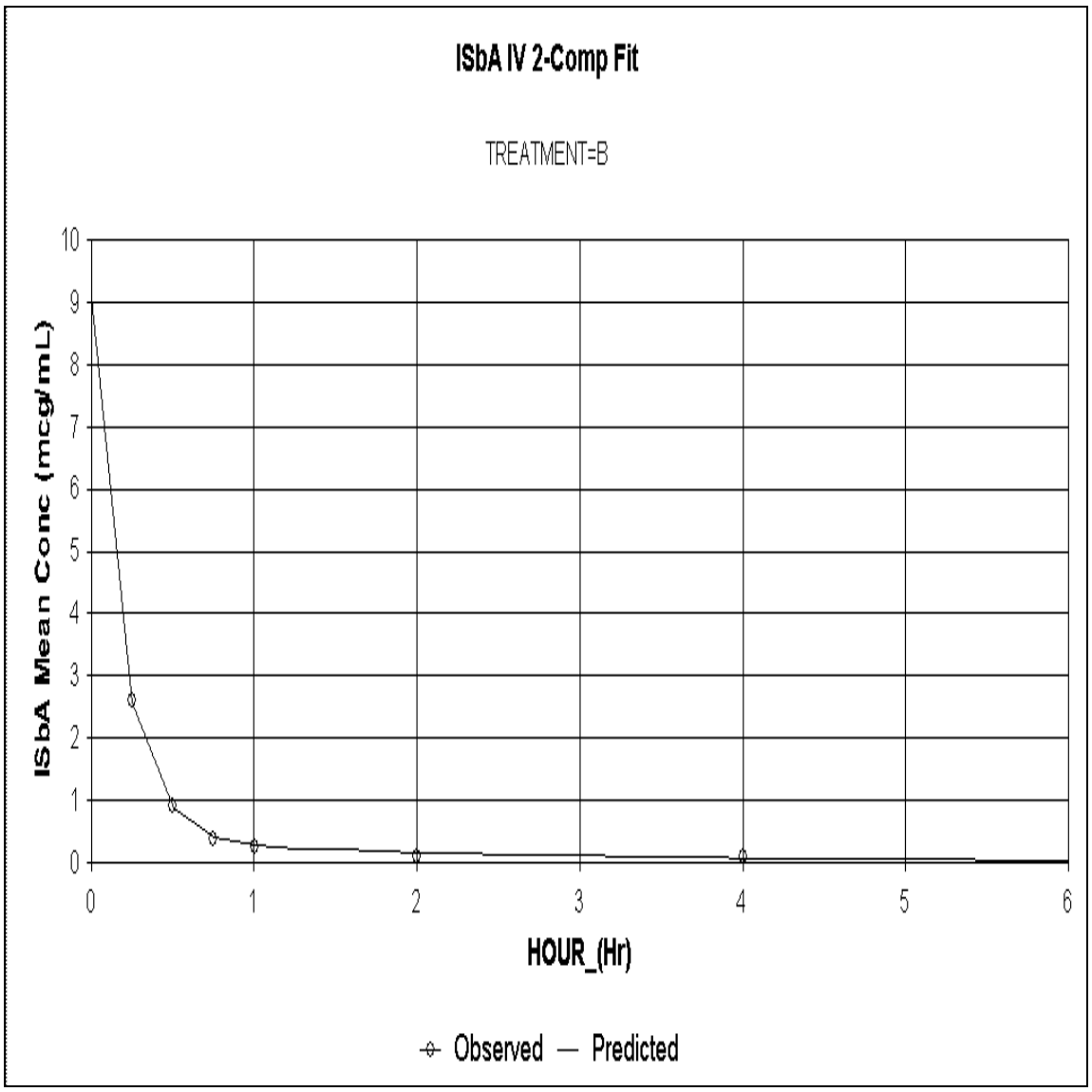


Figure 5.21: Representative Two-Compartmental Fit for Isosilybin A (ISbA; Treatment B: 4.11 mg/Kg) Following Intravenous Bolus Administration

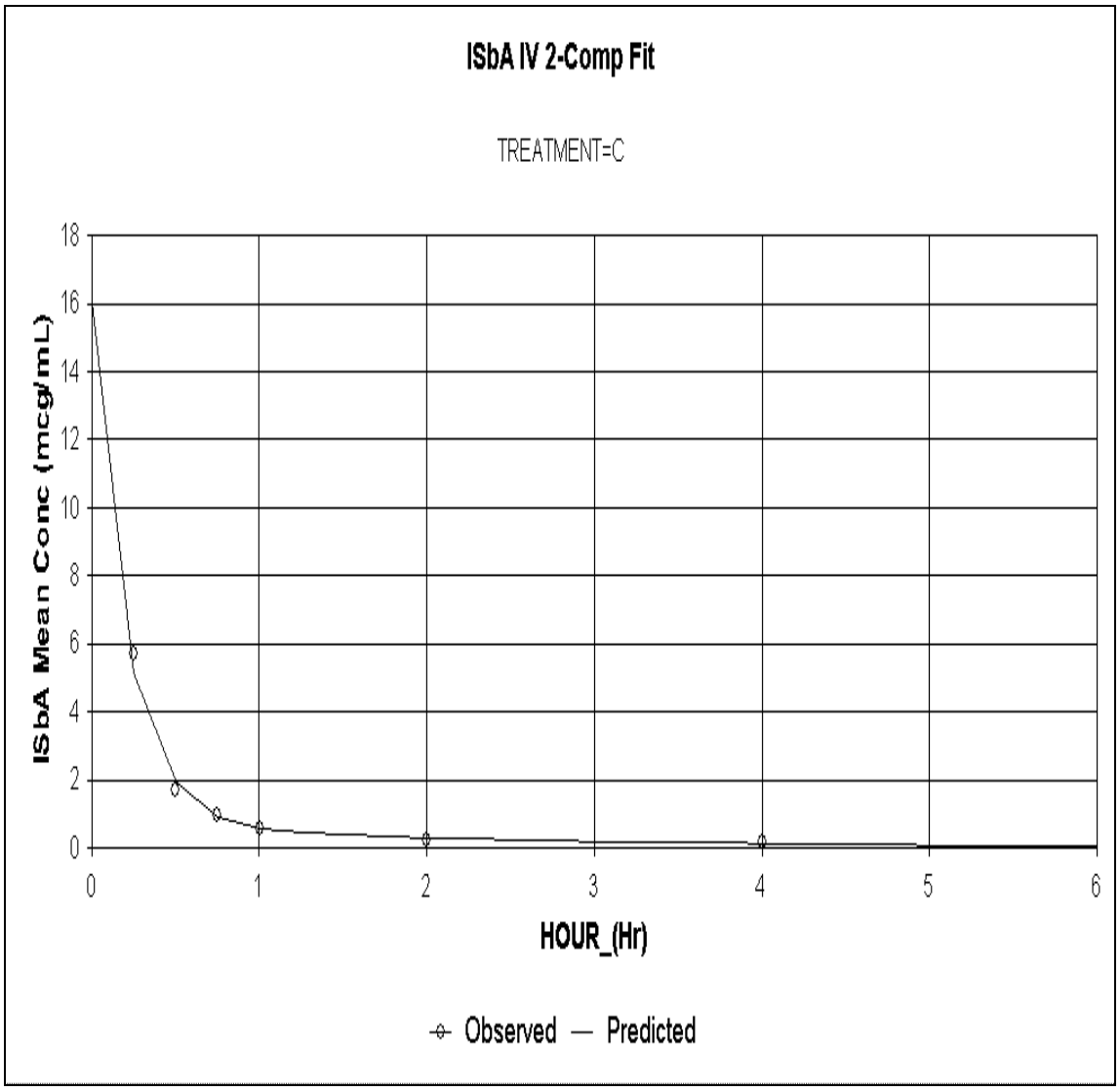


Figure 5.22: Representative Two-Compartmental Fit for Isosilybin A (ISbA; Treatment C: 8.22 mg/Kg) Following Intravenous Bolus Administration

Table 5.25: Isosilybin A I.V Bolus Dose and Mean $AUC_{0\text{-}inf}$

(*Mean of the individual AUC's calculated using NCA)

Treatment (I.V Bolus)	Dose (µg/Kg)	*$AUC_{0\text{-}inf}$ (µg.hr/mL)
A	2055.68	1.17
B	4111.35	2.51
C	8222.71	5.77

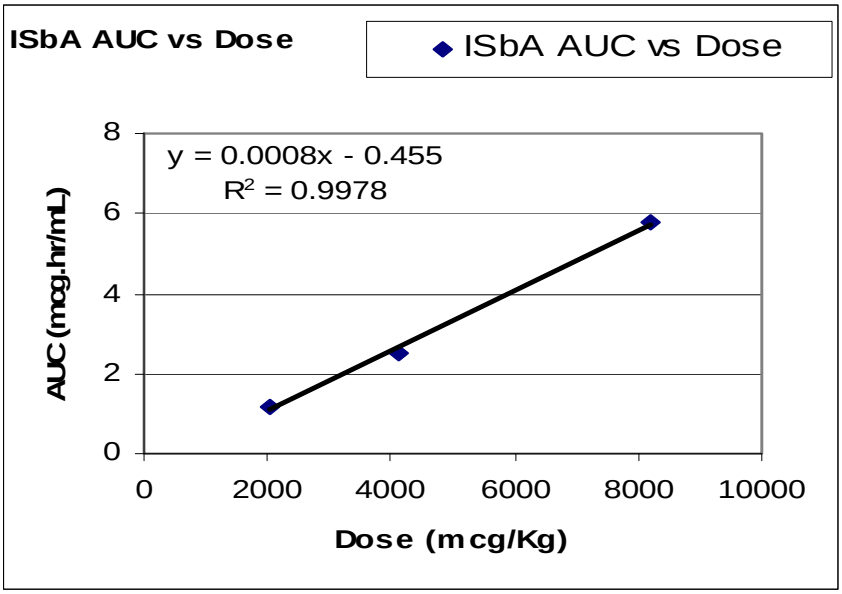


Figure 5.23: Dose Proportionality Plot for Isosilybin A (ISbA): $AUC_{0\text{-}inf}$ (µg.hr/mL) vs. Dose (µg/Kg) (ISbA = Isosilybin A)

5.3.9: Pharmacokinetics of Isosilybin A

A comparison of the mean pharmacokinetic parameters obtained for isosilybin A following intravenous administration, using non compartmental and 2-compartmental analysis, are presented in the following Table 5.24. The plasma concentration-time data post intravenous administration fit a two compartment model, representative graphs of which are shown in Figures 5.20-5.22. A plot of the mean of the individual $AUC_{0\text{-inf}}$ obtained using non compartmental analysis for each I.V treatment (A,B&C) plotted against the isosilybin A dose equivalent of silymarin, indicated dose proportionality (Figure 5.23).

The respective values of $_{AUC0\text{-inf}}$, CL_T, V_d, and half life calculated from the mean plasma concentration-time profile and the means obtained from the individual pharmacokinetic parameters, using NCA do are in close approximation with those calculated using a two-compartment model confirming the assumption that the isosilybin A plasma data confidently fits a two compartmental model. The mean half life for each of the three treatments calculated from the individual plasma data ranges between 1.27-1.32 hours indicating no significant change in elimination with increasing dose.

No levels of isosilybin A were detected in plasma after oral administration of silymarin Treatments A1, B1 and C1.

The hepatic extraction ratio (EH) for isosilybin A was found to be in the range of 0.30-0.37 indicating a maximum oral bioavailability between 0.63-0.70. After oral administration, no quantitative levels of isosilybin A were detected in plasma. Hence

bioavailability of isosilybin A can be improved with modified formulations as approximately 70% of the administered dose escapes hepatic metabolism.

The mean V_d values (Table 5.23:Mean of Ind PK.Param), obtained by NCA indicates a 18% decrease from Treatment A to Treatment C whereas the Vd obtained by 2-compartmental analysis shows a 11 % decrease from Treatment A to Treatment C. Thus, it is not clear about the extent of binding of isosilybin A to tissues or plasma proteins.

5.3.10: Results for Isosilybin B

Table 5.26: Mean Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg (A), 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 0.64 mg/Kg, 1.28 mg/Kg, 2.56 mg/Kg of Isosilybin B respectively, to Male Sprague Dawley Rats-Treatment A, B, C

	Mean Isosilybin B Plasma Concentration (µg/mL)					
	Treatment A (0.64 mg/Kg)		**Treatment B (1.28 mg/Kg)**		**Treatment C (2.56 mg/Kg)**	
Time (Hrs)	Mean (n=2)	SD	Mean (n=2)	SD	Mean (n=2)	SD
0	0	0	0	0	0	0
0.25	0.62	0.24	1.14	0.11	3.26	0.62
0.5	0.37	0.23	0.47	0.18	0.96	0.23
0.75	0.11	0.06	0.38	0.20	0.60	0.25
1	0.20	0.14	0.17	0.08	0.28	0.05
2	0.13	0.07	0.12	0.09	0.17	0.05
Weight (Kg)	0.28	0.01	0.29	0.01	0.31	0.00

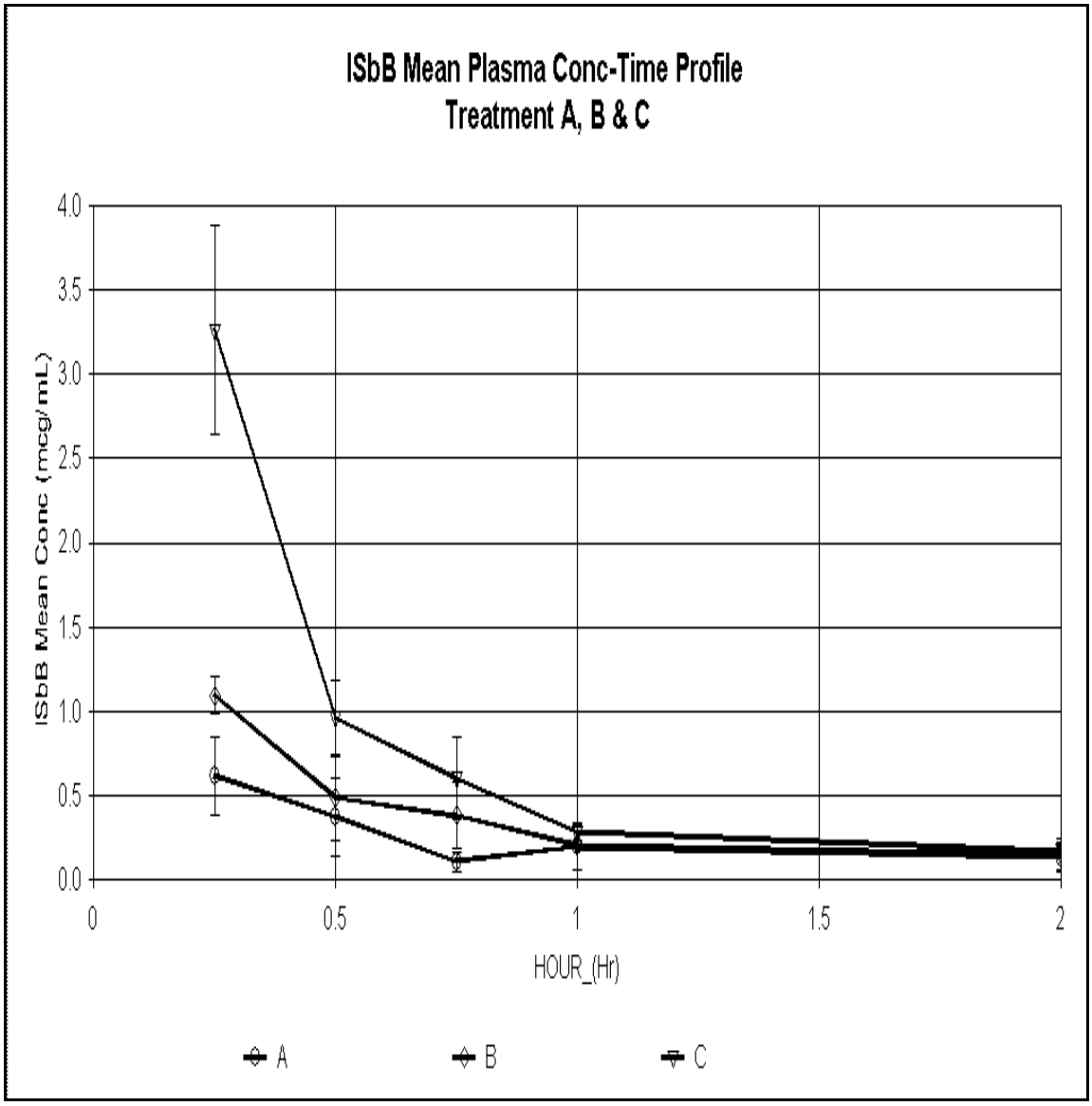


Figure 5.24: Mean Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg (A), 50 mg/Kg (B), 100 mg/Kg (C) Silymarin equivalent to 0.64 mg/Kg, 1.28 mg/Kg, 2.56 mg/Kg of Isosilybin B respectively, to Male Sprague Dawley Rats-Treatment A, B, C

Table 5.27: Comparison of Pharmacokinetic Parameters for Isosilybin B-(I.V Bolus Administration) from Mean Plasma Concentration-Time Data and Mean of Individual Pharmacokinetic Parameters

Non Compartmental Analysis (Cp= Plasma Conc.)						
Parameter	Treatment A Parameters n=2		Treatment B Parameters n= 2		Treatment C Parameters n= 2	
	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏	Mean Cp*	Mean of Ind. PK Param§∏
AUC_{0-t} (mcg-hr/ml)	0.59	0.60	1.01	1.03	2.86	2.99
$AUC_{0-\infty}$ (mcg-hr/ml)	0.92	1.03	1.17	1.29	3.21	3.24
Clearance (ml/Kg-hr)	693.39	629.54	1097.47	1031.64	797.88	844.83
Vd (ml/kg)	1744.58	2128.87	1458.84	1486.47	1631.63	1050.65
Vss (ml/kg)	1399.06	2688.90	897.34	984.10	552.62	422.05
$t_{1/2}$ (hr)	1.74	2.80	0.92	1.06	1.42	0.95
E_H	--	0.12	--	0.20	--	0.16
2-Compartmental Analysis						
AUCo-inf (mcg-hr/ml)	0.96	1.05	1.28	2.78	3.29	3.45
Clearance (ml/Kg-hr)	668.75	624.74	1000.95	573.88	779.25	802.89
Vd (ml/kg)	1671.87	2671.86	1334.59	984.15	916.84	979.25
Vss (ml/kg)	1306.57	2032.84	774.25	625.61	358.22	397.88
$t_{1/2}$ (hr)	1.73	2.75	0.92	1.05	0.82	0.93
A (mcg/mL)	0.91	0.95	4.30	33.41	18.08	19.00
Alfa (hr^{-1})	3.90	3.96	7.16	11.42	17.20	7.45
B (mcg/mL)	0.29	0.30	0.51	0.52	7.63	0.92
Beta (hr^{-1})	0.40	0.33	0.75	0.72	0.88	0.84

***** Mean of plasma concentration time profile; **§** Mean of individual pharmacokinetic parameters
∏ Standard deviations for the means are listed in the Addendum at the end of this chapter.

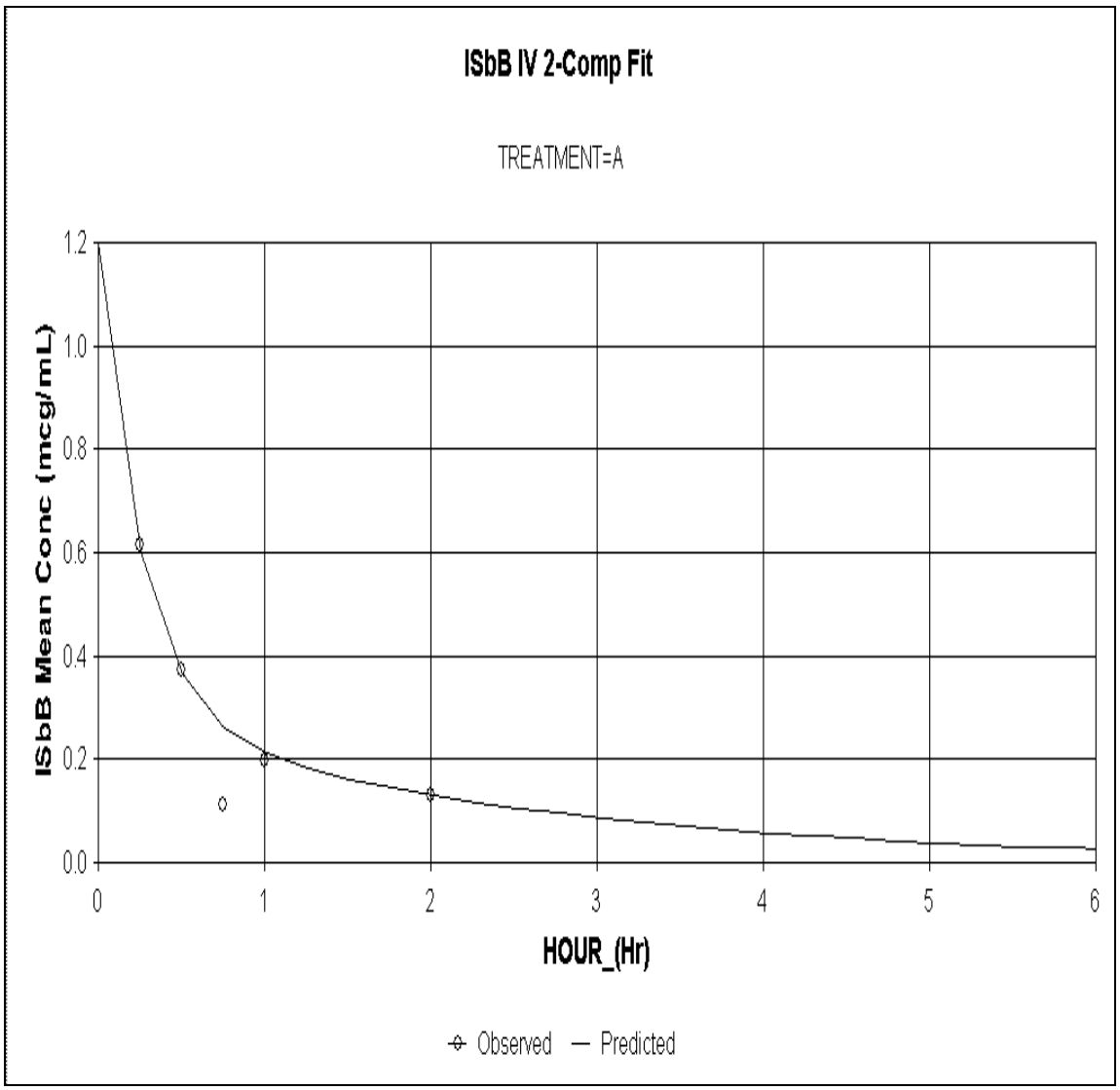


Figure 5.25: Representative Two-Compartmental Fit for Isosilybin B (ISbB; Treatment A: 0.640 mg/Kg) Following Intravenous Bolus Administration

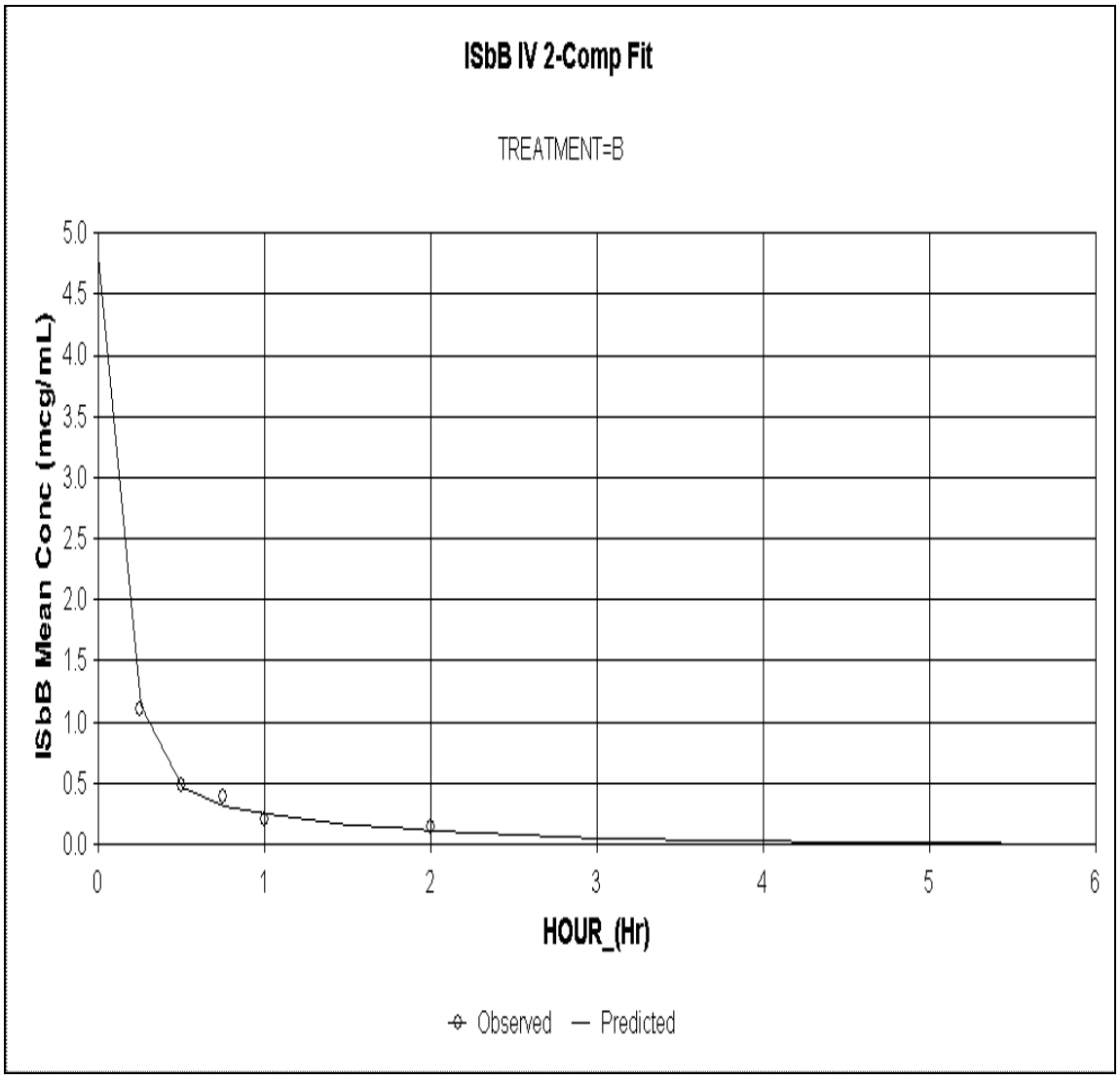


Figure 5.26: Representative Two-Compartmental Fit for Isosilybin B (ISbB; Treatment B: 1.28 mg/Kg) Following Intravenous Bolus Administration

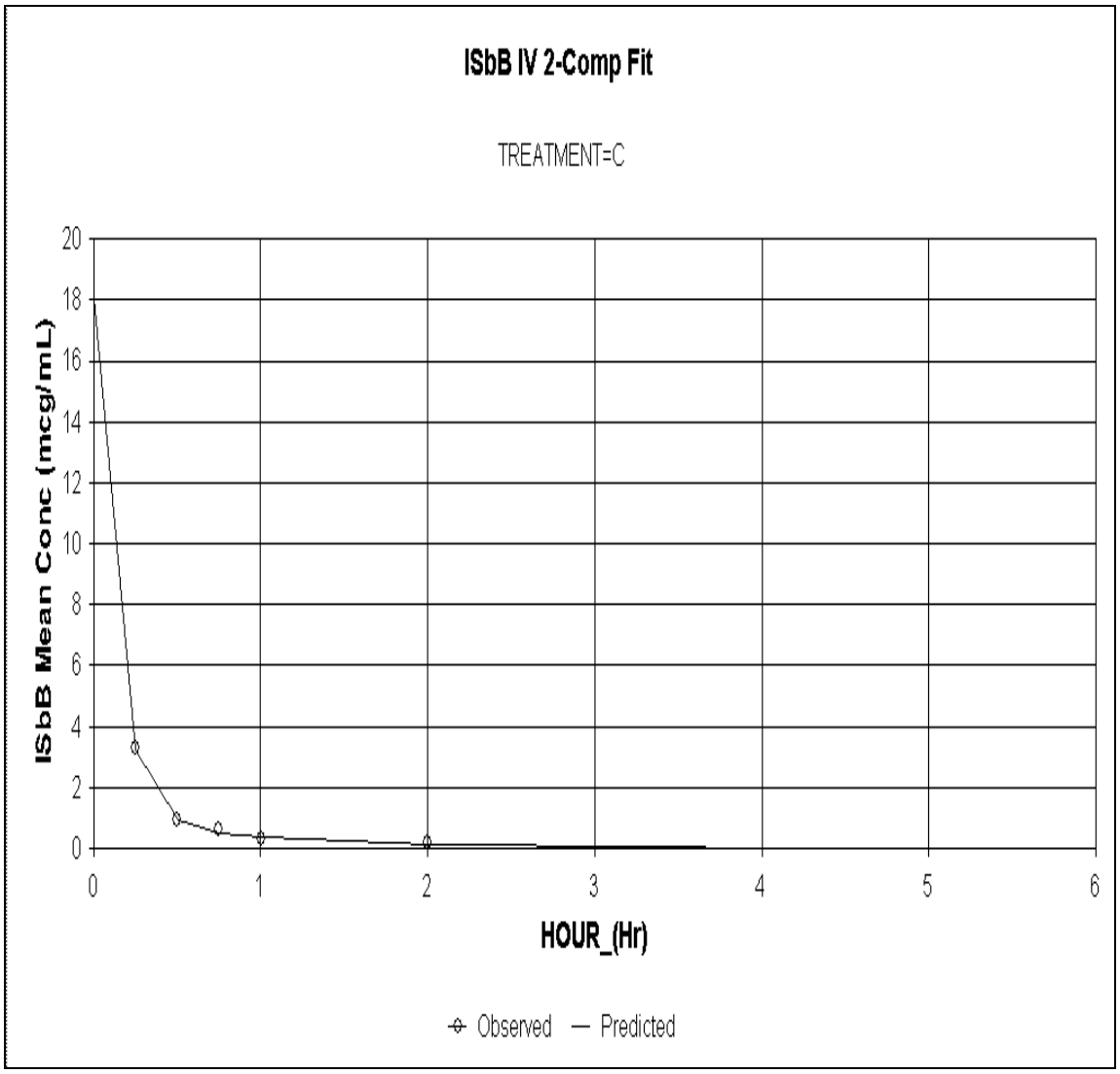


Figure 5.27: Representative Two-Compartmental Fit for Isosilybin B (ISbB; Treatment C: 2.56 mg/Kg) Following Intravenous Bolus Administration

5.3.11: Pharmacokinetics of Isosilybin B

A comparison of the mean pharmacokinetic parameters obtained for isosilybin B following intravenous administration, using non compartmental and 2-compartmental analysis, are presented in the following Table 5.27. The plasma concentration-time data post intravenous administration fit a two compartment model, representative graphs of which are shown in Figures 5.25-5.27. Mean $AUC_{0\text{-}inf}$ for Isosilybin B did not increase proportionally with the increasing intravenous dose (Treatment A: $AUC_{0\text{-}inf}$=1.03µg.hr/mL; Treatment B: $AUC_{0\text{-}inf}$=1.29µg.hr/mL; Treatment C $AUC_{0\text{-}inf}$=3.24µg.hr/mL).

Plasma levels of Isosilybin B were observed only in two rats out of the minimum six tested, leading to a significant degree of variation observed between pharmacokinetic parameters obtained using non compartmental analysis and a two-compartment model.

The respective values of $AUC_{0\text{-}inf}$ and half life calculated from the mean plasma concentration-time profile and the means obtained from the individual pharmacokinetic parameters, using NCA are in fair approximation with those calculated using a two-compartment model indicating a two-compartment fit for the isosilybin B plasma data following intravenous administration. The mean half life for the three treatments calculated from the individual plasma data ranges between 0.92-2.80 hours.

No levels of isosilybin B were detected in plasma after oral administration of silymarin treatments A1, B1 and C1.

The hepatic extraction ratio (E_H) for isosilybin B was found to be in the range 0.12-0.2 indicating very low elimination by the liver. This also implies that the maximum oral bioavailability for isosilybin B is in the range of 0.78-0.8.

The mean V_d values (Table 5.27:Mean of Ind PK.Param), obtained by NCA and 2-compartmental analysis, indicated a ~50% decrease from Treatment A to Treatment C, indicating a decrease in the V_d with increasing dose. Thus, based on this observation, isosilybin B shows probable saturability in binding to tissues.

The clearance obtained for treatment B using NCA shows a higher value (1031.64 ml/Kg-hr) which could be attributed to the low n=2 sample size. Using the equation Dose=CL_T x $AUC_{0\text{-inf}}$, the value of the dose calculated is within 2-10% of the administered dose for Treatments A and C and within 25% of the administered dose for Treatment B, indicating the uniformity of clearance with the increasing dose.

5.3.12: Comparison of Pharmacokinetic Parameters

The comparison of pharmacokinetic parameters calculated using non compartmental analysis and 2-compartmental analysis, for the different silymarin isomers is presented in Table 5.28 and 5.29 respectively. The parameters presented are the mean of the individual pharmacokinetic parameters calculated from the individual plasma concentration-time profile.

Table 5.30 represents the comparison of absolute bioavailability for the different silymarin isomers.

It is interesting to note that, even though all the active components in silymarin are isomers and diastereomers there are significant differences observed between their

pharmacokinetic parameters. Based on non compartmental analysis, diastereomer silybin B is found to have the longest elimination half life (~2.2 hours) compared to silybin A (~0.9 hour). Silybin B also has the highest volume of distribution among all the isomers. Thus, previous and current studies, *in vitro* and *in vivo* which are conducted based on the measure of silybin as a single component, should not neglect the differences found between these major diastereomers of silymarin. Similarly significant differences are also found between diastereomers isosilybin A and isosilybin B.

The values of absolute bioavailability (F) for silycristin, silybin A and silybin B indicate dissolution limited absorption due to the very low solubility of the silymarin isomers. This is evident from the decrease in the F values for the isomers with the increasing dose and significantly differing values of F obtained for Treatment A1, which is the lowest oral dose. Thus considering the values of F obtained for Treatment A1, silycristin (F_{A1}= 0.15) is the least bioavailable, followed silybin A (F_{A1}=0.20) and then silybin B (F_{A1}=0.62) being the most bioavailable. Thus, considering the values of maximum oral bioavailability calculated from E_H, the observations made in this study for Treatment A1 and from previous related studies about increasing F by modification of formulations, the overall bioavailability of silymarin isomers can be increased to give meaningful therapeutic levels.

Table 5.28: Non-Compartmental Pharmacokinetic Parameters for Silymarin Isomers

Treatment A						
Parameter	Silycristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
AUC_{0-t} (µg-hr/ml)	5.45	ND	ND	4.57	1.04	0.60
$AUC_{0-\infty}$ (µg-hr/ml)	5.58	ND	ND	7.17	1.17	1.03
Clearance (ml/Kg-hr)	1351.81	ND	ND	1314.30	1918.90	629.54
Vd (ml/kg)	1726.07	ND	ND	3837.18	3375.78	2128.87
Vss (ml/kg)	566.53	ND	ND	2469.51	1824.19	2688.90
$t_{1/2}$ (hr)	0.86	ND	ND	2.30	1.32	2.80
E_H	0.26	ND	ND	0.26	0.37	0.12
Treatment B						
	Silycristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
AUC_{0-t} (µg-hr/ml)	13.66	ND	7.39	11.56	2.33	1.03
$AUC_{0-\infty}$ (µg-hr/ml)	13.77	ND	7.93	14.08	2.51	1.29
Clearance (ml/Kg-hr)	1062.14	ND	1279.19	1272.04	1780.37	1031.64
Vd (ml/kg)	1007.75	ND	1561.42	3565.72	3011.19	1486.47
Vss (ml/kg)	464.85	ND	602.03	2459.83	1294.44	984.10
$t_{1/2}$ (hr)	0.65	ND	0.87	2.27	1.28	1.06
E_H	0.18	ND	0.25	0.25	0.35	0.20
Treatment C						
	Silycristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
AUC_{0-t} (µg-hr/ml)	29.04	ND	15.14	24.07	5.47	2.99
$AUC_{0-\infty}$ (µg-hr/ml)	29.65	ND	15.36	26.48	5.77	3.24
Clearance (ml/Kg-hr)	908.86	ND	1316.52	1381.00	1518.61	844.83
Vd (ml/kg)	1134.76	ND	1768.92	4538.23	2780.47	1050.65
Vss (ml/kg)	422.51	ND	484.38	1818.23	1100.04	422.05
$t_{1/2}$ (hr)	0.90	ND	0.95	2.24	1.27	0.95
E_H	0.18	ND	0.26	0.27	0.30	0.16

Table 5.29: Two-Compartmental Pharmacokinetic Parameters for Silymarin Isomers

Treatment A						
Parameter	Silycristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
$AUC_{0-\infty}$ (µg-hr/ml)	6.49	ND	ND	7.71	1.38	1.05
Clearance (ml/Kg-hr)	1192.22	ND	ND	1217.57	1671.09	624.74
Vd (ml/kg)	1500.78	ND	ND	3545.23	3253.49	2671.86
Vss (ml/kg)	474.34	ND	ND	2161.34	1775.52	2032.84
$t_{1/2}$ (hr)	0.86	ND	ND	2.27	1.33	2.75
A (mcg/mL)	55.36	ND	ND	25.70	6.32	0.95
Alfa (hr^{-1})	8.93	ND	ND	7.38	6.40	3.96
B (mcg/mL)	1.11	ND	ND	1.20	0.34	0.30
Beta (hr^{-1})	0.97	ND	ND	0.42	0.70	0.33
Treatment B						
	Silycristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
$AUC_{0-\infty}$ (µg-hr/ml)	13.98	ND	8.33	13.94	2.40	2.78
Clearance (ml/Kg-hr)	1110.58	ND	1192.37	1275.31	1797.01	573.88
Vd (ml/kg)	1058.22	ND	1473.66	3997.97	3112.39	984.15
Vss (ml/kg)	470.68	ND	569.56	2365.02	1368.77	625.61
$t_{1/2}$ (hr)	0.65	ND	0.90	2.25	1.27	1.05
A (mcg/mL)	69.92	ND	36.90	48.59	8.87	33.41
Alfa (hr^{-1})	6.32	ND	5.84	6.02	5.83	11.42
B (mcg/mL)	5.36	ND	1.31	2.06	0.51	0.52
Beta (hr^{-1})	1.33	ND	0.88	0.55	0.71	0.72
Treatment C						
	Silycristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
$AUC_{0-\infty}$ (µg-hr/ml)	28.71	ND	14.71	24.39	5.54	3.45
Clearance (ml/Kg-hr)	1007.84	ND	1429.35	1553.70	1578.40	802.89
Vd (ml/kg)	1308.42	ND	1849.47	5169.78	2900.06	979.25
Vss (ml/kg)	559.25	ND	634.39	2212.85	1298.48	397.88
$t_{1/2}$ (hr)	0.93	ND	0.90	2.22	1.26	0.93
A (mcg/mL)	106.25	ND	73.13	89.12	22.95	19.00
Alfa (hr^{-1})	4.75	ND	5.63	5.14	5.70	7.45
B (mcg/mL)	5.48	ND	2.47	3.63	1.33	0.92
Beta (hr^{-1})	0.81	ND	0.94	0.59	0.73	0.84

Table 5.30: Average Bioavailability Comparison of Silymarin Isomers

	Silycristin			Sily-dianin	Silybin A			Silybin B			Iso-silybin A&B
Treat-ment	A1 n=5	B 1 n=3	C1 n=3	A1, B1, C1	A1 n=3	B1 n=2	C1 n=3	A1 n=2	B1 n=5	C1 n=4	A1, B1, C1
F	**0.15**	**0.06**	**0.04**	**ND**	**0.20**	**0.12**	**0.03**	**0.62**	**0.09**	**0.03**	**ND**

5.3.13: Selection of a Bioavailability and Bioequivalence Marker

As discussed previously in Chapter II of this dissertation we set three main criteria that need to be fulfilled when selecting a bioavailability and bioequivalence marker for an herbal extract. These criteria are:

1. The selected marker should have one of the least permeability among its active components in the extract.

2. The proportion of the selected marker in the extract should be sufficient for its precise and quantitative determination in biological fluids after oral administration (e.g. silycristin in Milk thistle, kawain in Kava)

3. The selected marker should be easily available as a reference standard, at a reasonable cost and acceptable purity for routine analysis.

Based on these criteria, and the inter parameter relationships observed between the *in silico* descriptors and the *in vitro* permeability, the following markers were selected for Kava, Ginkgo biloba and Milk thistle (Chapter II)

I: Kawain as a marker for Kava

II: Ginkgolide B as a marker for the ginkgo terpenes, and quercetin as a marker for the flavonol glycosides.

III: Silycristin as a marker for Milk thistle (silymarin).

Milk thistle (silymarin) was selected as a representative extract for the verification of marker prediction and further experiments with regards to solubility, partition coefficient, *in vitro* dissolution and in vivo pharmacokinetic studies were conducted. The presence of isomers and diastereomers in silymarin further presented a challenge in the analytical separation and quantitation of these components in chemical and biological matrices. Further most previous related studies concerning silymarin were always done with a measure for silybin as a single component representing the extract, and not as separate diastereomers or even as separate isomers of silymarin.

Silycristin was predicted as a bioavailability marker for Milk thistle (silymarin) based on its low permeability, low lipophilicity and a relatively high minimal cross-sectional area. Further, the proportion of silycristin in silymarin is high enough for its quantitation in biological fluids. Based on the correlations between *in silico* descriptors and *in vitro* permeability, silydianin can also be selected as a marker for silymarin, but the proportion of silydianin in the extract is significantly low for its quantitation in biological matrices. Thus, not selecting silydianin as a marker for silymarin proved to be true as no levels of silydianin were detected in plasma after intravenous and oral administration of any of the doses. Hence, silydianin may be considered as a 'false

positive' during marker selection, leading to a wrong bioavailability/bioequivalence marker.

Post oral administration, plasma levels were observed only for silycristin, silybin A and silybin B. Table 5.28 shows the average absolute bioavailability for silycristin, silybin A and silybin B following three oral treatments of silymarin. The value of F is seen to decrease for each isomer with the increasing dose, indicating dissolution limited absorption. Based on the values of F for the lowest oral dose, silycristin is found to have the lowest bioavailability (F=0.15±0.1) followed by silybin A (F=0.20±0.04) followed by silybin B (F=0.62±0.08). Thus, even though the value of F decreases with the increasing dose, based on the lowest oral dose administered, silycristin is found to have the least bioavailability among the detected isomers.

Table 4.3 in Chapter IV of this dissertation, presents the Biopharmaceutic Classification of isomers of silymarin based on their experimental and calculated partition coefficient, experimentally determined solubility and its comparison to the partition coefficient values of metoprolol as a model reference drug. Silymarin isomers having a CLogP and experimental LogP value less than that of Metoprolol were classified as low permeability drugs and vice versa. Based on this classification, silybin A, silybin B, Isosilybin A and Isosilybin B were classified as High Permeability-Low Solubility (Class II drugs), silydianin was classified as a Low Permeability-Low Solubility (Class IV drug) and only silycristin due to its intermediate CLogP and experimental LogP shuffled between the High-Permeability-Low Solubility class and Low Permeability-Low Solubility class. Thus, silycristin is a good choice for a bioavailability marker, as it can be safely assumed that it has a permeability nature which is intermediate between High Permeability and the Low Permeability Class. Thus, being in a low permeability class like silydianin, during prediction, silycristin would pose as a

risk towards marker selection (posing as a false negative) for being so less permeable, that it would not be detected in biological fluids at all. But its simultaneous placement into the Low Permeability and High Permeability class indicates that silycristin can be one of the least permeable compounds, having a low permeability than most of the other isomers but at the same time permeable enough to be detected in biological fluids as a representative marker for the Milk thistle extract.

CHAPTER 6:CONCLUSIONS

Due to their rising popularity, herbal supplements have created a specific niche for themselves between the food and the drug industry. Due to their categorization as dietary supplements, they lack scientific seriousness where as on the other hand they act like unregulated drugs with potential therapeutic effects. Development of bioavailability marker compounds is necessary for the quality control and therapeutic efficacy of herbal supplements. Solubility, partition coefficient and intestinal permeability are the fundamental factors that affect bioavailability of a drug after oral administration. Though accurate quantitative predictions of human intestinal permeability or bioavailability are difficult, an effort has been made to get a qualitative idea about these parameters with the aid of *in silico* descriptors such as the polar surface area (PSA), minimal cross-sectional area (MCSA) and the predicted log of octanol-water partition coefficient (CLogP). Eight herbal extracts comprising of 37 active components were selected for this investigation. Conclusions based on the various experiments conducted on some or all of these 37 active components are listed below:

1. The main objective of this dissertation was to select the least permeable compound as a performance and bioavailability marker, which is the most conservative approach to ensure the bioavailability of a herbal extract or supplement. The estimation of *in silico* descriptors and their correlation with *in vitro* permeability enables the selection of a performance marker for establishing the bioavailability and bioequivalence of herbal extracts.

2. *In silico* descriptors like the MCSA, CLogP and polar surface area (PSA) were estimated for 37 active compounds in 8 herbal extract. The MCSA gives an idea of the permeability of the compound based on its size and orientation, while permeating the lipid bilayer. The PSA and CLogP relate to the (size and polar nature) and the lipophilicity of the compound respectively all of which are direct predictors of permeability. Hence, the use of *in silico* descriptors to predict the intestinal permeability of the compounds gives a certain directional focus in the selection of bioavailability/bioequivalence markers.

3. *In vitro* permeability data for the compounds in each herbal extract was obtained using SimBioDAS®, an *in vitro* epithelial cell based assay developed by Kinetana Inc. Permeability coefficients for two diastereomers, silybin A and silybin B present in Milk thistle were estimated using the CaCo-2 cell model where in silybin B (P_{eff} = 2.62 x 10^{-6} cm/sec) was found to have a higher permeability than silybin A (P_{eff} = 1.6 x 10^{-6} cm/sec).

4. Plots (CLogP vs PSA and P_{eff} vs PSA) indicate that the increase in the PSA leads to a decrease in the lipophilicity of the compounds indicating lower permeability values. The MCSA (P_{eff} vs MCSA) and the PSA (P_{eff} vs PSA) combined, provide a good estimate towards the selection of the least permeable compound in selected herbal extracts, thus enabling us to differentiate between isomers and diastereomers. The qualitative predictions are valid for compounds with molecular weight less than 500. Based on conservative limits of CLogP (-1.5 to +2.2), MCSA (100 Å^2 to 160 Å^2) and permeability (<5 x 10^{-6} cm/sec) a

rectangular box could be drawn containing an observed cluster of compounds that included the selected markers.

5. Though intestinal permeability and bioavailability are two different processes, the extent of intestinal permeability is a primary predictor of the bioavailability of that compound. Based on the inter parameter relationships between PSA, MCSA, CLogP and *in vitro* permeability the following markers were selected:

- Kawain selected as a bioavailability marker for the active kava lactones in Kava extract.
- In Ginkgo biloba, Ginkgolide B is selected as a bioavailability marker for the ginkgo terpenes and quercetin as a marker for the flavonol glycosides.
- In Milk thistle silycristin is selected as a bioavailability marker for the silymarin isomers.

Due to the presence of isomers and diastereomers as active components of Milk thistle (silymarin), lack of sufficient bioavailability data on the isomers in literature, Milk thistle (silymarin) was selected as a representative extract for further verification of the hypothesis through experimental techniques.

6. A reproducible, sensitive and accurate, binary gradient reverse phase HPLC method was developed and validated for the complete separation and quantitative determination of silymarin isomers in chemical matrices and rat plasma (LOD: 0.02-0.03µg/mL; LOQ: 0.05-0.08µg/mL).

7. Solubility of the silymarin isomers was determined at pH 7.2. All isomers were found to be practically insoluble with values of solubility <0.1 mg/mL. Silydianin (0.019 mg/mL) and silycristin (0.015 mg/mL) had relatively highest solubilies and isosilybin B (0.00045 mg/mL) had the lowest solubility among the isomers.

8. Apparent octanol-water partition coefficient values indicated that all the isomers were moderately lipophilic with values between the range 1.05 to 2.19. The correlation coefficient (r^2)obtained between the predicted CLogP and the apparent LogP was 0.835.

9. Classification of the isomers based on the BCS (Biopharmaceutic Classification System) indicated that silybin A, silybin B, isosilybin A and isosilybin B were Class II (High Permeability-Low Solubility) compounds, silydianin was a Class IV (Low Permeability-Low Solubility) compound and silycristin was classified as an intermediate between Class II and Class IV

10. Assay for content of isomers in three commercially available products having the same label claim indicated a high degree of variability between formulations. Content of silybin A and silybin B in one commercial product (Company C) was more than twice when compared to the other two commercial products (Company A and Company B). Such variation in isomer content for formulations with the same label claim is questionable and may arise due to many factors ranging from geographical location of the plant and the time of harvest, to the type and efficiency of the extraction method used obtain the standardized silymarin extract.

11. *In vitro* dissolution of one commercial product (Company A) for the release (%) of silybin A (32.09±1.18), silybin B (46.91±1.57)and silydianin (50.26±1.5) indicated incomplete dissolution of the dosage form. Considering that the formulation did not have any solubility enhancers and wettability was not a limiting factor towards dissolution, these results are in agreement with the results obtained from the solubility experiments for silybin A, silybin B and silydianin, where silydianin was the most soluble isomer, followed by silybin B ad silybin A.

The pharmacokinetics of silymarin isomers was studied in male Sprague Dawley rats after intravenous and oral administration. Quantitative levels of silydianin could not be detected post intravenous or oral administration. Intravenous plasma concentration time profiles for each detected isomer fit a two-compartment model. The half lives for the silymarin isomers ranged from 0.8-2.27 hours, and the apparent volume of distribution was in the range from 1.55-4.55 L/Kg. Following intravenous administration, the area under the plasma concentration-time curve was found to increase proportionally with the increasing dose for all the isomers (except isosilybin B) indicating dose proportionality (r^2=0.99). Post oral administration, plasma levels were detected only for silycristin, silybin A and silybin B with a high degree of variation. Silycristin was found to have the lowest bioavailability among the three isomers, indicating solubility limited absorption. Silybin A was found to be less bioavailable than silybin B indicating a significant difference between the two diastereomers.

Thus, based on the most conservative approach that selection of the least bioavailable compound from the extract as a marker, will ensure the bioavailability of the rest of the extract, silycristin was selected as a bioavailability and bioequivalence marker.

Hence, bioavailability testing of silycristin will ensure the bioavailability of silybin A and silybin B, which are considered as the most active components of Milk thistle.

Appendix

A1: INDIVIDUAL PHARMACOKINETIC DATA FOR SILYCRISTIN

Table 5.31:Plasma Concentrations (μg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg Silymarin equivalent to 6.13 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment A

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	SD
0	0	0	0	0	0	0	0	0
0.25	4.13	7.64	4.83	7.65	3.12	3.33	5.12	2.05
0.5	2.27	1.65	1.37	0.84	0.62	0.74	1.25	0.64
0.75	0.58	0.73	0.42	0.33	0.28	0.51	0.47	0.17
1	0.46	0.61	0.32	0.28	0.19	0.53	0.40	0.16
2	0.22	0.08	0.08	0.11	0.06	0.27	0.14	0.09
4	0.00	0.00	0.06	0.00	0.00	0.09	0.07	0.02
Weight (Kg)	0.274	0.295	0.290	0.288	0.324	0.288	0.293	0.017

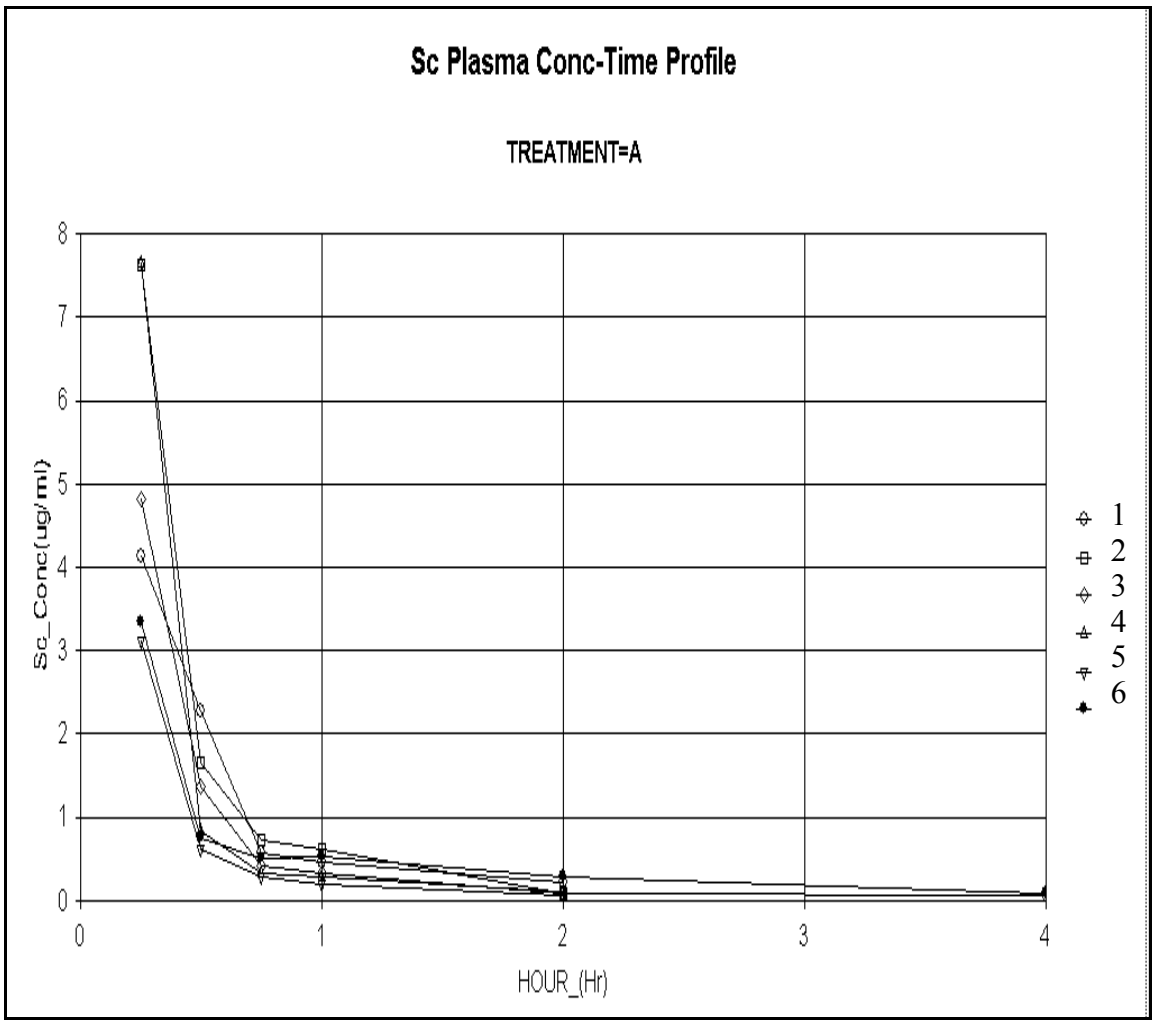


Figure 5.28: Plasma Concentrations (μg/mL) Time Profile after Intravenous Bolus Administration of 25 mg/Kg Silymarin equivalent to 6.13 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment A

Table 5.32: Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 50 mg/Kg Silymarin equivalent to 12.26 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment B

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Mean	SD
0	0	0	0	0	0	0	0	0	0
0.25	25.98	16.34	17.80	22.06	8.07	15.26	5.44	15.85	7.24
0.5	13.08	2.62	4.50	13.42	2.20	4.82	1.86	6.07	5.03
0.75	4.13	1.28	1.42	1.10	0.87	4.13	1.04	2.00	1.47
1	0.44	0.72	1.15	0.98	0.32	1.55	1.07	0.89	0.43
2	0.29	0.16	0.33	0.18	0.06	1.80	0.97	0.54	0.63
4	0	0	0.10	0	0	0.18	0.10	0.13	0.04
6	0	0	0	0	0	0.07	0	0.07	0
Weight (Kg)	0.29	0.31	0.29	0.30	0.31	0.30	0.30	0.30	0.01

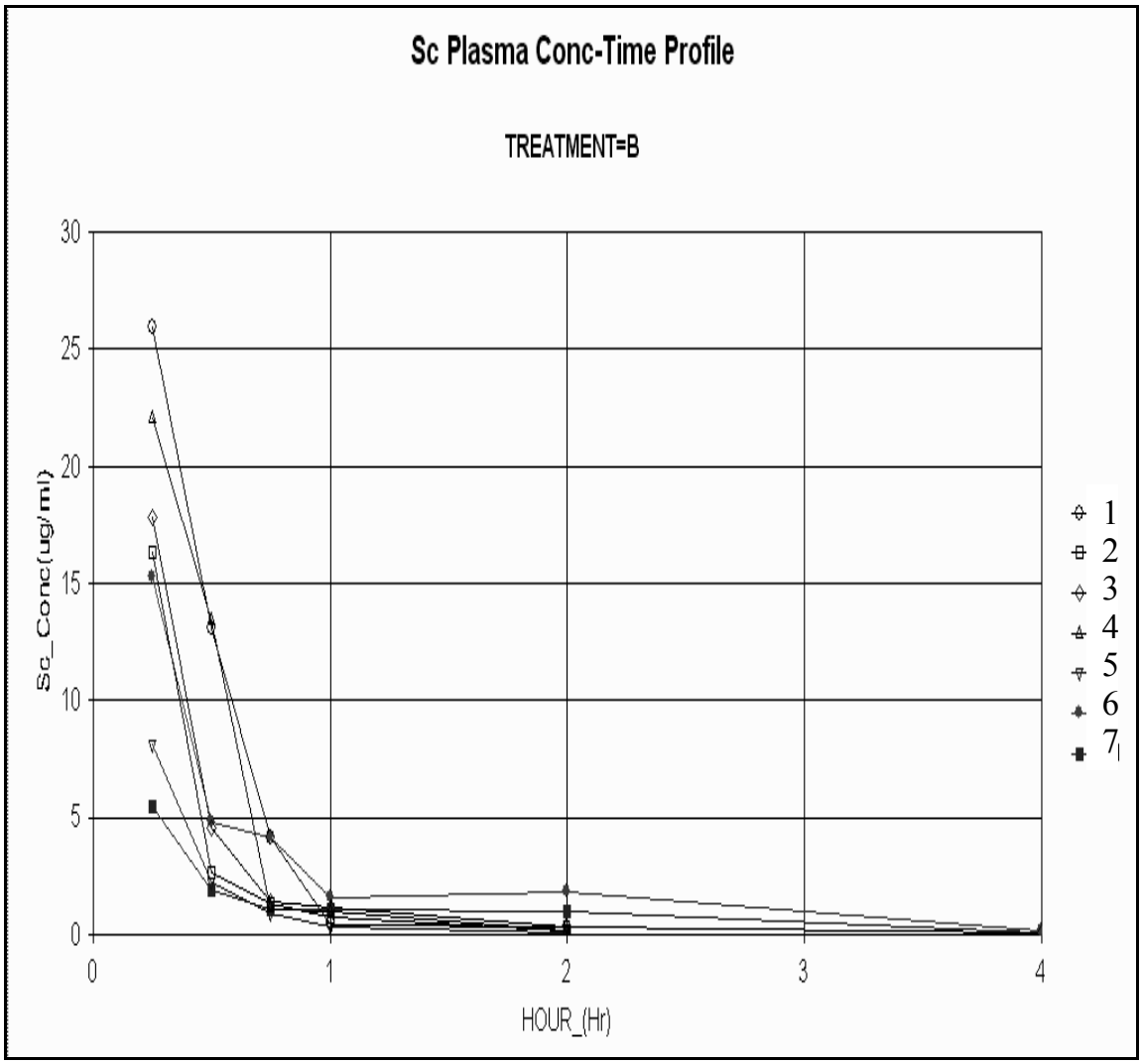


Figure 5.33: Plasma Concentrations (μg/mL) Time Profile after Intravenous Bolus Administration of 50 mg/Kg Silymarin equivalent to 12.26 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment B

Table 5.33:Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 100 mg/Kg Silymarin equivalent to 24.52 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment C

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	SD
0	0	0	0	0	0	0	0	0
0.25	15.47	35.64	23.99	33.06	13.32	53.25	33.96	13.32
0.5	3.23	9.61	9.89	17.72	7.65	25.22	13.52	7.65
0.75	4.10	8.51	6.98	4.56	3.47	13.23	7.03	3.47
1	2.39	6.06	1.74	5.88	1.83	6.06	3.95	1.83
2	1.24	1.34	0.57	0.67	0.32	1.34	0.95	0.32
4	0	0.41	0	0.07	0.16	0.41	0.28	0.16
6	0	0.08	0	0	0	0.08	0.08	0
Weight (Kg)	0.32	0.31	0.29	0.31	0.32	0.29	0.30	0.01

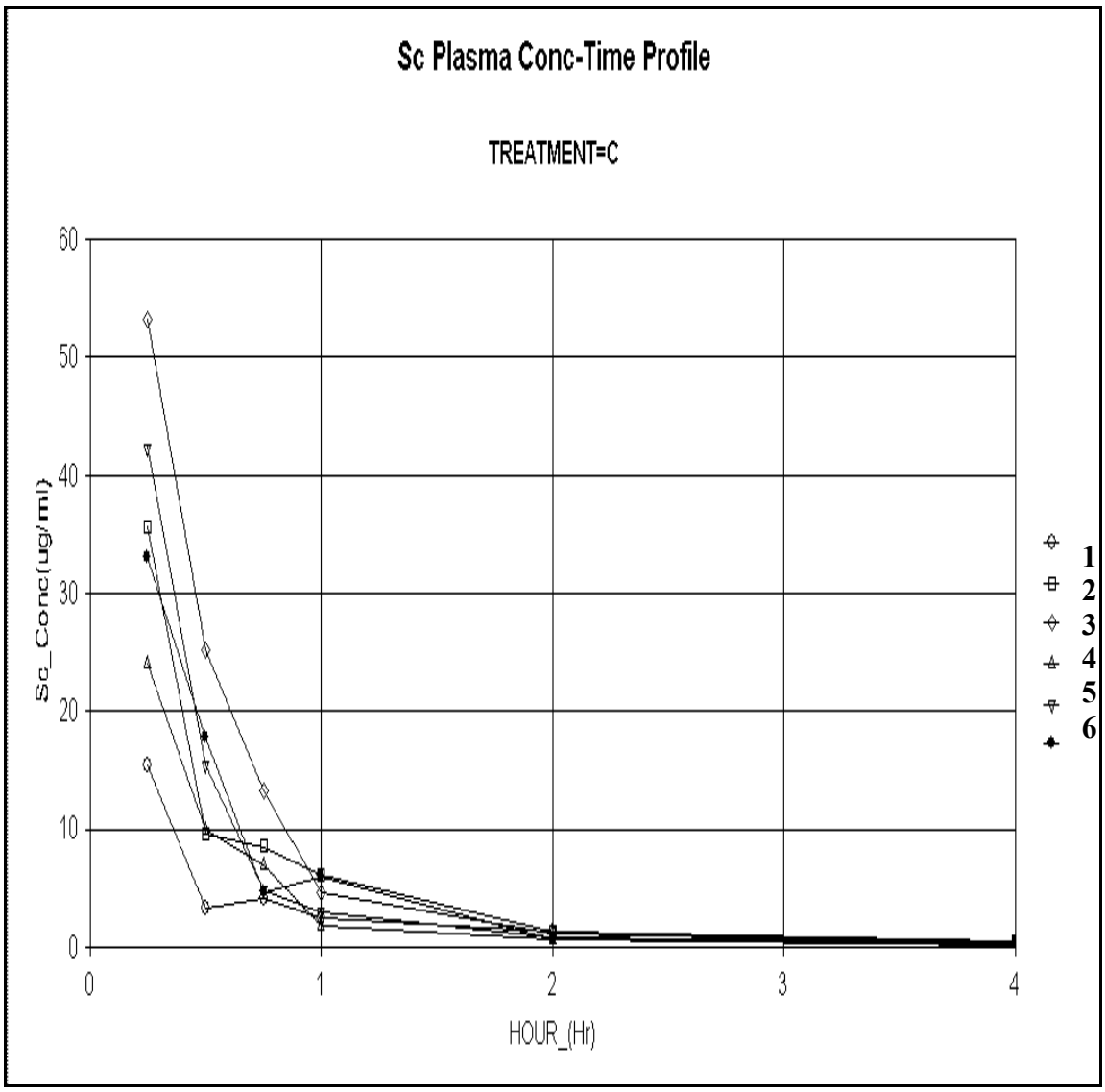


Figure 5.30: Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 100 mg/Kg Silymarin equivalent to 24.52 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment C

Table 5.34: Pharmacokinetic Parameters for Silycristin (6.13 mg/Kg) equivalent to Silymarin (25 mg/Kg) after Intravenous Administration (Treatment A) using Non-Compartmental and 2-Compartmental Analysis

Non Compartmental Analysis								
Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	SD
AUC_{0-t} (mcg-hr/ml)	3.08	7.35	4.16	11.17	3.10	3.86	5.45	3.22
$AUC_{0-\infty}$ (mcg-hr/ml)	3.36	7.39	4.27	11.31	3.15	4.00	5.58	3.20
Clearance (ml/Kg-hr)	1823.25	829.26	1436.77	542.24	1946.45	1532.89	1351.81	555.76
Vd (ml/kg)	2363.73	459.40	2761.06	630.21	1562.36	2579.67	1726.07	1003.85
Vss (ml/kg)	1131.03	157.21	622.51	64.79	386.78	1036.86	566.53	446.00
$t_{1/2}$ (hr)	0.90	0.38	1.33	0.81	0.56	1.17	0.86	0.36
2-Compartmental Analysis								
AUCo-inf (mcg-hr/ml)	3.49	9.74	3.97	11.40	3.41	6.90	6.49	3.45
Clearance (ml/Kg-hr)	1756.71	629.64	1543.19	537.64	1797.64	888.51	1192.22	573.68
Vd (ml/kg)	2196.01	346.71	2805.87	640.12	1382.82	1633.16	1500.78	926.35
Vss (ml/kg)	1162.47	108.87	697.80	85.42	377.67	413.81	474.34	405.29
$t_{1/2}$ (hr)	0.87	0.38	1.26	0.83	0.53	1.27	0.86	0.37
A (mcg/mL)	6.72	88.00	19.00	120.62	26.80	71.01	55.36	44.88
Alfa (hr^{-1})	3.00	11.00	5.76	11.30	9.30	13.22	8.93	3.84
B (mcg/mL)	1.00	3.15	0.37	0.61	0.69	0.83	1.11	1.02
Beta (hr^{-1})	0.80	1.82	0.55	0.84	1.30	0.54	0.97	0.50

Table 5.35:Pharmacokinetic Parameters for Silycristin (12.26 mg/Kg) equivalent to Silymarin (50 mg/Kg) after Intravenous Administration (Treatment B) using Non-Compartmental and 2-Compartmental Analysis

Non Compartmental Analysis									
Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Mean	SD
AUC_{0-t} (mcg-hr/ml)	17.66	18.32	16.05	14.38	6.71	16.18	6.29	13.66	5.05
$AUC_{0-\infty}$ (mcg-hr/ml)	17.80	18.42	16.18	14.50	6.75	16.30	6.43	13.77	5.06
Clearance (ml/Kg-hr)	688.85	665.79	757.88	845.70	1817.22	752.30	1907.21	1062.14	550.17
Vd (ml/kg)	323.40	411.27	972.91	555.76	1045.14	1213.00	2532.73	1007.75	752.63
Vss (ml/kg)	190.86	88.90	222.55	254.96	345.75	528.08	1622.88	464.85	528.90
$t_{1/2}$ (hr)	0.33	0.43	0.89	0.46	0.40	1.12	0.92	0.65	0.32
2-Compartmental Analysis									
AUCo-inf (mcg-hr/ml)	18.42	22.37	15.24	15.07	6.02	14.21	6.52	13.98	5.94
Clearance (ml/Kg-hr)	671.60	593.92	780.60	755.78	1953.45	997.04	2021.68	1110.58	612.08
Vd (ml/kg)	302.52	336.34	1005.63	508.56	1132.05	1437.92	2684.56	1058.22	835.83
Vss (ml/kg)	243.08	80.68	266.97	343.34	487.79	553.56	1319.37	470.68	406.01
$t_{1/2}$ (hr)	0.32	0.43	0.87	0.43	0.39	1.16	0.99	0.65	0.34
A (mcg/mL)	32.99	222.00	84.00	33.00	28.80	58.27	30.40	69.92	70.05
Alfa (hr^{-1})	3.71	11.15	6.59	2.61	5.80	6.00	8.40	6.32	2.85
B (mcg/mL)	20.97	4.00	2.00	3.90	1.89	2.70	2.03	5.36	6.94
Beta (hr^{-1})	2.20	1.63	0.80	1.60	1.80	0.60	0.70	1.33	0.63

Table 5.36: Pharmacokinetic Parameters for Silycristin (24.52 mg/Kg) equivalent to Silymarin (100 mg/Kg) after Intravenous Administration (Treatment C) using Non-Compartmental and 2-Compartmental Analysis

Non Compartmental Analysis								
Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	SD
AUC_{0-t} (mcg-hr/ml)	17.09	36.65	41.86	18.87	33.48	26.29	29.04	9.96
$AUC_{0-\infty}$ (mcg-hr/ml)	19.03	36.74	42.59	19.38	33.83	26.34	29.65	9.64
Clearance (ml/Kg-hr)	1288.92	667.37	575.78	1265.36	724.87	930.85	908.86	308.26
Vd (ml/kg)	2014.55	817.32	1098.14	1134.10	940.99	803.44	1134.76	452.43
Vss (ml/kg)	798.66	313.52	282.82	469.30	269.85	400.91	422.51	199.42
$t_{1/2}$ (hr)	1.08	0.85	1.32	0.62	0.90	0.60	0.90	0.28
2-Compartmental Analysis								
AUCo-inf (mcg-hr/ml)	13.50	42.42	40.55	18.72	31.47	25.58	28.71	11.63
Clearance (ml/Kg-hr)	1816.18	578.08	604.80	1310.30	779.17	958.53	1007.84	478.90
Vd (ml/kg)	2595.26	722.59	1099.69	1191.16	1443.03	798.78	1308.42	683.28
Vss (ml/kg)	1428.36	276.89	322.77	542.52	342.99	441.95	559.25	436.30
$t_{1/2}$ (hr)	0.99	0.87	1.26	0.63	1.28	0.58	0.93	0.30
A (mcg/mL)	35.00	262.48	107.00	58.00	119.00	56.00	106.25	83.07
Alfa (hr^{-1})	4.95	9.15	3.07	4.20	4.34	2.80	4.75	2.30
B (mcg/mL)	4.50	11.00	3.10	5.40	2.20	6.70	5.48	3.14
Beta (hr^{-1})	0.70	0.80	0.55	1.10	0.54	1.20	0.81	0.28

Table 5.37:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg Silymarin equivalent to 30.65 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment A1

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
0	0	0	0	0	0	0	0
0.25	0	0	0.52	0	0	0.52	0
0.5	0	0.25	0.33	0	0	0.29	0.06
0.75	0	0.37	0.12	0	0	0.24	0.17
1	0.18	0.69	0.08	0	0	0.31	0.33
2	0.29	0.83	0.08	0.33	0.31	0.37	0.28
4	0.08	1.34	0	0.44	0.31	0.54	0.55
6	0.47	0.55	0	0.65	0.39	0.52	0.11
8	0.15	0.36	0	0.48	0.70	0.42	0.23
12	0.11	0	0	0.39	0.38	0.29	0.16
16	0	0	0	0.25	0.30	0.27	0.04
Weight(Kg)	0.285	0.308	0.24	0.264	0.242	0.27	0.03

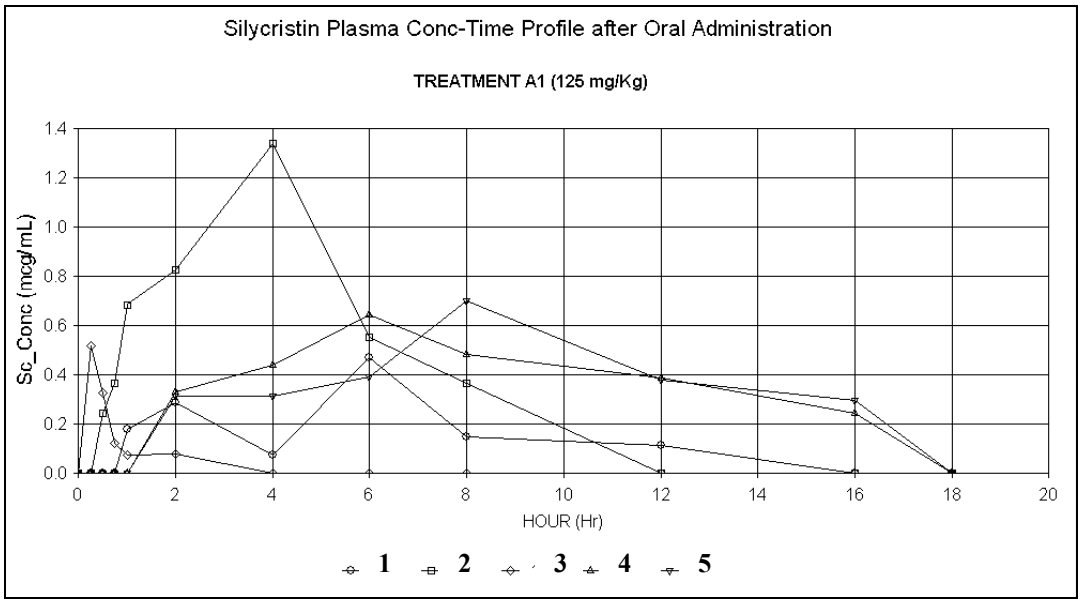


Figure 5.31: Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg Silymarin equivalent to 30.65 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment A1

Table 5.38: Plasma Concentrations (μg/mL) Time Profile after Oral Administration of 250 mg/Kg Silymarin equivalent to 61.30 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment B1

Time (Hrs)	Rat 1	Rat 2	Rat 3	Mean	SD
0	0	0	0	0	0
0.25	0.12	0	0.58	0.35	0.32
0.5	0.15	0.13	0.43	0.21	0.15
0.75	0	0.25	0.25	0.28	0.05
1	0.10	0.23	0.00	0.14	0.08
2	0.08	0.12	0.82	0.28	0.36
4	0.35	0.09	1.14	0.43	0.48
6	0.45	0	0.62	0.53	0.12
8	0.29	0	0.49	0.30	0.19
12	0.14	0	0.29	0.22	0.11
Weight (Kg)	0.308	0.264	0.242	0.27	0.03

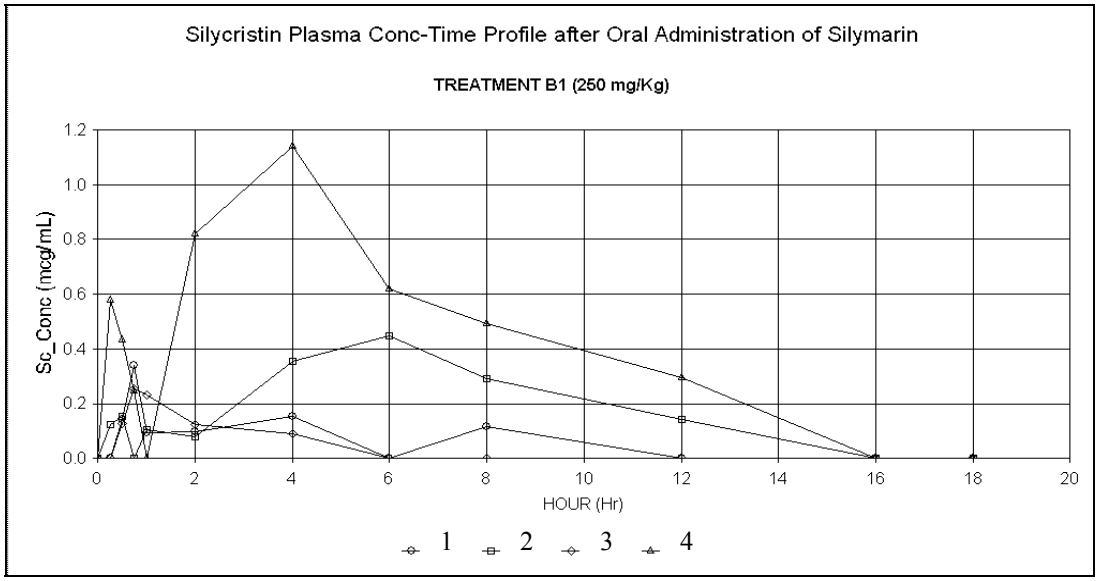


Figure 5.32: Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 250 mg/Kg Silymarin equivalent to 61.30 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment B1

Table 5.39: Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 500 mg/Kg Silymarin equivalent to 122.61 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment C1

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
0	0	0	0	0	0	0
0.25	0	0.59	0	0.43	0.51	0.11
0.5	0	0.36	0.18	0.61	0.38	0.22
0.75	0	0.44	0.25	0.41	0.37	0.10
1	0.62	0.46	0.49	0.22	0.45	0.17
2	0.66	0.37	0.45	0.11	0.40	0.23
4	0.74	0.32	0.22	0	0.43	0.28
6	1.78	0.86	0.12	0	0.92	0.83
8	1.13	0.73	0	0	0.93	0.29
12	0.34	0.41	0	0	0.38	0.05
Weight (Kg)	0.29	0.31	0.26	0.24	0.27	0.03

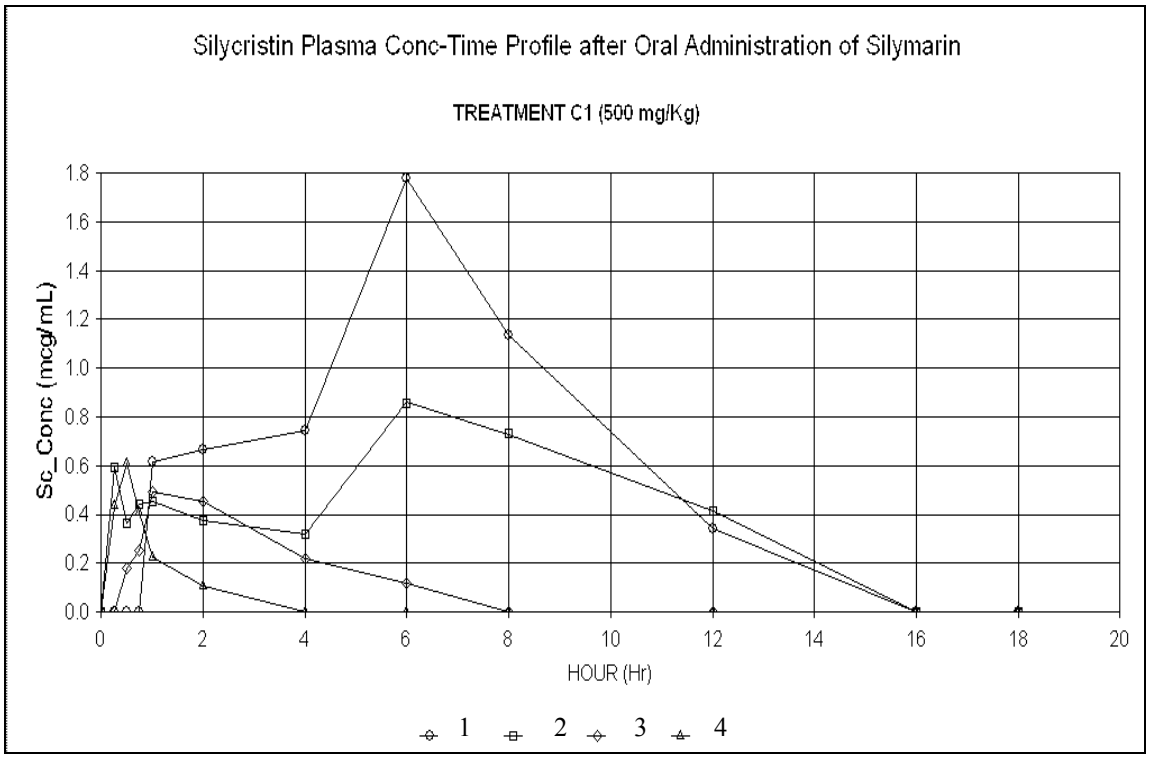


Figure 5.33:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 500 mg/Kg Silymarin equivalent to 122.61 mg/Kg of Silycristin to Male Sprague Dawley Rats-Treatment C1

Table 5.40:Non Compartmental Pharmacokinetic Parameters for Silycristin (30.65 mg/Kg) following Oral Administration of Silymarin (125 mg/Kg) Treatment A1

Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
AUC_{0-t} (mcg-hr/ml)	2.31	5.97	0.33	6.17	6.08	4.17	0.03
$AUC_{0-\infty}$ (mcg-hr/ml)	2.82	7.10	0.64	8.31	8.86	5.55	2.70
C_{max} (µg/mL)	0.47	1.34	0.52	0.65	0.70	0.73	3.62
T_{max} (Hr)	6	4	0.25	6	8	4.85	0.35
F	0.08	0.19	0.02	0.22	0.24	0.15	0.10

Table 5.41: Non Compartmental Pharmacokinetic Parameters for Silycristin (61.30 mg/Kg) following Oral Administration of Silymarin (250 mg/Kg) Treatment B1

Parameter	Rat 1	Rat 2	Rat 3	Mean	SD
AUC_{0-t} (mcg-hr/ml)	3.00	0.51	7.13	3.55	3.34
$AUC_{0-\infty}$ (mcg-hr/ml)	3.74	0.83	9.50	4.69	4.41
C_{max} (µg/mL)	0.45	0.25	1.14	0.61	0.47
T_{max} (Hr)	6.00	0.75	4.00	3.58	2.65
F	0.05	0.01	0.13	0.06	0.06

Table 5.42:Non Compartmental Pharmacokinetic Parameters for Silycristin (122.61 mg/Kg) following Oral Administration of Silymarin (500 mg/Kg) Treatment C1

Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
AUC_{0-t} (mcg-hr/ml)	10.52	6.57	1.65	0.56	4.82	4.61
$AUC_{0-\infty}$ (mcg-hr/ml)	11.76	9.89	2.00	0.71	6.09	5.54
C_{max} (µg/mL)	1.78	0.86	0.49	0.61	0.94	0.58
T_{max} (Hr)	6.00	6.00	1.00	0.50	3.38	3.04
F	0.08	0.07	0.01	0.005	0.04	0.04

A2: INDIVIDUAL PHARMACOKINETIC DATA FOR SILYBIN A

Table 5.43: Plasma Concentrations (μg/mL) Time Profile after Intravenous Bolus Administration of 50 mg/Kg Silymarin equivalent to 9.45 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment B

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
0	0	0	0	0	0	0	0
0.25	9.23	8.09	14.95	7.68	6.53	9.29	3.30
0.5	1.13	2.89	5.71	4.53	1.69	3.19	1.92
0.75	0.64	0.56	0.49	2.18	0.53	0.88	0.73
1	0.52	0.28	0.48	1.39	0.48	0.63	0.43
2	0.12	0.11	0.17	0.86	0.12	0.28	0.33
Weight (Kg)	0.305	0.293	0.295	0.304	0.304	0.299	0.006

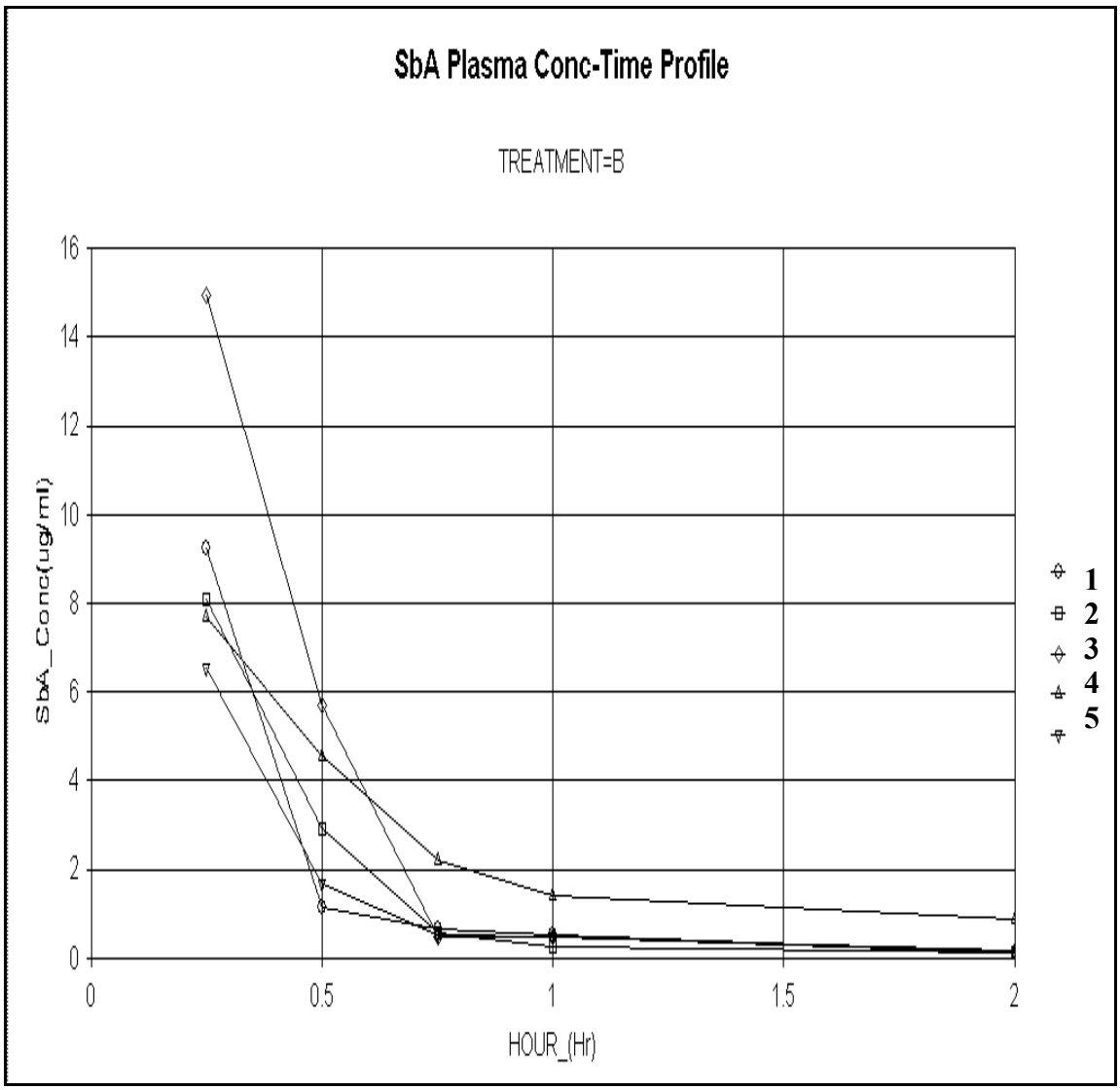


Figure 5.34: Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 50 mg/Kg Silymarin equivalent to 9.45 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment B

Table 5.44:Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 100 mg/Kg Silymarin equivalent to 18.90 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment C

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	SD
0	0	0	0	0	0	0	0	0
0.25	15.39	12.17	24.90	11.84	17.66	21.20	17.19	5.15
0.5	3.12	4.36	8.12	3.57	5.25	6.05	5.08	1.83
0.75	1.10	3.98	4.33	1.77	1.35	1.33	2.31	1.45
1	1.19	2.49	1.51	0.62	1.04	1.24	1.35	0.63
2	0.75	0.38	0.70	0.17	0.21	0.19	0.40	0.26
4	0.07	0.12	0.34	0	0.09	0	0.16	0.13
6	0	0.07	0	0	0	0	0.07	0
Weight (Kg)	0.315	0.308	0.286	0.312	0.317	0.29	0.305	0.013

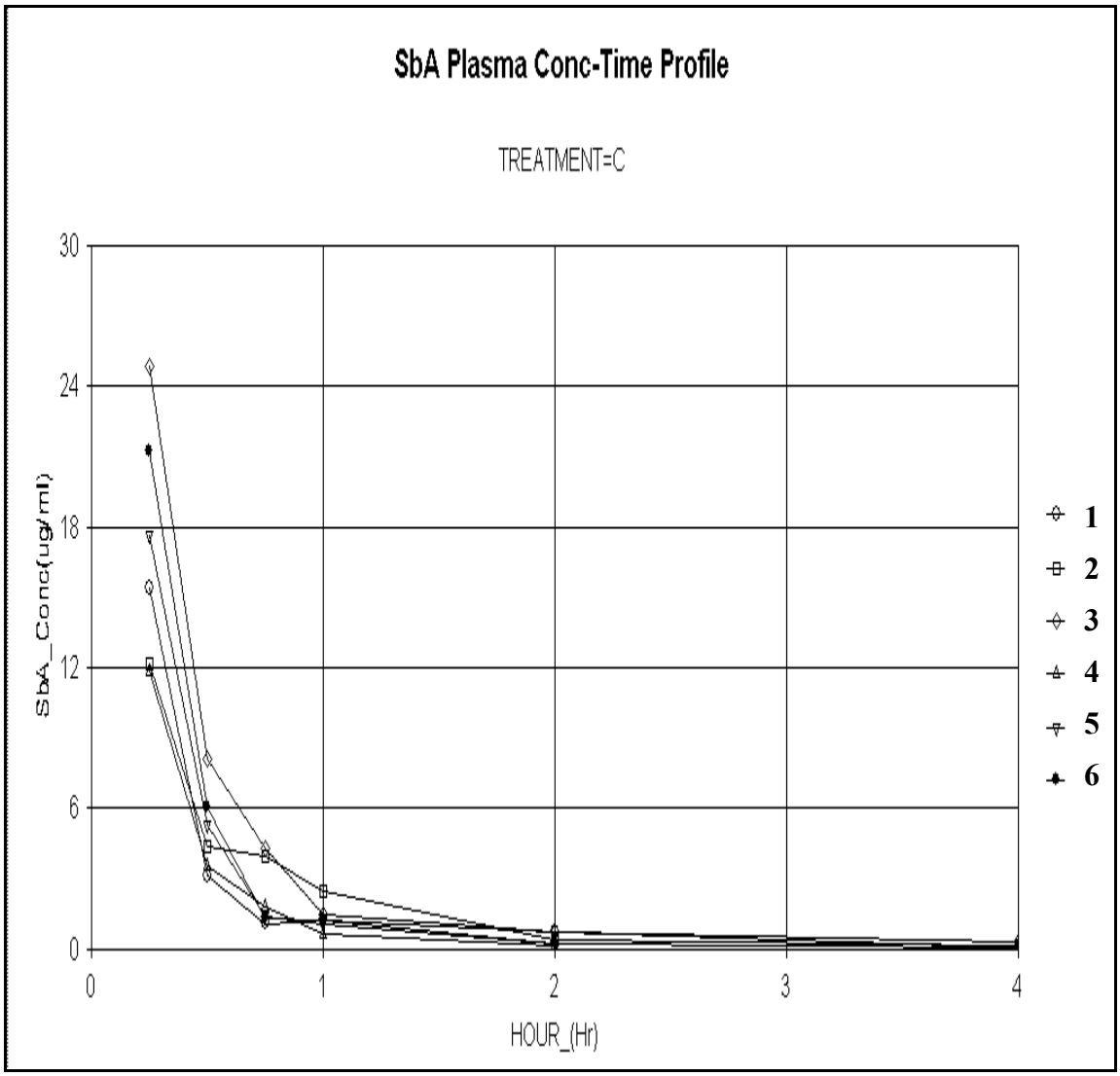


Figure 5.35:Plasma Concentrations (µg/mL) Time Profile after Intravenous Bolus Administration of 100 mg/Kg Silymarin equivalent to 18.90 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment C

Table 5.45:Pharmacokinetic Parameters for Silybin A (9.45 mg/Kg) equivalent to Silymarin (50 mg/Kg) after Intravenous Administration (Treatment B) using Non-Compartmental and 2-Compartmental Analysis

Non Compartmental Analysis							
Parameter	Rat 1*	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
AUC_{0-t} (mcg-hr/ml)	12.57	5.94	10.56	6.52	5.70	7.39	2.28
$AUC_{0-\infty}$ (mcg-hr/ml)	12.65	6.07	10.74	8.32	5.80	7.93	2.30
Clearance (ml/Kg-hr)	747.39	1557.78	880.33	1136.73	1630.55	1279.19	355.20
Vd (ml/kg)	534.91	1767.20	948.12	2377.70	1323.74	1561.42	614.81
Vss (ml/kg)	89.39	427.02	226.06	1440.43	400.07	602.03	551.92
$t_{1/2}$ (hr)	0.50	0.79	0.75	1.45	0.56	0.87	0.39
2-Compartmental Analysis							
AUCo-inf (mcg-hr/ml)	26.57	8.22	10.46	8.96	5.70	8.33	1.99
Clearance (ml/Kg-hr)	355.80	1150.39	904.06	1055.09	1659.93	1192.37	327.79
Vd (ml/kg)	260.92	1437.98	951.64	2228.10	1276.91	1473.66	542.12
Vss (ml/kg)	35.22	270.85	289.38	1288.63	429.37	569.56	484.58
$t_{1/2}$ (hr)	0.51	0.87	0.73	1.46	0.53	0.90	0.40
A (mcg/mL)	394.03	56.00	39.50	19.00	33.08	36.90	15.35
Alfa (hr^{-1})	15.65	7.50	4.20	4.50	7.16	5.84	1.73
B (mcg/mL)	1.90	0.60	1.00	2.24	1.40	1.31	0.70
Beta (hr^{-1})	1.36	0.80	0.95	0.47	1.30	0.88	0.34

* Rat 1 considered as an outlier, not included in the calculation of the Mean and SD.

Table 5.46:Pharmacokinetic Parameters for Silybin A (18.90 mg/Kg) equivalent to Silymarin (100 mg/Kg) after Intravenous Administration (Treatment C) using Non-Compartmental and 2-Compartmental Analysis

Non Compartmental Analysis								
Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	SD
AUC_{0-t} (mcg-hr/ml)	16.31	11.80	21.21	9.67	14.54	17.3016	15.14	4.11
$AUC_{0-\infty}$ (mcg-hr/ml)	16.39	11.95	21.94	9.81	14.66	17.4216	15.36	4.28
Clearance (ml/Kg-hr)	1153.51	1582.00	861.82	1927.02	1289.44	1085.33	1316.52	382.28
Vd (ml/kg)	1299.89	3602.25	1820.98	1518.35	1704.10	667.926	1768.92	986.20
Vss (ml/kg)	367.17	971.04	480.95	484.41	377.51	225.208	484.38	256.59
$t_{1/2}$ (hr)	0.78	1.58	1.46	0.55	0.92	0.4266	0.95	0.47
2-Compartmental Analysis								
AUCo-inf (mcg-hr/ml)	17.48	9.28	18.90	8.44	17.54	16.62	14.71	4.60
Clearance (ml/Kg-hr)	1081.71	2037.23	1000.20	2241.40	1077.99	1137.60	1429.35	555.43
Vd (ml/kg)	1201.91	4074.84	2000.42	1724.16	1426.31	669.18	1849.47	1181.71
Vss (ml/kg)	309.99	1567.85	632.42	730.63	294.17	271.28	634.39	496.68
$t_{1/2}$ (hr)	0.77	1.39	1.39	0.53	0.92	0.41	0.90	0.42
A (mcg/mL)	133.00	15.80	61.00	32.00	112.00	84.99	73.13	45.52
Alfa (hr^{-1})	9.26	2.11	4.15	4.80	7.20	6.27	5.63	2.50
B (mcg/mL)	2.80	0.90	2.10	2.30	1.50	5.20	2.47	1.49
Beta (hr^{-1})	0.90	0.50	0.50	1.30	0.76	1.70	0.94	0.48

Table 5.47:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg Silymarin equivalent to 23.63 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment A1

Time (Hrs)	Rat 1	Rat 2	Rat 3	Mean	SD
0	0	0	0	0	0
0.25	0	0.69	0.32	0.50	0.26
0.5	0	0.48	0.48	0.48	0
0.75	0	0.25	0.08	0.17	0.12
1	0	0.24	0.14	0.19	0.07
2	0.21	0.23	0.32	0.25	0.06
4	0.14	0.20	0.30	0.21	0.08
6	0.22	0.43	0.17	0.27	0.14
8	0.15	0.16	0.18	0.16	0.02
12	0.13	0	0	0.13	0
Weight (Kg)	0.285	0.240	0.247	0.26	0.02

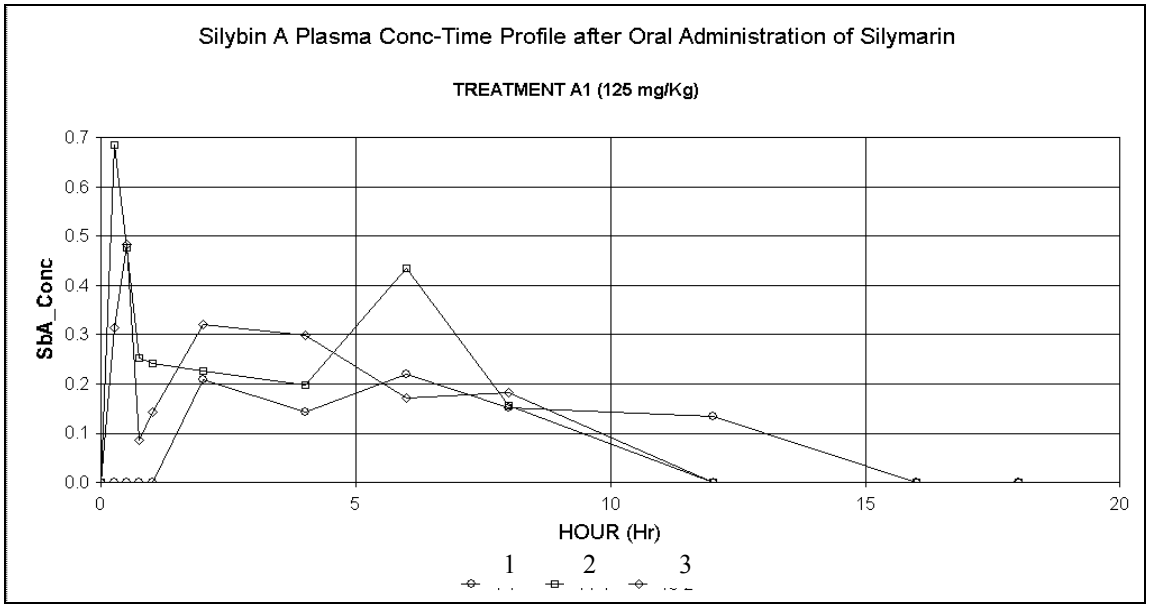


Figure 5.36: Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg Silymarin equivalent to 23.63 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment A1

Table 5.48:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 250 mg/Kg Silymarin equivalent to 47.27 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment B1

Time (Hrs)	Rat 1	Rat 2	Mean	SD
0	0	0	0	0
0.25	0.31	0	0.31	0
0.5	0.14	0.22	0.18	0.06
0.75	0	0.24	0.24	0
1	0.28	0.16	0.22	0.08
2	0.19	0.24	0.22	0.03
4	0.48	0.19	0.34	0.20
6	0.17	0.16	0.16	0.01
8	0.13	0	0.13	0
Weight (Kg)	0.308	0.264	0.29	0.03

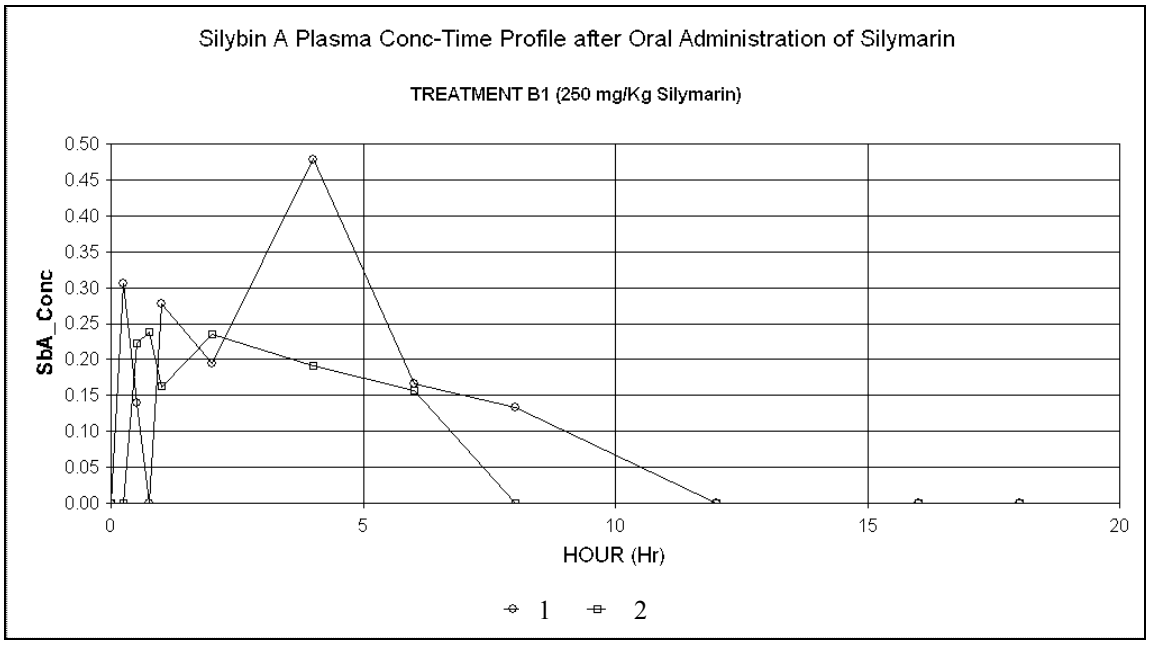


Figure 5.37:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 250 mg/Kg Silymarin equivalent to 47.27 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment B1

Table 5.49:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 500 mg/Kg Silymarin equivalent to 94.54 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment C1

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
0	0	0	0	0	0	0
0.25	0.27	0.60	0.72	0.41	0.50	0.20
0.5	0	0.37	0.60	0.22	0.39	0.19
0.75	0.34	0.27	0.68	0.33	0.40	0.18
1	0.18	0.31	0.35	0.18	0.25	0.09
2	0.19	0.19	0.25	0.15	0.19	0.04
4	0.14	0.17	0.15	0.63	0.27	0.24
6	0.29	0	0	0.18	0.24	0.08
8	0.13	0	0.20	0	0.17	0.05
12	0.12	0	0	0	0.12	0
Weight (Kg)	0.285	0.300	0.247	0.242	0.27	0.03

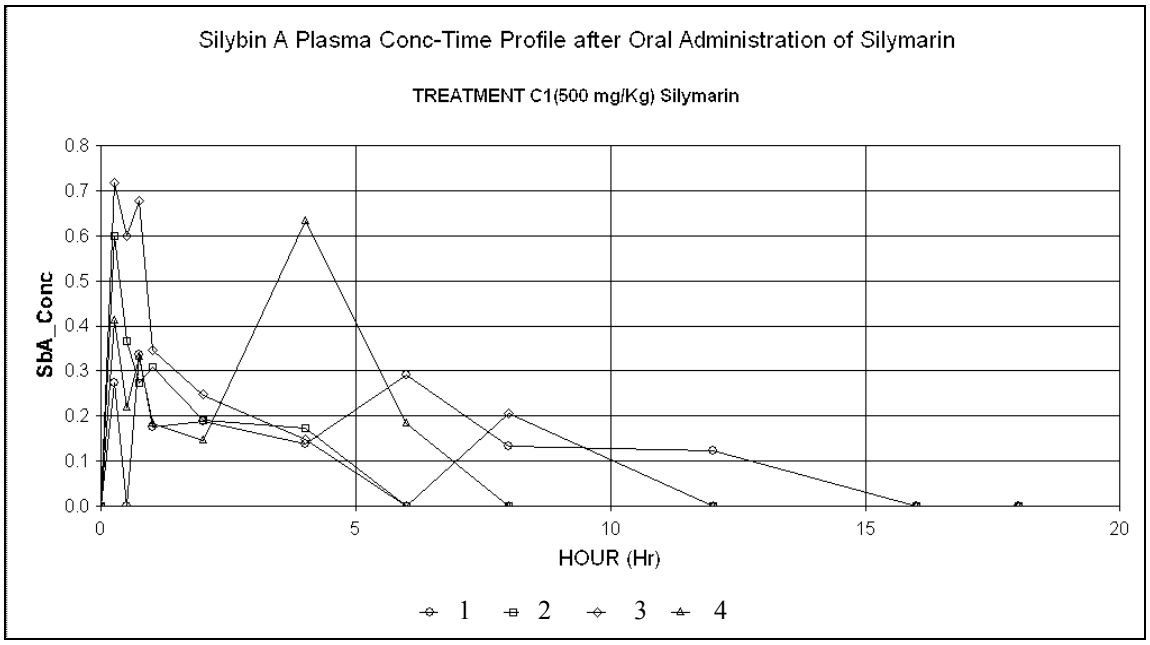


Figure 5.38: Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 500 mg/Kg Silymarin equivalent to 94.54 mg/Kg of Silybin A to Male Sprague Dawley Rats-Treatment C1

Table 5.50: Non Compartmental Pharmacokinetic Parameters for Silybin A (23.63 mg/Kg) following Oral Administration of Silymarin (125 mg/Kg) Treatment A1

Parameter	Rat 1	Rat 2	Rat 3	Mean	SD
AUC_{0-t} (mcg-hr/ml)	1.76	2.26	1.91	1.98	0.26
$AUC_{0-\infty}$ (mcg-hr/ml)	3.55	4.80	3.40	3.92	0.77
C_{max} (µg/mL)	0.22	0.69	0.48	0.46	0.23
T_{max} (Hr)	6.00	0.25	0.50	2.25	3.25
F	0.19	0.25	0.18	0.20	0.04

Table 5.51: Non Compartmental Pharmacokinetic Parameters for Silybin A (47.27 mg/Kg) following Oral Administration of Silymarin (250 mg/Kg) Treatment B1

Parameter	Rat 1	Rat 2	Mean	SD
AUC_{0-t} (mcg-hr/ml)	2.00	1.11	1.55	0.63
$AUC_{0-\infty}$ (mcg-hr/ml)	2.42	2.64	2.53	0.16
C_{max} (µg/mL)	0.48	0.24	0.36	0.17
T_{max} (Hr)	4.00	0.75	2.38	2.30
F	0.11	0.12	0.12	0.01

Table 5.52: Non Compartmental Pharmacokinetic Parameters for Silybin A (94.54 mg/Kg)following Oral Administration of Silymarin (500 mg/Kg) Treatment C1

Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
AUC_{0-t} (mcg-hr/ml)	2.05	0.96	1.59	2.02	1.66	0.51
$AUC_{0-\infty}$ (mcg-hr/ml)	3.04	1.99	2.32	2.80	2.54	0.47
C_{max} (µg/mL)	0.34	0.60	0.72	0.63	0.57	0.16
T_{max} (Hr)	0.75	0.25	0.25	4.00	1.31	1.81
F	0.04	0.03	0.03	0.04	0.03	0.01

A3: INDIVIDUAL PHARMACOKINETIC DATA FOR SILYBIN B

Table 5.53:Plasma Concentrations (μg/mL) Time Profile after Intravenous Administration of 25 mg/Kg Silymarin equivalent to 8.64 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	SD
0	0	0	0	0	0	0	0	0
0.25	6.98	5.90	4.40	3.77	3.84	2.84	4.62	1.53
0.5	6.15	1.25	1.72	1.30	1.84	0.85	2.18	1.97
0.75	0.91	0.47	1.23	0.74	1.30	0.57	0.87	0.34
1	0.63	0.46	1.25	0.65	1.23	0.32	0.76	0.39
2	0.39	0.17	1.00	0.45	1.02	0.21	0.54	0.38
4	0	0	0	0	0	0.20	0.20	0
Weight (Kg)	0.274	0.295	0.344	0.29	0.324	0.288	0.303	0.026

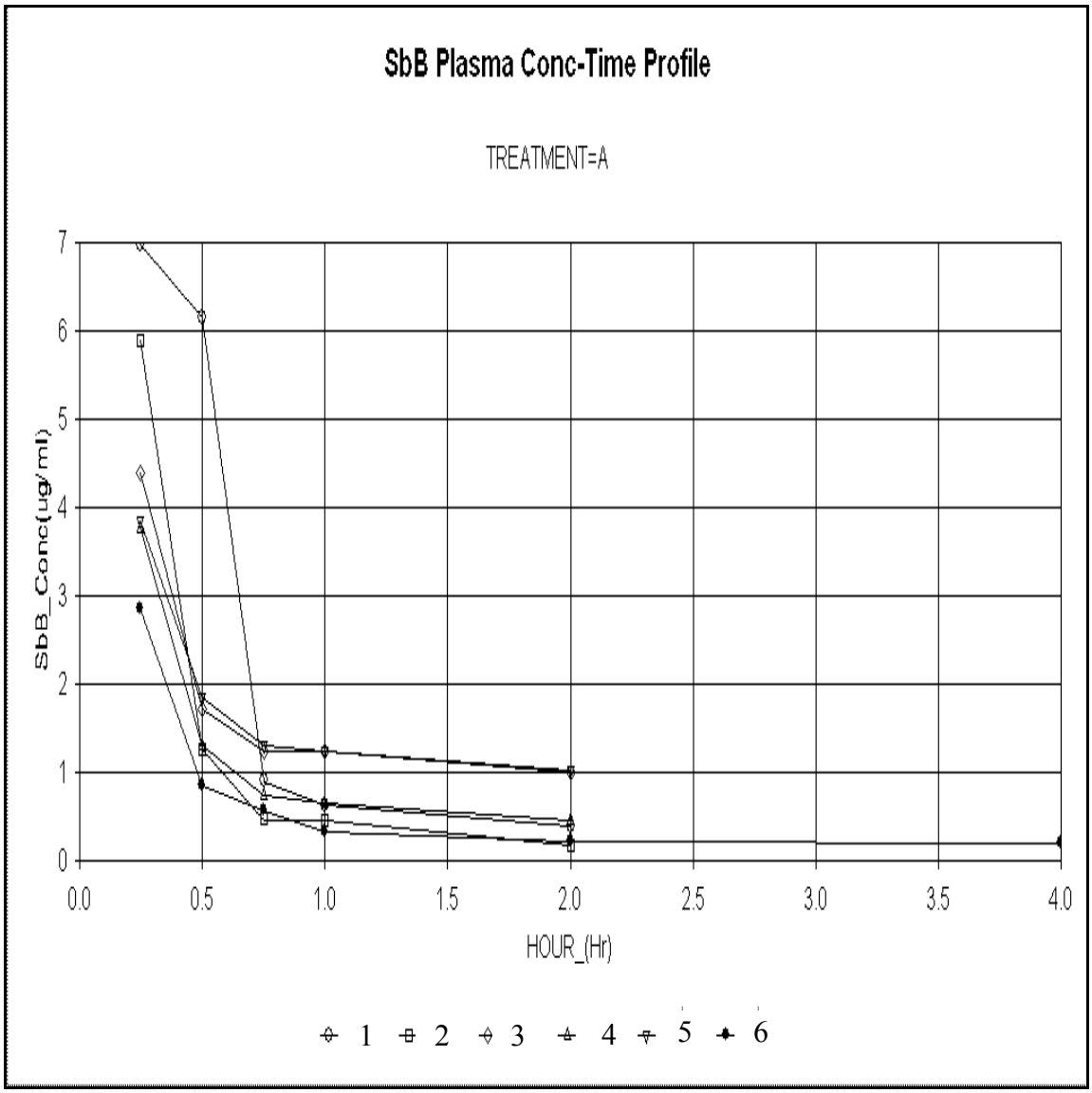


Figure 5.39: Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 25 mg/Kg Silymarin equivalent to 8.64 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment

Table 5.54:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 50 mg/Kg Silymarin equivalent to 17.29 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8	Mean	SD
0	0	0	0	0	0	0	0	0	0	0
0.25	15.76	9.53	11.46	25.74	6.70	9.38	11.11	6.79	12.06	6.23
0.5	5.91	2.49	3.82	8.97	2.67	6.13	2.06	2.65	4.34	2.44
0.75	2.66	1.57	1.00	0.65	1.91	3.10	1.06	2.39	1.79	0.88
1	0.50	1.37	0.77	0.92	1.86	1.91	0.57	1.86	1.22	0.60
2	0.20	1.17	0.68	0.19	1.16	1.19	0.55	1.27	0.80	0.45
4	0	1.02	0.53	0	1.05	0.20	0.07	1.04	0.65	0.45
Weight (Kg)	0.293	0.305	0.293	0.295	0.307	0.304	0.304	0.284	0.298	0.008

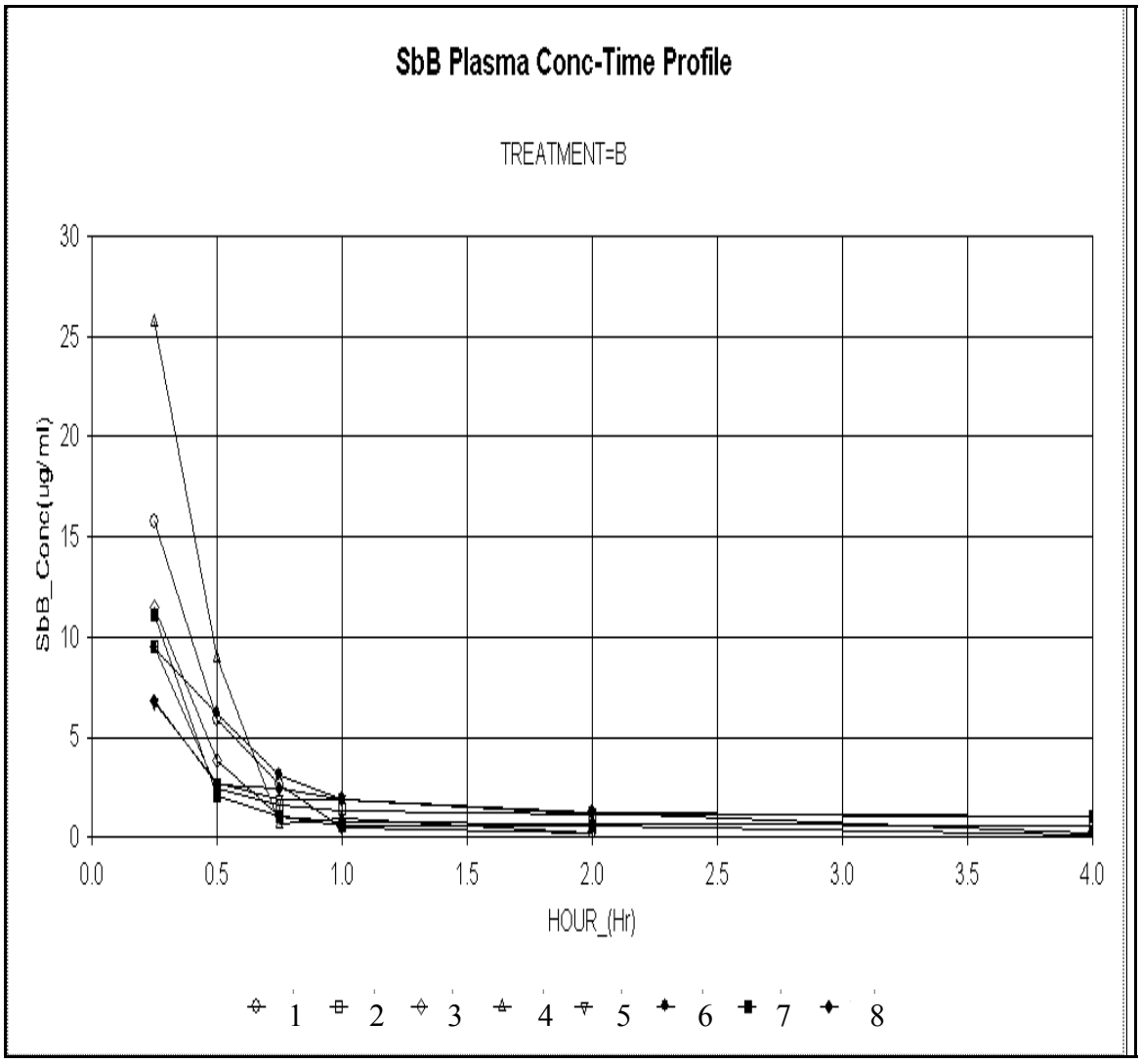


Figure 5.40:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 50 mg/Kg Silymarin equivalent to 17.29 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment

Table 5.55:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 100 mg/Kg Silymarin equivalent to 34.58 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	SD
0	0	0	0	0	0	0	0	0
0.25	19.07	17.29	38.01	17.29	24.40	36.17	25.37	9.46
0.5	5.12	5.28	15.94	6.42	6.02	8.81	7.93	4.14
0.75	2.78	4.68	10.33	4.47	1.79	1.98	4.34	3.18
1	1.88	2.48	4.31	2.51	1.38	2.09	2.44	1.01
2	1.64	0.58	1.38	1.71	0.73	0.23	1.04	0.61
4	1.10	0.33	0.80	1.04	0	0	0.82	0.35
6	1.03	0.24	0	0	0	0	0.63	0.56
8	0	0.13	0	0	0	0	0.13	0
Weight (Kg)	0.32	0.31	0.29	0.31	0.32	0.29	0.30	0.01

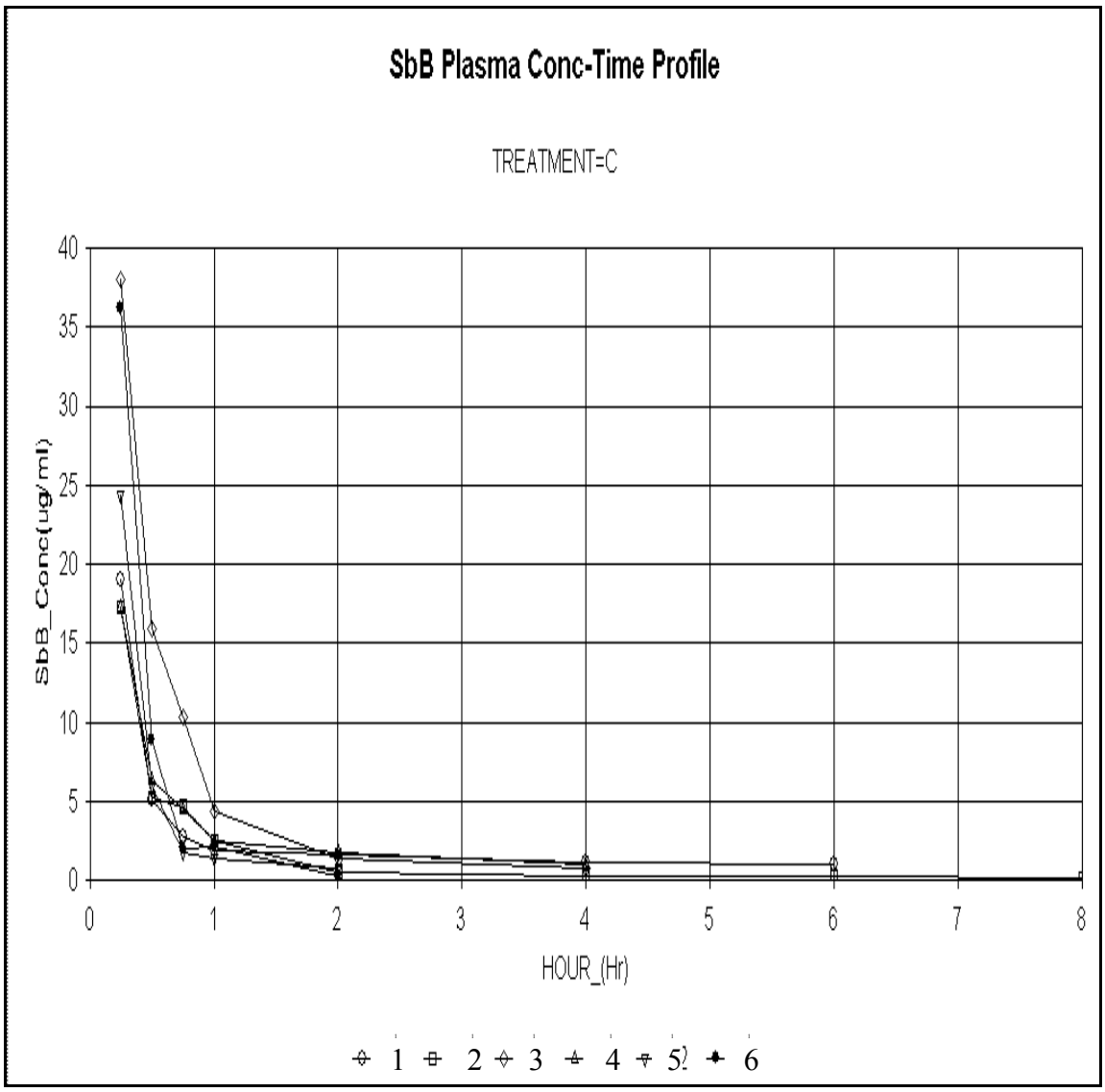


Figure 5.41: Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 100 mg/Kg Silymarin equivalent to 34.58 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment C

Table 5.56:Pharmacokinetic Parameters for Silybin B (8.64 mg/Kg) after Intravenous Administration of Silymarin (25 mg/Kg) using Non-Compartmental and 2-Compartmental Analysis-Treatment A

Non Compartmental Analysis								
Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6*	Mean	SD
AUC_{0-t} (mcg-hr/ml)	5.09	5.75	4.53	3.45	4.03	2.98	4.57	0.90
$AUC_{0-\infty}$ (mcg-hr/ml)	5.92	5.94	10.12	4.64	9.24	4.34	7.17	2.37
Clearance (ml/Kg-hr)	1461.86	1455.13	854.58	1864.36	935.56	1993.17	1314.30	418.02
Vd (ml/kg)	3089.44	1665.37	4759.85	4878.96	4792.30	13747.83	3837.18	1425.20
Vss (ml/kg)	1375.62	410.14	3785.45	2764.57	4011.78	7735.68	2469.51	1552.01
$t_{1/2}$ (hr)	1.46	0.79	3.86	1.81	3.55	4.78	2.30	1.34
2-Compartmental Analysis								
AUCo-inf (mcg-hr/ml)	7.34	6.13	10.84	4.85	9.42	3.78	7.71	2.43
Clearance (ml/Kg-hr)	1178.02	1411.70	797.72	1782.31	918.12	2286.06	1217.57	395.01
Vd (ml/kg)	2506.16	1604.21	4432.04	4570.01	4613.73	15247.53	3545.23	1398.71
Vss (ml/kg)	917.02	428.19	3296.96	2443.64	3720.91	9229.80	2161.34	1445.14
$t_{1/2}$ (hr)	1.47	0.79	3.85	1.78	3.48	4.62	2.27	1.33
A (mcg/mL)	34.00	42.40	23.10	16.80	12.20	6.80	25.70	12.41
Alfa (hr^{-1})	6.80	8.46	8.14	7.03	6.49	4.40	7.38	0.86
B (mcg/mL)	1.10	0.98	1.44	0.96	1.50	0.34	1.20	0.26
Beta (hr^{-1})	0.47	0.88	0.18	0.39	0.20	0.15	0.42	0.28

*Rat 6 considered as an outlier, not included in the calculation of the Mean and SD.

Table 5.57:Pharmacokinetic Parameters for Silybin B (17.29 mg/Kg) after Intravenous Administration of Silymarin (50 mg/Kg) using Non-Compartmental and 2-Compartmental Analysis- Treatment B

Non Compartmental Analysis										
Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8	Mean	SD
AUC_{0-t} (µg-hr/ml)	11.75	11.58	10.40	18.74	8.87	9.63	12.30	9.24	11.56	3.16
$AUC_{0-\infty}$ (µg-hr/ml)	11.97	16.34	13.66	18.91	14.40	9.89	12.40	15.05	14.08	2.79
Clearance (ml/Kg-hr)	1444.50	1058.16	1265.98	914.59	1200.99	1747.87	1394.87	1149.33	1272.04	257.71
Vd (ml/kg)	1573.22	1573.22	7742.07	803.39	6330.46	2284.49	1825.78	6393.09	3565.72	2759.90
Vss (ml/kg)	411.87	3296.29	3616.36	198.59	5106.01	1576.16	444.99	5028.36	2459.83	2062.75
$t_{1/2}$ (hr)	0.75	3.24	4.24	0.61	3.65	0.91	0.91	3.86	2.27	1.60
2-Compartmental Analysis										
AUCo-inf (µg-hr/ml)	11.19	16.37	13.15	17.97	14.72	10.55	13.35	14.25	13.94	2.48
Clearance (ml/Kg-hr)	1545.76	1056.35	1315.05	962.31	1174.72	1639.83	1295.12	1213.33	1275.31	229.24
Vd (ml/kg)	1680.21	5030.08	7735.62	801.92	6182.81	2129.84	1681.97	6741.30	3997.97	2719.64
Vss (ml/kg)	547.20	2653.93	3566.52	264.17	4570.69	1351.67	393.75	5572.19	2365.02	2046.21
$t_{1/2}$ (hr)	0.75	3.30	4.08	0.58	3.65	0.90	0.90	3.85	2.25	1.59
A (mcg/mL)	39.00	68.88	38.10	75.00	28.00	22.40	106.71	10.60	48.59	32.16
Alfa (hr^{-1})	3.97	8.69	5.20	4.60	7.10	4.90	9.60	4.10	6.02	2.17
B (µg/mL)	1.26	1.77	0.99	2.00	2.05	4.60	1.72	2.10	2.06	1.10
Beta (hr^{-1})	0.92	0.21	0.17	1.20	0.19	0.77	0.77	0.18	0.55	0.41

Table 5.58: Pharmacokinetic Parameters for Silybin B (34.58 mg/Kg) after Intravenous Administration of Silymarin (100 mg/Kg) using Non-Compartmental and 2-Compartmental Analysis- Treatment C

Non Compartmental Analysis								
Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	SD
AUC_{0-t} (mcg-hr/ml)	22.48	17.57	32.97	18.03	21.63	31.72	24.07	6.71
$AUC_{0-\infty}$ (mcg-hr/ml)	28.64	18.15	35.93	21.64	22.67	31.84	26.48	6.78
Clearance (ml/Kg-hr)	1207.49	1906.07	962.53	1598.14	1525.56	1086.22	1381.00	356.61
Vd (ml/kg)	7217.54	8127.38	3557.32	5559.95	2182.57	584.62	4538.23	2940.99
Vss (ml/kg)	4101.12	2049.98	1049.19	2995.75	522.71	190.61	1818.23	1521.24
$t_{1/2}$ (hr)	4.14	2.96	2.56	2.41	0.99	0.37	2.24	1.36
2-Compartmental Analysis								
AUCo-inf (mcg-hr/ml)	28.03	13.46	32.41	19.83	21.88	30.72	24.39	7.27
Clearance (ml/Kg-hr)	1234.08	2570.07	1067.28	1743.78	1581.06	1125.93	1553.70	564.21
Vd (ml/kg)	7259.27	10951.84	3930.18	6013.40	2258.67	605.34	5169.78	3724.38
Vss (ml/kg)	3743.29	3812.02	1326.57	3545.89	616.13	233.19	2212.85	1669.17
$t_{1/2}$ (hr)	4.08	2.95	2.55	2.39	0.99	0.37	2.22	1.35
A (mcg/mL)	98.30	26.20	68.70	35.10	121.40	185.00	89.12	59.35
Alfa (hr^{-1})	7.07	2.73	2.90	4.00	6.85	7.30	5.14	2.16
B (mcg/mL)	2.40	0.91	2.37	3.21	2.91	10.00	3.63	3.22
Beta (hr^{-1})	0.17	0.23	0.27	0.29	0.70	1.86	0.59	0.65

Table 5.59:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg Silymarin equivalent to 43.23 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment A1

Time (Hrs)	Rat 1	Rat 2	Mean	SD
0	0	0	0	0
0.25	0.19	0	0.19	0
0.5	0.11	0	0.11	0
0.75	0.17	0	0.17	0
1	0.19	0	0.19	0
2	0.46	0.13	0.30	0.23
4	1.38	0.24	0.81	0.81
6	0.25	0.19	0.22	0.04
8	0.12	0.16	0.14	0.02
12	0.11	0.14	0.12	0.02
Weight (Kg)	0.308	0.264	0.29	0.03

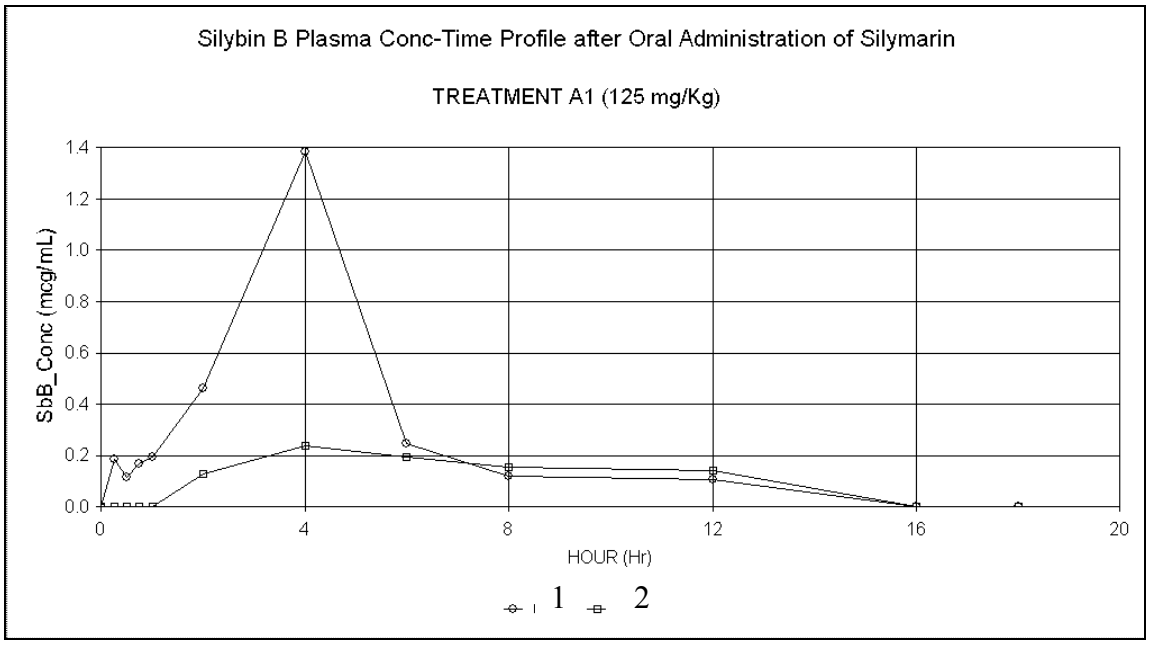


Figure 5.42: Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 125 mg/Kg Silymarin equivalent to 43.23 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment A1

Table 5.60:Plasma Concentrations (μg/mL) Time Profile after Oral Administration of 250 mg/Kg Silymarin equivalent to 86.46 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment B1

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
0	0	0	0	0	0	0	0
0.25	0	0.17	0.39	0	0.19	0.25	0.12
0.5	0	0.07	0.36	0.22	0.11	0.19	0.13
0.75	0	0	0.27	0.32	0.13	0.24	0.09
1	0	0.11	0.11	0.33	0	0.18	0.12
2	0.10	0.26	0.08	0.19	0.46	0.22	0.15
4	0.14	1.24	0.33	0.12	0.71	0.51	0.47
6	0.07	0.36	0.44	0.08	0.19	0.23	0.17
8	0.20	0.10	0.15	0	0.14	0.15	0.04
12	0.38	0	0	0	0.10	0.24	0.19
16	0.16	0	0	0	0	0.16	0
Weight (Kg)	0.285	0.308	0.24	0.264	0.242	0.27	0.03

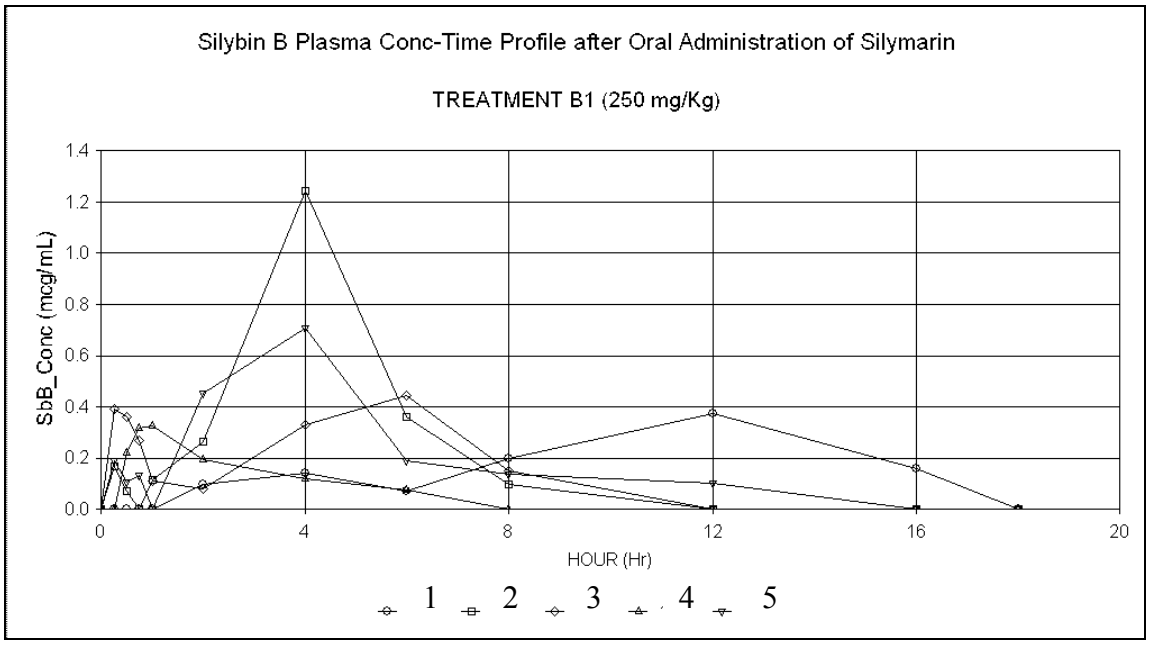


Figure 5.43:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 250 mg/Kg Silymarin equivalent to 86.46 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment B1

Table 5.61:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 500 mg/Kg Silymarin equivalent to 172.93 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment C1

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
0	0	0	0	0	0	0
0.25	0.46	0.50	0.67	0.81	0.63	0.14
0.5	0.19	0.77	0.52	0.19	0.49	0.30
0.75	0.19	0.34	0.33	0.38	0.43	0.19
1	0.22	0.15	0.15	0.21	0.21	0.07
2	0.29	0	0.23	0	0.34	0.26
4	0.48	0.07	0.12	1.98	0.80	0.84
6	1.66	0	0.34	0.41	0.80	0.74
8	0.65	0	0.28	0.35	0.43	0.19
12	0.13	0	0.11	0	0.12	0.01
Weight (Kg)	0.285	0.300	0.31	0.242	0.28	0.03

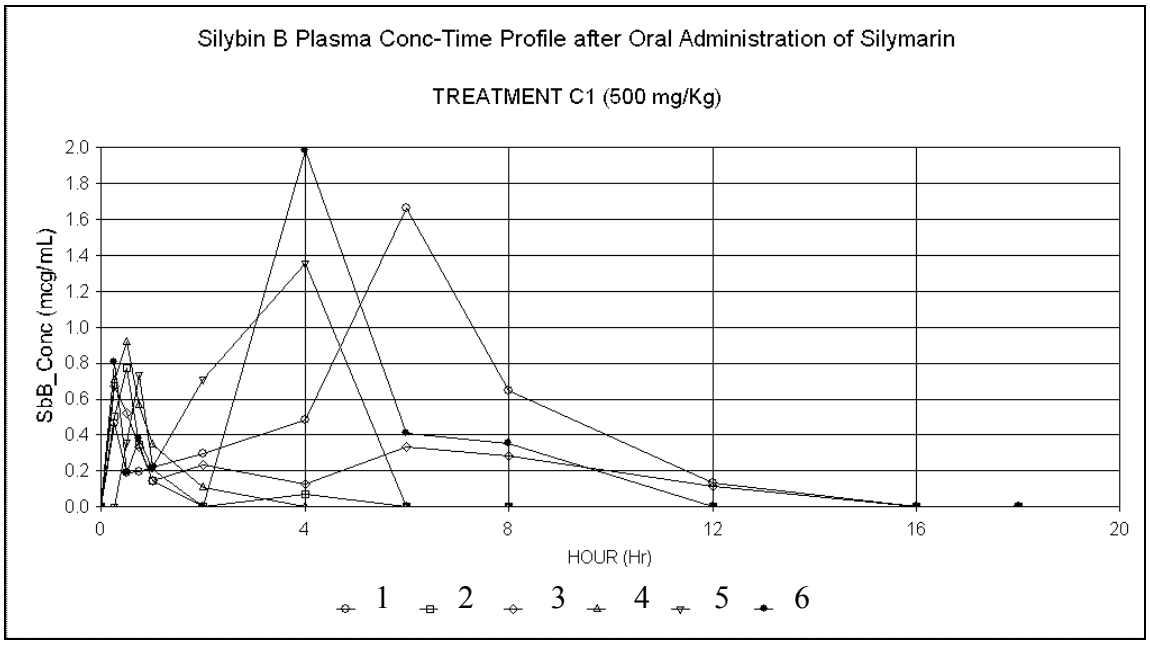


Figure 5.44:Plasma Concentrations (µg/mL) Time Profile after Oral Administration of 500 mg/Kg Silymarin equivalent to 172.93 mg/Kg of Silybin B to Male Sprague Dawley Rats-Treatment C1

Table 5.62:Non Compartmental Pharmacokinetic Parameters for Silybin B (43.23 mg/Kg) following Oral Administration of Silymarin (125 mg/Kg) Treatment A1

Parameter	Rat 1	Rat 2	Mean	SD
AUC_{0-t} (mcg-hr/ml)	4.76	1.81	3.29	2.09
$AUC_{0-\infty}$ (mcg-hr/ml)	5.65	4.69	5.17	0.68
C_{max} (µg/mL)	1.38	0.24	0.81	0.81
T_{max} (Hr)	4.00	4.00	4.00	0.00
F	0.67	0.56	0.62	0.08

Table 5.63:Non Compartmental Pharmacokinetic Parameters for Silybin B (86.46 mg/Kg) following Oral Administration of Silymarin (250 mg/Kg) Treatment B1

Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
AUC_{0-t} (mcg-hr/ml)	2.98	3.83	2.13	0.94	3.20	2.62	1.12
$AUC_{0-\infty}$ (mcg-hr/ml)	3.71	3.98	2.40	1.28	4.26	3.13	1.25
C_{max} (µg/mL)	0.38	1.24	0.44	0.33	0.71	0.62	0.38
T_{max} (Hr)	12.00	4.00	6.00	1.00	4.00	5.40	4.10
F	0.11	0.11	0.07	0.04	0.12	0.09	0.04

Table 5.64:Non Compartmental Pharmacokinetic Parameters for Silybin B (172.93 mg/Kg) following Oral Administration of Silymarin (500 mg/Kg) Treatment C1

Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
AUC_{0-t} (mcg-hr/ml)	7.29	0.57	2.81	5.61	4.07	2.98
$AUC_{0-\infty}$ (mcg-hr/ml)	7.62	0.75	3.43	6.43	4.56	3.09
C_{max} (µg/mL)	1.66	0.77	0.67	1.98	1.27	0.65
T_{max} (Hr)	6.00	0.50	0.25	4.00	2.69	2.79
F	0.06	0.01	0.03	0.05	0.03	0.02

A4: INDIVIDUAL PHARMACOKINETIC DATA FOR ISOSILYBIN A

Table 5.65:Plasma Concentrations (μg/mL) Time Profile after Intravenous Administration of 25 mg/Kg Silymarin equivalent to 2.05 mg/Kg of Isosilybin A to Male Sprague Dawley Rats-Treatment-Treatment A

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
0	0	0	0	0	0	0	0
0.25	1.10	1.64	3.81	0.79	0.96	1.66	1.24
0.5	0.89	0.47	0.31	0.29	0.29	0.45	0.26
0.75	0.19	0.28	0.17	0.17	0.32	0.23	0.07
1	0.13	0.21	0.25	0.11	0.17	0.18	0.06
2	0.06	0.07	0.06	0.06	0.12	0.07	0.03
4	0	0	0	0	0.08	0.08	0
Weight (Kg)	0.274	0.295	0.258	0.324	0.288	0.30	0.02

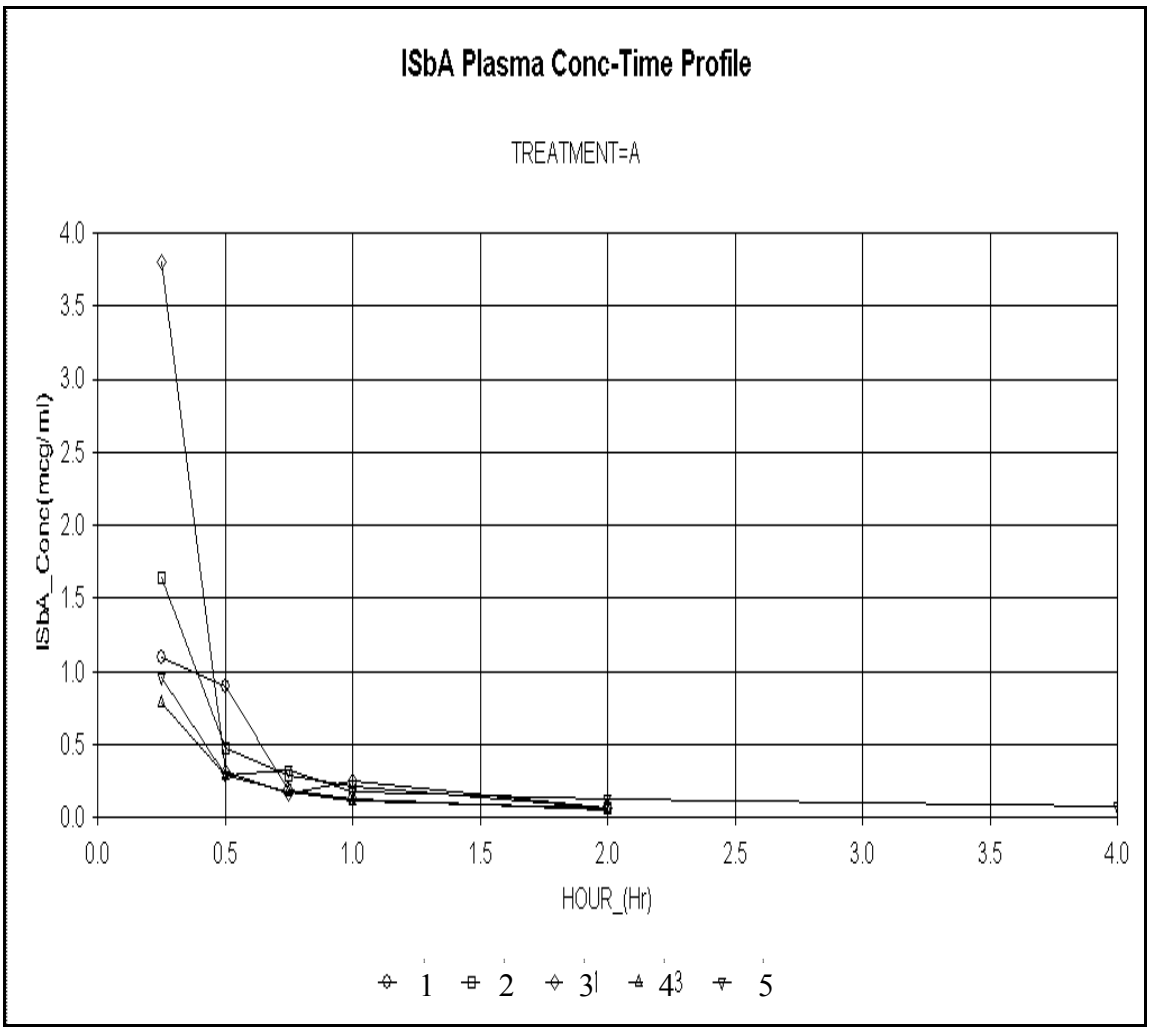


Figure 5.45:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 25 mg/Kg Silymarin equivalent to 2.05 mg/Kg of Isosilybin A to Male Sprague Dawley Rats-Treatment A

Table 5.66:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 50 mg/Kg Silymarin equivalent to 4.11 mg/Kg of Isosilybin A to Male Sprague Dawley Rats-Treatment B

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Mean	SD
0	0	0	0	0	0	0	0	0	0
0.25	3.72	2.98	2.43	3.38	1.52	2.41	1.77	2.60	0.81
0.5	1.58	0.79	0.90	1.38	0.57	0.37	0.66	0.89	0.44
0.75	0.33	0.46	0.50	0.18	0.35	0.36	0.41	0.37	0.10
1	0.21	0.29	0.27	0.25	0.27	0.17	0.35	0.26	0.06
2	0.12	0.11	0.15	0.07	0.06	0.10	0.08	0.10	0.03
4	0.00	0.10	0.12	0.00	0.00	0.00	0.07	0.10	0.03
Weight (Kg)	0.293	0.305	0.293	0.295	0.307	0.304	0.284	0.30	0.008

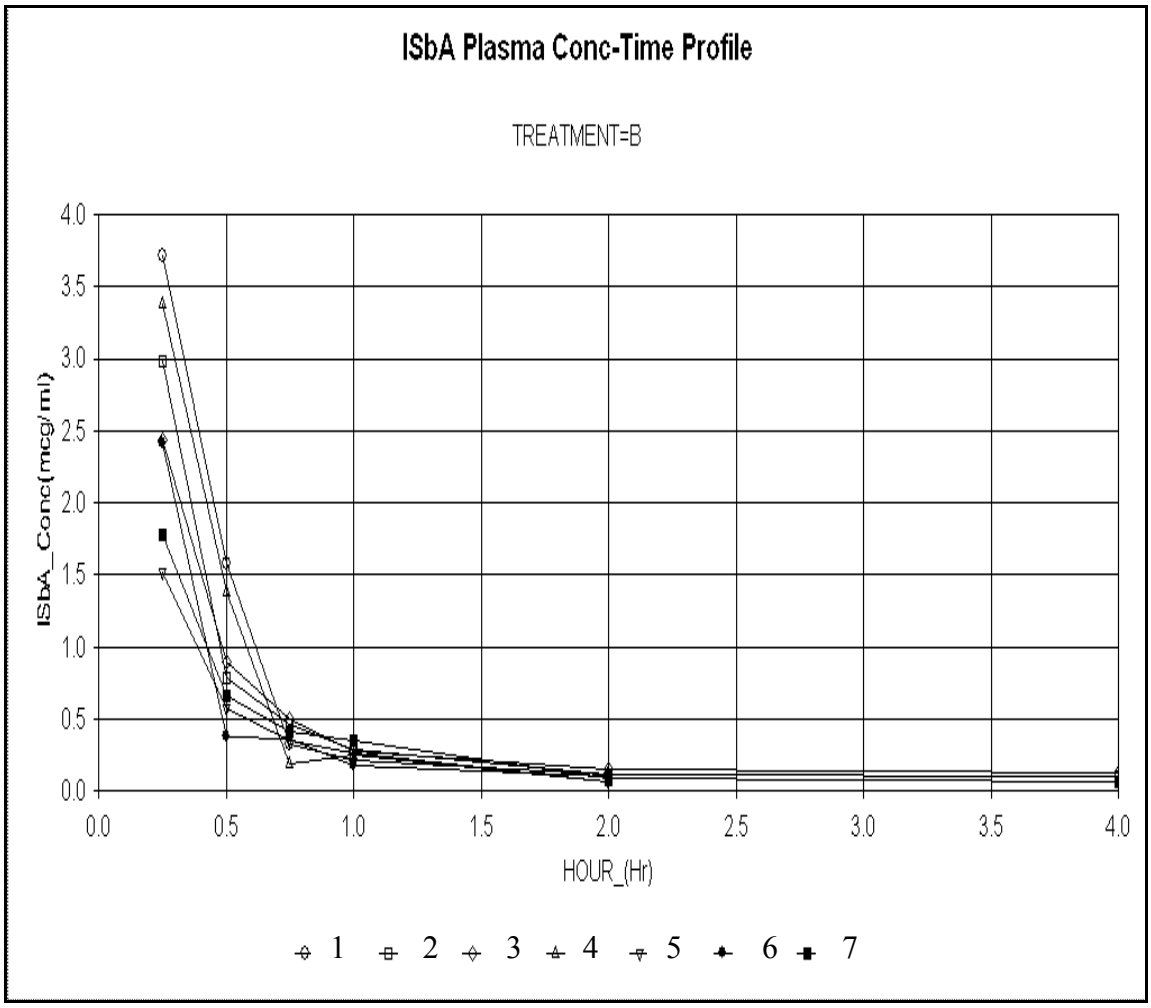


Figure 5.46:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 50 mg/Kg Silymarin equivalent to 4.11 mg/Kg of Isosilybin A to Male Sprague Dawley Rats-Treatment B

Table 5.67:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 100 mg/Kg Silymarin equivalent to 8.22 mg/Kg of Isosilybin A to Male Sprague Dawley Rats-Treatment C

Time (Hrs)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
0	0	0	0	0	0	0	0
0.25	5.20	4.46	8.08	3.95	6.89	5.71	1.73
0.5	1.18	1.60	2.81	1.46	1.31	1.67	0.65
0.75	0.82	1.45	0.83	0.98	0.78	0.97	0.28
1	0.43	0.92	0.55	0.51	0.51	0.59	0.19
2	0.34	0.22	0.10	0.15	0.27	0.22	0.09
4	0	0.18	0	0.09	0.16	0.14	0.04
6	0	0.11	0	0	0	0.11	0.00
Weight (Kg)	0.32	0.31	0.29	0.31	0.32	0.31	0.01

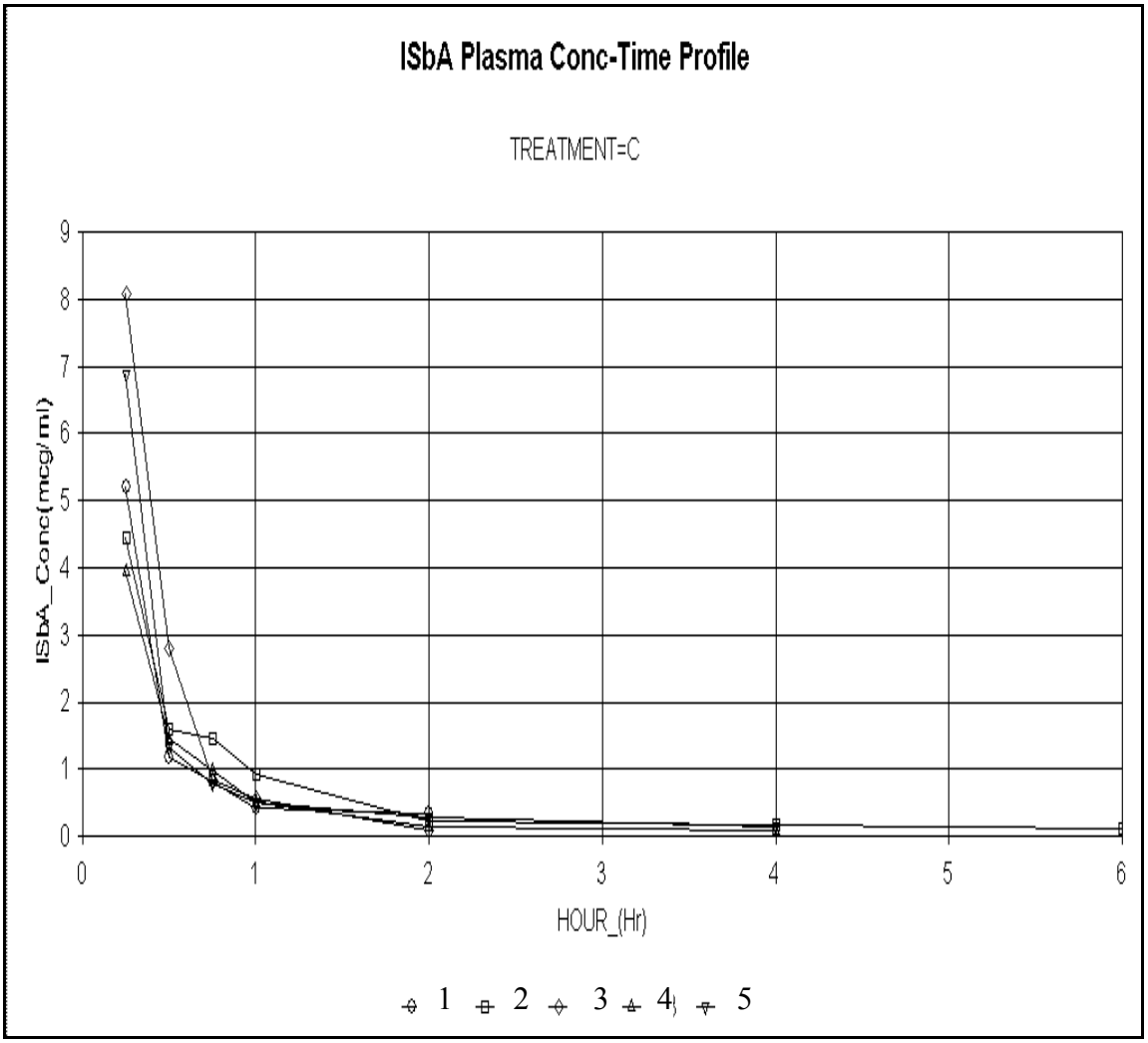


Figure 5.47: Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 100 mg/Kg Silymarin equivalent to 8.22 mg/Kg of Isosilybin A to Male Sprague Dawley Rats-Treatment C

Table 5.68: Pharmacokinetic Parameters for Isosilybin A (2.05 mg/Kg) following Intravenous Administration of Silymarin (25 mg/Kg) using Non-Compartmental and 2-Compartmental Analysis- Treatment A

Non Compartmental Analysis							
Parameter	Rat 1	Rat 2	Rat 3*	Rat 4	Rat 5	Mean	SD
AUC_{0-t} (mcg-hr/ml)	0.83	1.48	7.15	0.68	1.16	1.04	0.36
$AUC_{0-\infty}$ (mcg-hr/ml)	0.89	1.54	7.21	0.78	1.49	1.17	0.40
Clearance (ml/Kg-hr)	2307.15	1334.14	284.74	2650.00	1384.29	1918.90	661.57
Vd (ml/kg)	2563.08	1197.34	300.23	4168.34	5574.35	3375.78	1903.33
Vss (ml/kg)	1443.78	520.55	28.77	2004.40	3328.04	1824.19	1174.48
$t_{1/2}$ (hr)	0.77	0.62	0.73	1.09	2.79	1.32	1.00
2-Compartmental Analysis							
$AUC_{0-\infty}$ (mcg-hr/ml)	1.49	1.95	24.03	0.76	1.35	1.38	0.49
Clearance (ml/Kg-hr)	1380.02	1055.00	85.54	2722.04	1527.32	1671.09	727.89
Vd (ml/kg)	1709.16	941.97	91.00	4253.19	6109.62	3253.49	2372.41
Vss (ml/kg)	563.89	355.64	6.10	2200.47	3982.11	1775.52	1686.59
$t_{1/2}$ (hr)	0.86	0.62	0.74	1.08	2.77	1.33	0.98
A (mcg/mL)	7.80	13.50	461.13	2.24	1.72	6.32	5.52
Alfa (hr^{-1})	6.90	9.87	19.58	5.42	3.40	6.40	2.72
B (mcg/mL)	0.29	0.65	0.45	0.22	0.21	0.34	0.21
Beta (hr^{-1})	0.81	1.12	0.94	0.64	0.25	0.70	0.36

* Rat 3 considered as an outlier, not included in the calculation of the Mean and SD.

Table 5.69:Pharmacokinetic Parameters for Isosilybin A (4.11 mg/Kg) following Intravenous Administration of Silymarin (50 mg/Kg) using Non-Compartmental and 2-Compartmental Analysis-Treatment B

Non-Compartmental Analysis									
Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Mean	SD
AUC_{0-t} (µg-hr/ml)	2.70	2.92	2.30	2.45	1.31	2.90	1.72	2.33	0.61
$AUC_{0-\infty}$ (µg-hr/ml)	2.85	3.14	2.80	2.52	1.36	3.07	1.84	2.51	0.67
Clearance (ml/Kg-hr)	1441	1310.85	1467.31	1631.53	3033.99	1339.11	2238.73	1780.37	636.67
Vd (ml/kg)	1911	2991.15	6018.28	1680.13	2211.23	2294.60	3971.56	3011.19	1532.98
Vss (ml/kg)	641.06	1111.91	2901.24	543.88	1318.44	472.57	2071.97	1294.44	902.40
$t_{1/2}$ (hr)	0.92	1.58	2.84	0.71	0.51	1.19	1.23	1.28	0.77
2-Compartmental Analysis									
AUCo-inf (µg-hr/ml)	2.88	3.21	2.62	2.49	1.68	2.01	1.90	2.40	0.56
Clearance (ml/Kg-hr)	1425	1281.53	1570.04	1647.94	2444.46	2049.33	2160.62	1797.01	426.77
Vd (ml/kg)	1875.2	2912.58	6280.27	1681.71	1772.78	3473.44	3790.72	3112.39	1634.93
Vss (ml/kg)	716.53	1060.40	3075.83	653.21	906.25	1200.58	1968.56	1368.77	870.69
$t_{1/2}$ (hr)	0.91	1.58	2.77	0.71	0.50	1.17	1.22	1.27	0.75
A (mcg/mL)	8.80	15.70	6.10	8.22	9.38	7.70	6.20	8.87	3.25
Alfa (hr^{-1})	4.00	7.23	4.30	4.10	9.80	5.20	6.15	5.83	2.12
B (mcg/mL)	0.52	0.46	0.30	0.48	1.00	0.31	0.51	0.51	0.23
Beta (hr^{-1})	0.76	0.44	0.25	0.98	1.38	0.59	0.57	0.71	0.37

Table 5.70:Pharmacokinetic Parameters for Isosilybin A (8.22 mg/Kg) after Intravenous Administration of Silymarin (100 mg/Kg) using Non-Compartmental and 2-Compartmental Analysis- Treatment C

Non Compartmental Analysis							
Parameter	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
AUC_{0-t} (mcg-hr/ml)	5.10	4.81	6.23	3.57	7.64	5.47	1.54
$AUC_{0-\infty}$ (mcg-hr/ml)	5.69	5.06	6.29	3.75	8.05	5.77	1.59
Clearance (ml/Kg-hr)	1444.72	1626.51	1307.98	2192.21	1021.61	1518.61	436.52
Vd (ml/kg)	2517.25	3706.48	769.27	4225.23	2684.12	2780.47	1329.32
Vss (ml/kg)	871.69	1972.32	334.73	1660.49	660.98	1100.04	690.21
$t_{1/2}$ (hr)	1.21	1.58	0.41	1.34	1.82	1.27	0.54
2-Compartmental Analysis							
$AUC_{0-\infty}$ (mcg-hr/ml)	6.49	5.21	6.16	3.36	6.50	5.54	1.33
Clearance (ml/Kg-hr)	1266.33	1579.09	1335.94	2446.38	1264.25	1578.40	502.06
Vd (ml/kg)	2183.88	3588.86	781.25	4704.64	3241.68	2900.06	1487.44
Vss (ml/kg)	685.47	2232.77	393.96	2176.53	1003.68	1298.48	855.09
$t_{1/2}$ (hr)	1.20	1.58	0.41	1.33	1.78	1.26	0.53
A (mcg/mL)	44.11	8.34	24.50	7.60	30.20	22.95	15.43
Alfa (hr^{-1})	9.28	3.74	5.56	3.60	6.31	5.70	2.32
B (mcg/mL)	1.01	1.31	2.99	0.65	0.67	1.33	0.97
Beta (hr^{-1})	0.58	0.44	1.71	0.52	0.39	0.73	0.55

A5: INDIVIDUAL PHARMACOKINETIC DATA FOR ISOSILYBIN B

Table 5.71: Plasma Concentrations (μg/mL) Time Profile after Intravenous Administration of 25 mg/Kg Silymarin equivalent to 0.64 mg/Kg of Isosilybin B to Male Sprague Dawley Rats-Treatment A

Time (Hrs)	Rat 1	Rat 2	Mean	SD
0	0	0	0	0
0.25	0.78	0.45	0.62	0.24
0.5	0.54	0.21	0.37	0.23
0.75	0.15	0.07	0.11	0.06
1	0.30	0.10	0.20	0.14
2	0.18	0.08	0.13	0.07
Weight (Kg)	0.274	0.288	0.28	0.01

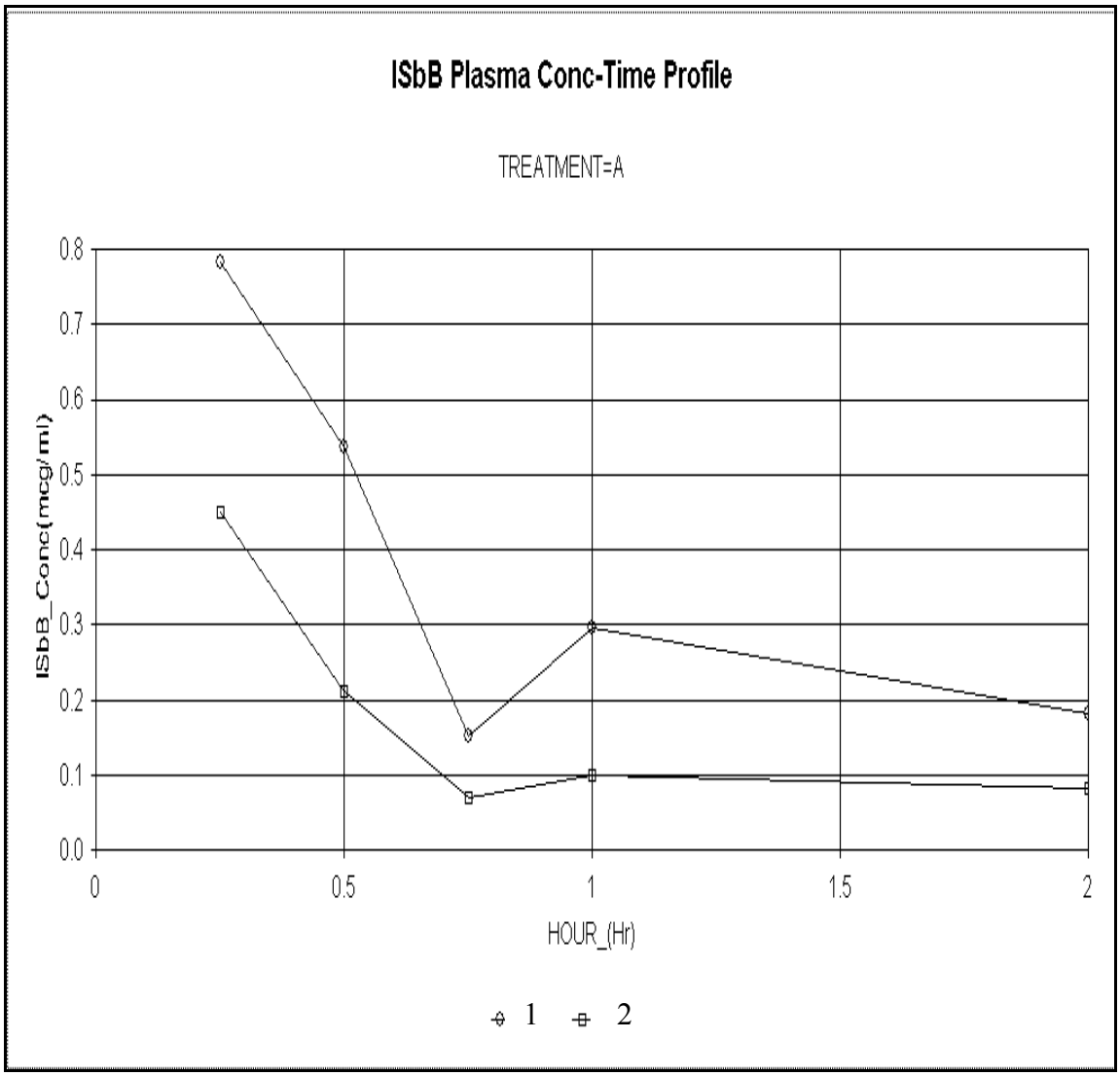


Figure 5.48:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 25 mg/Kg Silymarin equivalent to 0.64 mg/Kg of Isosilybin B to Male Sprague Dawley Rats-Treatment A

Table 5.72:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 50 mg/Kg Silymarin equivalent to 1.28 mg/Kg of Isosilybin B to Male Sprague Dawley Rats-Treatment B

Time (Hrs)	Rat 1	Rat 2	Mean	SD
0	0	0	0	0
0.25	1.18	1.01	1.14	0.11
0.5	0.67	0.31	0.47	0.18
0.75	0.52	0.24	0.38	0.20
1	0.23	0.19	0.17	0.08
2	0.22	0.08	0.12	0.09
Weight (Kg)	0.304	0.284	0.29	0.01

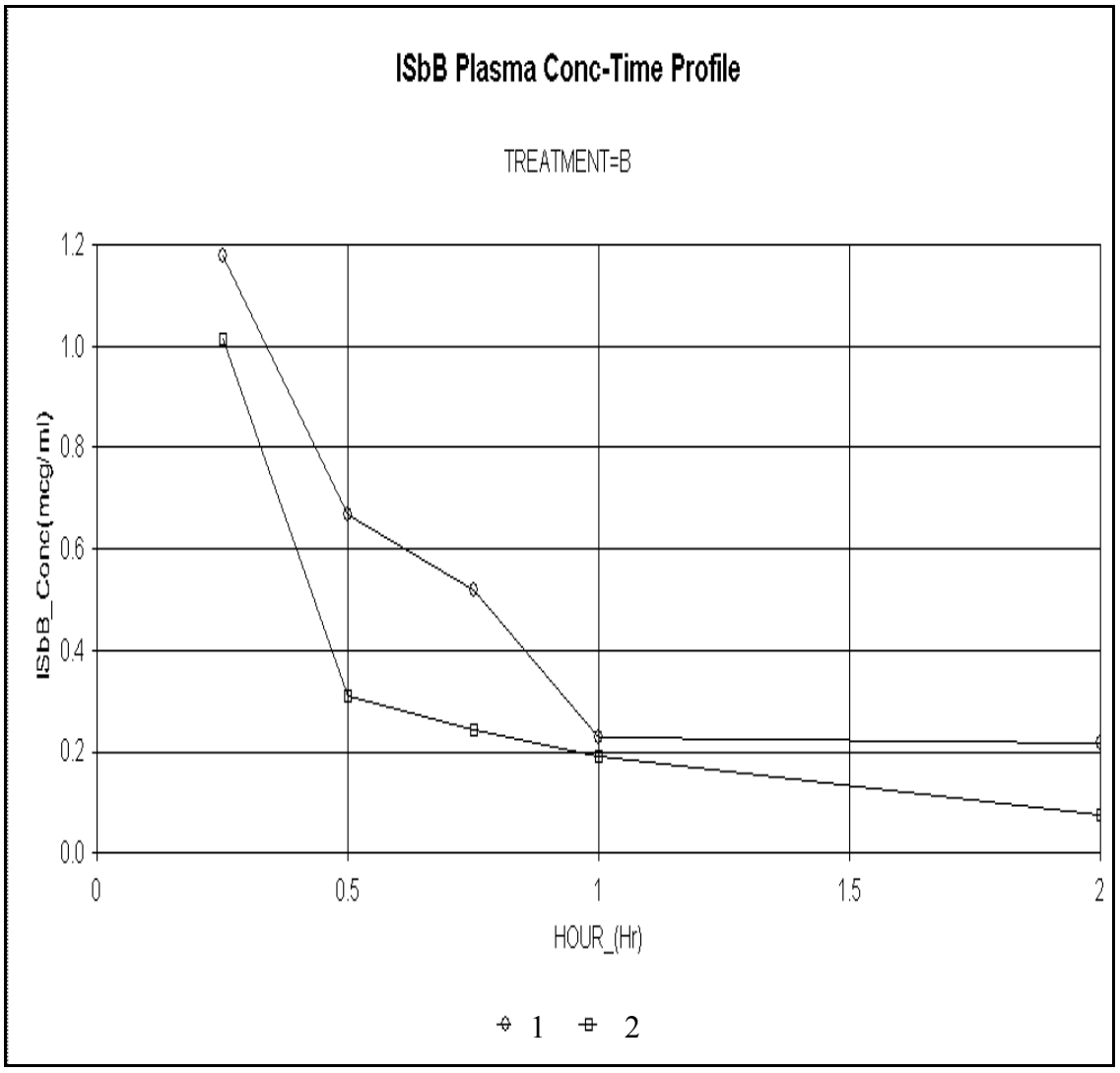


Figure 5.49:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 50 mg/Kg Silymarin equivalent to 1.28 mg/Kg of Isosilybin B to Male Sprague Dawley Rats-Treatment B

Table 5.73:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 100 mg/Kg Silymarin equivalent to 2.56 mg/Kg of Isosilybin B to Male Sprague Dawley Rats-Treatment C

Time (Hrs)	Rat 1	Rat 2	Mean	SD
0	0	0	0	0
0.25	3.70	2.82	3.26	0.62
0.5	0.80	1.12	0.96	0.23
0.75	0.43	0.77	0.60	0.25
1	0.32	0.25	0.28	0.05
2	0.21	0.14	0.17	0.05
Weight (Kg)	0.315	0.312	0.31	0.00

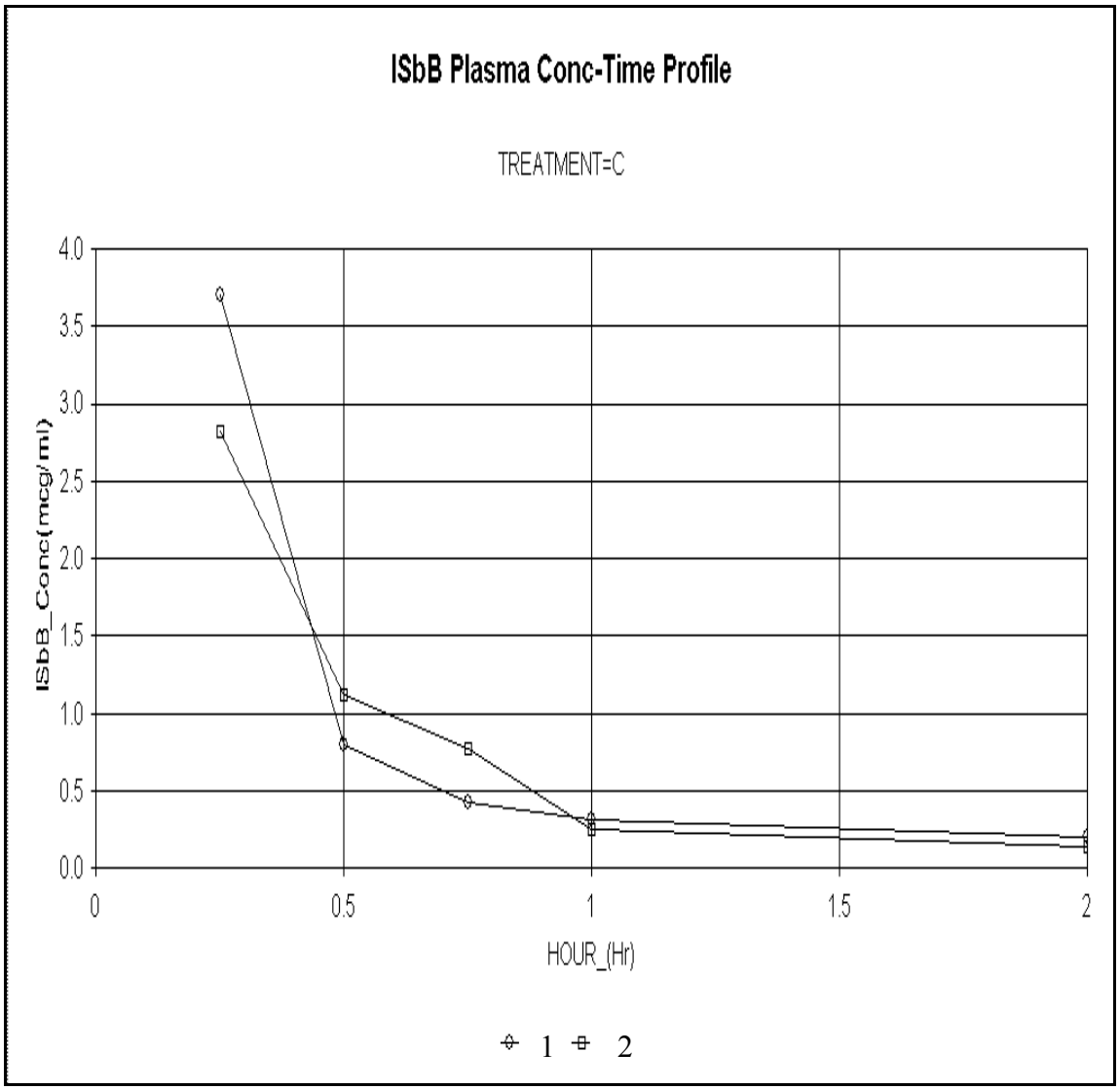


Figure 5.50:Plasma Concentrations (µg/mL) Time Profile after Intravenous Administration of 100 mg/Kg Silymarin equivalent to 2.56 mg/Kg of Isosilybin B to Male Sprague Dawley Rats-Treatment C

Table 5.74:Pharmacokinetic Parameters for Isosilybin B (0.640 mg/Kg) following Intravenous Administration of Silymarin (25 mg/Kg) using Non-Compartmental and 2-Compartmental Analysis- Treatment A

Non Compartmental Analysis				
Parameter	Rat 1	Rat 2	Mean	SD
AUC_{0-t} (mcg-hr/ml)	0.79	0.41	0.60	0.27
$AUC_{0-\infty}$ (mcg-hr/ml)	1.16	0.91	1.03	0.17
Clearance (ml/Kg-hr)	554.45	704.63	629.54	106.20
Vd (ml/kg)	963.81	3293.92	2128.87	1647.64
Vss (ml/kg)	1134.78	4243.02	2688.90	2197.86
$t_{1/2}$ (hr)	1.42	4.17	2.80	1.95
2-Compartmental Analysis				
$AUC_{0-\infty}$ (mcg-hr/ml)	1.23	0.88	1.05	0.24
Clearance (ml/Kg-hr)	522.21	727.26	624.74	144.99
Vd (ml/kg)	1065.74	4277.98	2671.86	2271.40
Vss (ml/kg)	883.72	3181.96	2032.84	1625.10
$t_{1/2}$ (hr)	1.41	4.08	2.75	1.88
A (mcg/mL)	0.79	1.11	0.95	0.23
Alfa (hr^{-1})	3.19	4.74	3.96	1.10
B (mcg/mL)	0.48	0.11	0.30	0.26
Beta (hr^{-1})	0.49	0.17	0.33	0.23

Table 5.75:Pharmacokinetic Parameters for Isosilybin B (1.28 mg/Kg) following Intravenous Administration of Silymarin (50 mg/Kg) using Non-Compartmental and 2-Compartmental Analysis- Treatment B

Non Compartmental Analysis				
Parameter	Rat 1	Rat 2	Mean	SD
AUC_{0-t} (mcg-hr/ml)	1.10	0.96	1.03	0.10
$AUC_{0-\infty}$ (mcg-hr/ml)	1.53	1.05	1.29	0.34
Clearance (ml/Kg-hr)	837.00	1226.28	1031.64	275.26
Vd (ml/kg)	1648.39	1324.55	1486.47	228.99
Vss (ml/kg)	1268.12	700.09	984.10	401.66
$t_{1/2}$ (hr)	1.37	0.75	1.06	0.44
2-Compartmental Analysis				
$AUC_{0-\infty}$ (mcg-hr/ml)	1.23	0.88	1.05	0.24
Clearance (ml/Kg-hr)	522.21	727.26	624.74	144.99
Vd (ml/kg)	1065.74	4277.98	2671.86	2271.40
Vss (ml/kg)	883.72	3181.96	2032.84	1625.10
$t_{1/2}$ (hr)	1.41	4.08	2.75	1.88
A (mcg/mL)	0.79	1.11	0.95	0.23
Alfa (hr^{-1})	3.19	4.74	3.96	1.10
B (mcg/mL)	0.48	0.11	0.30	0.26
Beta (hr^{-1})	0.49	0.17	0.33	0.23

Table 5.76:Pharmacokinetic Parameters for Isosilybin B (2.56 mg/Kg) following Intravenous Administration of Silymarin (100 mg/Kg) using Non-Compartmental and 2-Compartmental Analysis- Treatment C

Non Compartmental Analysis				
Parameter	Rat 1	Rat 2	Mean	SD
AUC_{0-t} (mcg-hr/ml)	3.68	2.30	2.99	0.98
$AUC_{0-\infty}$ (mcg-hr/ml)	4.06	2.42	3.24	1.16
Clearance (ml/Kg-hr)	631.24	1058.42	844.83	302.06
Vd (ml/kg)	1167.83	933.47	1050.65	165.72
Vss (ml/kg)	354.89	489.21	422.05	94.97
$t_{1/2}$ (hr)	1.28	0.61	0.95	0.47
2-Compartmental Analysis				
$AUC_{0-\infty}$ (mcg-hr/ml)	4.39	2.51	3.45	1.33
Clearance (ml/Kg-hr)	584.39	1021.39	802.89	309.00
Vd (ml/kg)	1062.54	895.97	979.25	117.78
Vss (ml/kg)	309.43	486.33	397.88	125.09
$t_{1/2}$ (hr)	1.26	0.61	0.93	0.46
A (mcg/mL)	29.50	8.50	19.00	14.85
Alfa (hr^{-1})	8.90	6.00	7.45	2.05
B (mcg/mL)	0.59	1.25	0.92	0.46
Beta (hr^{-1})	0.55	1.14	0.84	0.42

References

1. Wolters, B., *The oldest medicinal plants. Phytotherapy in the Stone Age.* Deutsche Apotheker Zeitung, 1999. **139**(39): p. 3675-3682.
2. Lietava, J., *Medicinal plants in a Middle Paleolithic grave Shanidar IV?* Journal of Ethnopharmacology, 1992. **35**(3): p. 263-6.
3. Capasso, L., *5300 years ago, the Ice Man used natural laxatives and antibiotics.* Lancet, 1998. **352**(9143): p. 1864.
4. Eisenberg, D.M., et al., *Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey.* JAMA, 1998. **280**(18): p. 1569-75.
5. Barnes Patricia, M., et al., *Complementary and alternative medicine use among adults: United States, 2002.* Advance data, 2004(343): p. 1-19.
6. Ebadi, M., *Pharmacodynamic Basis of Herbal Medicine*. 2001. 592 pp.
7. U. S. Food and Drug Administration, C.F.S.A.N. *Overview of Dietary Supplements*. 2001 [cited; Available from: http://www.cfsan.fda.gov/~dms/ds-oview.html#what.
8. Wagner, H., *Phytomedicine research in Germany.* Environmental Health Perspectives, 1999. **107**(10): p. 779-81.
9. Frauke Gaedcke, B.S., Helga Blasius., *Herbal medicinal products: scientific and reulatory basis for development, quality assurance and marketing authorisation /.* 2003, Stuttgart: Medpharm; Boca Raton: CRC Press.
10. Krueger, R.J., *The Handbook of Clinically Tested Herbal Remedies, Vols. I and II edited by M. Barrett*. Journal of Natural Products. Vol. 68. 2005. 154-155.
11. Moss, J. and C.-S. Yuan, *Herbal medicines and perioperative care.* Anesthesiology FIELD Full Journal Title:Anesthesiology, 2006. **105**(3): p. 441-2.

12. Blendon, R.J., et al., *Americans' views on the use and regulation of dietary supplements.* Arch Intern Med FIELD Full Journal Title:Archives of internal medicine, 2001. **161**(6): p. 805-10.

13. Eisenberg, D.M., et al., *Unconventional medicine in the United States. Prevalence, costs, and patterns of use.* The New England Journal of Medicine, 1993. **328**(4): p. 246-52.

14. Kaufman David, W., et al., *Recent patterns of medication use in the ambulatory adult population of the United States: the Slone survey.* The Journal of the American Medical Association, 2002. **287**(3): p. 337-44.

15. Vaughan, B. and W.J. Keller, *Botanical Dietary Supplements: Quality, Safety and Efficacy. By Gail B. Mahady, Harry H. S. Fong, and Norman R. Farnsworth (University of Illinois at Chicago). Swets & Zeitlinger Publishers, The Netherlands. 2001. v + 271 pp. 17 * 24 cm. $79.50. ISBN 90-265-1855-2*. Journal of Natural Products. Vol. 65. 2002. 949-950.

16. Halsted, C.H., *The ABC Clinical Guide to Herbs: Edited by Mark Blumenthal*. American Journal of Clinical Nutrition. Vol. 79. 2004. 1127-1128.

17. Blumental, M., *Herbal medicine: expanded Commission E monographs*, ed. M. Blumental. 2000.

18. Hammouda, F.M., et al., *Evaluation of the silymarin content in Silybum marianum (L.) Gaertn. cultivated under different agricultural conditions.* Phytotherapy Research, 1993. **7**(1): p. 90-1.

19. Walker, R., *Criteria for risk assessment of botanical food supplements.* Toxicology Letters, 2004. **149**(1-3): p. 187-195.

20. *STATEMENT OF ROBERT E. BRACKETT, PH.D. DIRECTOR, CENTER FOR FOOD SAFETY AND APPLIED NUTRITION*. 2004 [cited; Available from: http://www.fda.gov/ola/2004/dietarysupplements0324.html.

21. Wiesenauer, M., *Drugs for special therapies. Demonstration of the effectiveness of homeopathy drugs.* Fortschr Med FIELD Full Journal Title:Fortschritte der Medizin, 1989. **107**(35): p. 741-2, 752.

22. Srinivasan, V.S., *Considerations in the development of public standards for botanicals and their dosage forms.* Advances in Phytomedicine, 2002. **1**(Ethnomedicine and Drug Discovery): p. 259-265.

23. van Breemen, R.B., H.H.S. Fong, and N.R. Farnsworth, *The Role of Quality Assurance and Standardization in the Safety of Botanical Dietary Supplements.* Chemical Research in Toxicology, 2007. **20**(4): p. 577-582.

24. Bandaranayake, W.M., *Quality control, screening, toxicity, and regulation of herbal drugs.* Modern Phytomedicine, 2006: p. 25-57.

25. Rader Jeanne, I., P. Delmonte, and W. Trucksess Mary, *Recent studies on selected botanical dietary supplement ingredients.* Anal Bioanal Chem FIELD Full Journal Title:Analytical and bioanalytical chemistry, 2007. **389**(1): p. 27-35.

26. Lam, Y.W.F., et al., *Herbal Supplements-Drug Interactions: Scientific and Regulatory Perspectives. [In: Drugs Pharm. Sci., 2006; 162]*. 2006. 332 pp.

27. Khan Ikhlas, A., *Issues related to botanicals.* Life Sci FIELD Full Journal Title:Life sciences, 2006. **78**(18): p. 2033-8.

28. Campbell, M.H., et al., *Variation in Hypericum perforatum L. (St. John's wort) in New South Wales.* Plant Protection Quarterly, 1997. **12**(2): p. 64-66.

29. Southwell, I.A. and M.H. Campbell, *Hypericin content variation in Hypericum perforatum in Australia.* Phytochemistry, 1991. **30**(2): p. 475-8.

30. Hathcock, J., *Botanical Medicine: Efficacy, Quality Assurance, and Regulation, edited by D Eskinazi, M Blumenthal, N Farnsworth, and CW Riggins*. American Journal of Clinical Nutrition. Vol. 71. 2000. 605.

31. Bleadsdell, B., E.O. Espinosa, and M.J. Mann, *Toxic metals in selected traditional chinese medicinals.* Journal of Forensic Science, 1996. **41**: p. 453-456.

32. Farnsworth, N.R., *Relative safety of herbal medicines.* HerbalGram, 1993. **29**: p. 36A-36H.

33. Mclean, A., et al., *The value of bioassay in assessing the quality of botanical products.* Pharmacopoeial Forum 26. Vol. 3. 2000. 857-864.

34. Keledjian, J., et al., *Uptake into mouse brain of four compounds present in the psychoactive beverage kava.* Journal of Pharmaceutical Sciences, 1988. **77**(12): p. 1003-6.

35. Amidon, G.L., et al., *A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability.* Pharmaceutical Research, 1995. **12**(3): p. 413-20.

36. Ioannides, C., *Enzyme Systems that Metabolise Drugs and Other Xenobiotics*. 2002. 608 pp.

37. Ioannides, C., *Pharmacokinetic interactions between herbal remedies and medicinal drugs.* Xenobiotica FIELD Full Journal Title:Xenobiotica; the fate of foreign compounds in biological systems, 2002. **32**(6): p. 451-78.

38. Singh, Y.N., *Kava: an overview.* Journal of Ethnopharmacology, 1992. **37**(1): p. 13-45.

39. Wang, J., et al., *Immunosuppressive activity of the Chinese medicinal plant Tripterygium wilfordii. I. Prolongation of rat cardiac and renal allograft survival by the PG27 extract and immunosuppressive synergy in combination therapy with cyclosporine.* Transplantation, 2000. **70**(3): p. 447-455.

40. Izzo, A.A. and E. Ernst, *Interactions between herbal medicines and prescribed drugs: A systematic review.* Drugs, 2001. **61**(15): p. 2163-2175.

41. Cupp, M.J. and Editor, *Toxicology and Clinical Pharmacology of Herbal Products*. 2000. 325 pp.

42. Blumenthal, M., *Herbs continue slide in mainstream market. Sales down 14 percent.* HerbalGram, 2003. **58**(71).

43. Healing Herbs and Natural Remedies. *Top Selling Herbal Products in the United States*. 2005 [cited; Available from: http://www.herbsandnaturalremedies.com/top-sellers.htm.

44. *Physicians' desk reference for herbal medicines*. 2004, Montvale, N.J.: Medical Economics Co.

45. Bilia, A.R., S. Gallori, and F.F. Vincieri, *Kava-kava and anxiety: growing knowledge about the efficacy and safety.* Life Sciences, 2002. **70**(22): p. 2581-2597.

46. Haensel, R., P. Baehr, and J. Elich, *Isolation and characterization of two new pigments from Pipermethysticum rhizones.* Arch. Pharm., 1961. **294**: p. 739-43.

47. Gruenwald, J. *Kava Kava - Germany, UK To Review Ban*. 2004 [cited; Available from: http://www.newmediaexplorer.org/sepp/2004/04/07/kava_kava_germany_uk_to_review_ban.htm.

48. Gruenwald, J. *German Kava discussion re-opened: by The Executive Director of the International Kava Executive Council*. 2005 [cited; Available from: http://www.cropwatch.org/kava.htm.

49. Singh, Y.N., *Kava: From Ethnology to Pharmacology*. 2004. 176 pp.

50. Musser, S.M., *Kava (Piper methysticum).* Encyclopedia of Dietary Supplements, 2005: p. 373-380.

51. Jellin, J.M., *Natural medicines comprehensive database / compiled by the editors of Pharmacist's Letter, Prescriber's Letter; [Jeff M. Jellin, editor-in-chief].* 7th ed, ed. J.M. Jellin. 2006: Stockton, CA: Therapeutic Research Faculty, [2007], c2006.

52. Tarbah, F., et al., *Kinetics of kavain and its metabolites after oral application.* Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences, 2003. **789**(1): p. 115-130.

53. Mathews, J.M., et al., *Pharmacokinetics and disposition of the kavalactone kawain: Interaction with kava extract and kavalactones in vivo and in vitro.* Drug Metabolism and Disposition, 2005. **33**(10): p. 1555-1563.

54. Kemper, F.H., H. Schmid-Schoenbein, and Editors, *Roekan: Ginkgo biloba EGv 761, Vol. 1: Pharmacology*. 1991. 212 pp.

55. Van Beek, T.A. and Editor, *Ginkgo Biloba. [In: Med. Aromat. Plants--Ind. Profiles., 2000; 12]*. 2000. 548 pp.

56. Fraschini, F., G. Demartini, and D. Esposti, *Pharmacology of silymarin.* Clinical Drug Investigation, 2002. **22**(1): p. 51-65.

57. Halbach, G. and W. Trost, *Chemistry and pharmacology of silymarin. Studies on various metabolites of silybin.* Arzneimittelforschung FIELD Full Journal Title:Arzneimittel-Forschung, 1974. **24**(6): p. 866-7.

58. Morazzoni, P. and E. Bombardelli, *Silybum marianum (Carduus marianus).* Fitoterapia, 1995. **66**(1): p. 3-42.

59. Saller, R., R. Meier, and R. Brignoli, *The use of silymarin in the treatment of liver diseases.* Drugs, 2001. **61**(14): p. 2035-2063.

60. Wagner, H., P. Diesel, and M. Seitz, *Chemistry and analysis of silymarin from Silybum marianum.* Arzneimittel-Forschung, 1974. **24**(4): p. 466-71.

61. Burton, P.S., et al., *Predicting drug absorption: how nature made it a difficult problem.* Journal of Pharmacology and Experimental Therapeutics, 2002. **303**(3): p. 889-895.

62. Bergstroem, C.A.S., *In silico predictions of drug solubility and permeability: two rate-limiting barriers to oral drug absorption.* Basic & Clinical Pharmacology & Toxicology, 2005. **96**(3): p. 156-161.

63. Egan, W.J.L., Georgio., *Prediction of intestinal permeability.* Advanced Drug Delivery Reviews, 2002. **54**(3): p. 273-289.

64. Lipinski, C.A.L., Franco; Dominy, Beryl W.; Feeney, Paul J., *Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings.* Advanced Drug Delivery Reviews, 1997. **23(1-3), 3-25**.

65. *VCCLAB, Virtual Computational Chemistry Laboratory*. 2005.

66. Tetko, I.V.G., J.; Todeschini, R.; Mauri, A.; Livingstone, D.; Ertl, P.; Palyulin, V. A.; Radchenko, E. V.; Zefirov, N. S.; Makarenko, A. S.; Tanchuk, V. Y.; Prokopenko, V. V., *Virtual computational chemistry laboratory - design and description.* J. Comput. Aid. Mol. Des., 2005. **19**: p. 453-463.

67. Kramer, S.D., *Absorption prediction from physicochemical parameters.* Pharmaceutical Science & Technology Today, 1999. **2**(9): p. 373-380.

68. Palm, K., et al., *Correlation of Drug Absorption with Molecular Surface Properties.* Journal of Pharmaceutical Sciences, 1996. **85**(1): p. 32-9.

69. Deanda, F.S., Karl M.; Liu, Jie; Pearlman, Robert S., *GSSI, a General Model for Solute-Solvent Interactions. 1. Description of the Model.* 2004. **1**(1): p. 23-29.

70. Xiang, T.X. and B.D. Anderson, *The relationship between permeant size and permeability in lipid bilayer membranes.* Journal of Membrane Biology, 1994. **140**(2): p. 111-122.

71. van de Waterbeemd, H., *Intestinal permeability: prediction from theory.*, in *Drugs and the Pharmaceutical Sciences, (Oral Drug Absorption: Prediction and Assessment)*, H.L. Jennifer B. Dressman, Editor. (2000), Marcel Dekker, Inc.: Pfizer Central Research, Kent, UK. p. 31-49.

72. Moriguchi, I.H., S.; Liu, Q.; Nakagome, I.; Matsushita, Y. Simple, *Method of Calculating Octanol/Water Partition Coefficient.* Chem.

Pharm. Bull., 1992. **40**: p. 127-130.

73. Moriguchi, I.H., S.; Nakagome, I.; Hirano H., *Comparison of Reliability of log P Values for Drugs Calculated by Several Methods.* Chemical & pharmaceutical bulletin, 1994. **42**(4): p. 976-978.

74. Corrigan, O.I., L.E. Majella, and O.D.M. Caitriona, *The Relationship Between Rat Intestinal Permeability and Hydrophilic Probe Size.* 1996. **13**(10): p. 1554-1558.

75. Avdeef, A., et al., *Drug absorption in vitro model: filter-immobilized artificial membranes. 2. Studies of the permeability properties of lactones in Piper methysticum Forst.* Eur J Pharm Sci FIELD Full Journal Title:European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences, 2001. **14**(4): p. 271-80.

76. Campodonico, A., et al., *Dissolution test for silymarin tablets and capsules.* Drug Development and Industrial Pharmacy, 2001. **27**(3): p. 261-265.

77. Morazzoni, P., et al., *Comparative pharmacokinetics of silipide and silymarin in rats.* Eur J Drug Metab Pharmacokinet, 1993. **18**(3): p. 289-97.

78. Savio, D., P.C. Harrasser, and G. Basso, *Softgel capsule technology as an enhancer device for the absorption of natural principles in humans. A bioavailability cross-over randomised study on silybin.* Arzneimittelforschung, 1998. **48**(11): p. 1104-6.

79. Weyhenmeyer, R., H. Mascher, and J. Birkmayer, *Study on dose-linearity of the pharmacokinetics of silibinin diastereomers using a new stereospecific assay.* Int J Clin Pharmacol Ther Toxicol, 1992. **30**(4): p. 134-8.

80. *Herbal Medicine: Expanded Commission E monographs*. 1st Edition ed. 2000, Newton, MA.

81. Blumenthal, M., *Herb sales down 15% in mainstream market.* HerbalGram, 2001. **51**: p. 69.

82. Pearlman, R.S., *CONCORD User's Manual*: Inc. Tripos, St. Louis, MO.

83. Pearlman, R.S., *CONFORT*: University of Texas, Austin, Texas.

84. Pearlman, R.S., *Partition Coefficient: Determination and Estimation*. 1986: Pergamon Press NY. 3-20;154.

85. Pearlman, R.S., *SAVOL 3, Version 5.0*: University of Texas at Austin, Austin, Texas.

86. Deanda, F., *Development and application of software tools for computer assisted drug design.*, in *Division of Pharmaceutics*. 1999, University of Texas at Austin: Austin, Texas.

87. Tripos, I., *SYBYL 6.8*: 1699 South Hanley Road, St. Louis, MO 63144.

88. Leo, A.H., Corwin; Elkins, David., *Partition coefficients and their uses.* Chemical Reviews, 1971. **71**(6): p. 525-616.

89. Leo, A.H., Corwin., *Comprehensive Medicinal Chemistry*. Vol. 4. 1990: Pergamon, Oxford. 295-319.

90. Shefter, E. and T. Higuchi, *Dissolution behavior of crystalline solvated and nonsolvated forms of some pharmaceuticals.* Journal of Pharmaceutical Sciences, 1963. **52**(8): p. 781-91.

91. Gault, H., et al., *Influence of gastric pH on digoxin biotransformation. II. Extractable urinary metabolites.* Clin Pharmacol Ther FIELD Full Journal Title:Clinical pharmacology and therapeutics, 1981. **29**(2): p. 181-90.

92. Gault, H., et al., *Influence of gastric pH on digoxin biotransformation. I. Intragastric hydrolysis.* Clin Pharmacol Ther FIELD Full Journal Title:Clinical pharmacology and therapeutics, 1980. **27**(1): p. 16-21.

93. Shafer, R.B., R.A. Prentiss, and J.H. Bond, *Gastrointestinal transit in thyroid disease.* Gastroenterology FIELD Full Journal Title:Gastroenterology, 1984. **86**(5 Pt 1): p. 852-5.

94. Avdeef, A., *Absorption and drug development: solubility, permeability, and charge state / Alex Avdeef*. 2003: Hoboken, N.J.: J. Wiley.

95. Benet, L.Z. and Editor, *The Effect of Disease States on Drug Pharmacokinetics*. 1976. 252 pp.

96. Crouthamel, W., et al., *Effect of mesenteric blood flow on intestinal drug absorption.* Journal of Pharmaceutical Sciences, 1970. **59**(6): p. 878-9.

97. Teorell, T., *Transport processes and electrical phenomena in ionic membranes.* Progr. in Biophys. and Biophys. Chemistry, 1953. **3**: p. 305-69.

98. Teorell, T., *Transport phenomena in membranes.* Discussions Faraday Soc., 1956(No. 21): p. 9-26.

99. Rosenberg, T., *Accumulation and active transport in biological systems. I. Thermodynamic considerations.* Acta Chemica Scandinavica, 1948. **2**: p. 14-33.

100. Rosenberg, T., *The concept and definition of active transport.* Symposia of the Society for Experimental Biology, 1954. **8**(Active Transport and Secretion): p. 27-41.

101. Powell, D.W., *Intestinal Water and lectrolyte Transport*. Physiology of the Gastrointestinal Teact, ed. L.R. Johnson. 1977: Raven Press; NY.

102. Hardy., J.G., *Drug delivery to the gastrointestinal tract*, ed. S.S.D. J.G. Hardy, Clive G. Wilson. 1989: New York: Halsted Press.

103. Lowe, P.J., et al., *Hormonal regulation of hepatocyte tight junctional permeability.* American Journal of Physiology, 1988. **255**(4, Pt. 1): p. G454-G461.

104. Ballard, S.T., J.H. Hunter, and A.E. Taylor, *Regulation of tight-junction permeability during nutrient absorption across the intestinal epithelium.* Annual Review of Nutrition, 1995. **15**: p. 35-55.

105. Adson, A., et al., *Quantitative Approaches To Delineate Paracellular Diffusion in Cultured Epithelial Cell Monolayers.* Journal of Pharmaceutical Sciences, 1994. **83**(11): p. 1529-36.

106. Cussler, E.L., *Diffusion, Mass Transfer in Fluid Systems*. 1984. 525 pp.

107. Shore, P.A., B.B. Brodie, and C.A.M. Hogben, *The gastric secretion of drugs; a pH partition hypothesis.* Journal of Pharmacology and Experimental Therapeutics, 1957. **119**: p. 361-9.

108. Schanker, L.S., *Passage of drugs across body membranes.* Pharmacological Reviews, 1962. **14**: p. 501-30.

109. Binns, T.B. and Editor, *Physicochemical Factors in Drug Absorption*. Absorption and Distribution of drugs. 1964. 270 pp.

110. Turner, R.H., C.S. Mehta, and L.Z. Benet, *Apparent directional permeability coefficients for drug ions: in vitro intestinal perfusion studies.* Journal of Pharmaceutical Sciences, 1970. **59**(5): p. 590-5.

111. Ho, N.F.H., W.I. Higuchi, and J. Turi, *Theoretical model studies of drug absorption and transport in the gastrointestinal tract. III.* Journal of Pharmaceutical Sciences, 1972. **61**(2): p. 192-7.

112. Suzuki, A., W.I. Higuchi, and N.F.H. Ho, *Theoretical model studies of drug absorption and transport in the gastrointestinal tract. II.* Journal of Pharmaceutical Sciences, 1970. **59**(5): p. 651-9.

113. Dressman, J.B., H. Lennernas, and Editors, *In Vitro Assessment of Permeability*. Oral Drug Absorption: Prediction and Assessment. [In: Drugs Pharm. Sci., 2000; 106]. 2000. 330 pp.

114. Fogh, J. and Editor, *Human Tumor Cells in Vitro*. 1975. 557 pp.

115. Fogh, J., J.M. Fogh, and T. Orfeo, *One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice.* J Natl Cancer Inst FIELD Full Journal Title:Journal of the National Cancer Institute, 1977. **59**(1): p. 221-6.

116. Pade, V.S., *Prediction of drug transport across the gastrointestinal membrane based on solubility, and permeability measurements in caco-2 cells*. 1996. p. 314 pp.

117. Ucpinar, S.D., *Oligonucleotides and protease inhibitors transport across Caco-2 cell monolayers-permeability effects of dimethylsulfoxide and citicholine*. 2000. p. 288 pp.

118. Charles G. Smith, J.T.O.D., *The process of new drug discovery and development / edited by Charles G. Smith, James T. O'Donnell.*, ed. J.T.O.D. Charles G. Smith. 2006: New York: Informa Healthcare.

119. Stavchansky, S. and V. Pade, *Estimation of relative contribution of thetranscellular and paracellular pathway to the transport of passively absorbed drugs in the Caco-2 cell culture model.* Pharmaceutical Research, 1997. **14**(9): p. 1210-1215.

120. Lin, Y.-C.J.T., Yun K.; Semple, Hugh Alexander; Sloley, Brian Duff., *Establishment of intestinal epithelial cell culture and application in identifying absorbable active ingredients in natural health products.* 2004, PCT Int. Appl.: Kinetana Group Inc., Canada.

121. Ridgway D, L.Y.-C., Sloley BD, et al. *Assessment of SimBioDAS, a new system for in vitro permeability screening [abstract]*. in *American Association of Pharmaceutical Scientists Workshop on Lead Profiling*. 2003. Whippany, NJ.

122. Sloley BD, L.Y.-C., Tawfik S, et al. *Assessment of paracellular permeability in SimBioDAS and Caco-2 using polyethylene glycols (PEGs) as markers. [abstract].* in *American Association of Pharmaceutical Scientists Workshop on Lead Profiling*. 2003. Whippany, NJ.

123. Viswanadhan, V.N., A.K. Ghose, and J.J. Wendoloski, *See page 87 in: Estimating aqueous solvation and lipophilicity of small organic molecules: A comparative overview of atom/group contribution methods.* Perspectives in Drug Discovery and Design, 2000. **19**(Hydrophobicity and Solvation in Drug Design, Pt. 3): p. 85-98.

124. Hammerl, H., O. Pichler, and M. Studlar, *Silymarin action in liver diseases.* Med Klin FIELD Full Journal Title:Medizinische Klinik (1947), 1971. **66**(36): p. 1204-8.

125. Meyer-Burg, J., *Absorption of Silymarin in the rat.* Klin Wochenschr FIELD Full Journal Title:Klinische Wochenschrift, 1972. **50**(22): p. 1060-1.

126. Bulles, H., et al., *Studies of the metabolism and excretion of silybin in the rat.* Arzneimittelforschung FIELD Full Journal Title:Arzneimittel-Forschung, 1975. **25**(6): p. 902-5.

127. Tittel, G. and H. Wagner, *High-performance liquid chromatographic separation of silymarins and their determination in raw extracts of Silybum marianum Gaertn.* Journal of Chromatography, 1977. **135**(2): p. 499-501.

128. Tittel, G. and H. Wagner, *High-performance liquid chromatography of silymarin. II. Quantitative determination of silymarin from Silybum marianum by high-performance liquid chromatography.* Journal of Chromatography, 1978. **153**(1): p. 227-32.

129. Ding, T., et al., *Determination of active component in silymarin by RP-LC and LC/MS.* J Pharm Biomed Anal, 2001. **26**(1): p. 155-61.

130. Quaglia, M.G., et al., *Determination of silymarine in the extract from the dried silybum marianum fruits by high performance liquid chromatography and capillary electrophoresis.* J Pharm Biomed Anal FIELD Full Journal Title:Journal of pharmaceutical and biomedical analysis, 1999. **19**(3-4): p. 435-42.

131. Lee, J.I., M. Narayan, and J.S. Barrett, *Analysis and comparison of active constituents in commercial standardized silymarin extracts by liquid chromatography-electrospray ionization mass spectrometry.* Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences, 2007. **845**(1): p. 95-103.

132. Quercia, V., et al., *Different isomeric and diastereoisomeric products contained in silymarin. New chemical and analytical aspects.* Analytical Chemistry

Symposia Series, 1983. **13**(Chromatogr. Biochem., Med. Environ. Res. 1): p. 1-13.

133. Bilia, A.R., et al., *Stability of the constituents of Calendula, milk-thistle and passionflower tinctures by LC-DAD and LC-MS.* J Pharm Biomed Anal, 2002. **30**(3): p. 613-24.

134. Minakhmetov, R.A., et al., *Analysis of flavonoids in Silybum marianum fruit by HPLC.* Chemistry of Natural Compounds (Translation of Khimiya Prirodnykh Soedinenii), 2002. **37**(4): p. 318-321.

135. Martinelli, E.M., et al., *Liquid chromatographic assay of Silybin in human plasma and urine.* Journal of Liquid Chromatography, 1991. **14**(7): p. 1285-1296.

136. Morazzoni, P., et al., *Comparative bioavailability of Silipide, a new flavanolignan complex, in rats.* Eur J Drug Metab Pharmacokinet, 1992. **17**(1): p. 39-44.

137. Rickling, B., et al., *Two high-performance liquid chromatographic assays for the determination of free and total silibinin diastereomers in plasma using column switching with electrochemical detection and reversed-phase chromatography with ultraviolet detection.* J Chromatogr B Biomed Appl, 1995. **670**(2): p. 267-77.

138. Xiao, Y.S., Yunmei; Chen, Zhipeng; Qineng, Ping., *The preparation of silybin-phospholipid complex and the study on its pharmacokinetics in rats.* International Journal of Pharmaceutics, 2006. **307**(1): p. 77-82.

139. Mascher, H., C. Kikuta, and R. Weyhenmeyer, *Diastereomeric separation of free and conjugated silibinin in plasma by reversed phase HPLC after specific extraction.* Journal of Liquid Chromatography, 1993. **16**(13): p. 2777-89.

140. Skottova, N., et al., *Pharmacokinetic study of iodine-labeled silibinins in rat.* Pharmacol Res, 2001. **44**(3): p. 247-53.

141. Lee, J.I.H., Bih H.; Wu, Di; Barrett, Jeffrey S., *Separation and characterization of silybin, isosilybin, silydianin and silychristin in milk thistle extract by liquid*

chromatography-electrospray tandem mass spectrometry. Journal of Chromatography, A, 2006. **1116**(1-2): p. 57-68.

142. Kim, N.-C., et al., *Complete isolation and characterization of silybins and isosilybins from milk thistle (Silybum marianum).* Organic & Biomolecular Chemistry, 2003. **1**(10): p. 1684-1689.

143. *Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System*. 2000 [cited; Available from: http://www.fda.gov/cder/guidance/3618fnl.htm.

144. Kasim, N.A., et al., *Molecular Properties of WHO Essential Drugs and Provisional Biopharmaceutical Classification.* Molecular Pharmaceutics, 2004. **1**(1): p. 85-96.

145. Koch, H. and G. Zinsberger, *Physicochemical properties of silymarin. II. Solubility parameters of silybin, silydianin, and silychristin.* Archiv der Pharmazie (Weinheim, Germany), 1980. **313**(6): p. 526-33.

146. Fujita, T., J. Iwasa, and C. Hansch, *A new substituent constant, p, derived from partition coefficients.* Journal of the American Chemical Society, 1964. **86**(23): p. 5175-80.

147. Koch, H. and K. Hecht, *Physicochemical properties of silymarin. III: Partition coefficients of silybin, silydianin, and silychristin.* Archiv der Pharmazie (Weinheim, Germany), 1980. **313**(6): p. 533-7.

148. Kim, Y.C.K., E. J.; Lee, E. D.; Kim, J. H.; Jang, S. W.; Kim, Y. G.; Kwon, J. W.; Kim, W. B.; Lee, M. G. (2003), 41(12), 593-596. Publisher, *Comparative bioavailability of silibinin in healthy male volunteers.* International Journal of Clinical Pharmacology and Therapeutics, 2003. **41**(12): p. 593-596.

149. Arcari, M., et al., *New silibinin-b cyclodextrin inclusion complex: In vitro dissolution rate and in vivo absorption in comparison with standard formulations.* Bollettino Chimico Farmaceutico, 1992. **131**(5): p. 205-9.

150. Barzaghi, N., et al., *Pharmacokinetic studies on IdB 1016, a silybin-phosphatidylcholine complex, in healthy human subjects.* European Journal of Drug Metabolism and Pharmacokinetics, 1990. **15**(4): p. 333-8.

151. Filburn, C.R., R. Kettenacker, and D.W. Griffin, *Bioavailability of a silybin-phosphatidylcholine complex in dogs.* J Vet Pharmacol Ther FIELD Full Journal Title:Journal of veterinary pharmacology and therapeutics, 2007. **30**(2): p. 132-8.

152. Park, M.S., N.C. Yu, and K.H. Kim, *Clinical pharmacokinetic profiles of Hanmi SMEDDS silymarin soft capsule preparation.* Journal of Applied Pharmacology, 2000. **8**(3): p. 269-275.

153. Wu, W.W., Yang; Que, Li., *Enhanced bioavailability of silymarin by self-microemulsifying drug delivery system.* European Journal of Pharmaceutics and Biopharmaceutics, 2006. **63**(3): p. 288-294.

154. Yanyu, X., et al., *The preparation of silybin-phospholipid complex and the study on its pharmacokinetics in rats.* Int J Pharm FIELD Full Journal Title:International journal of pharmaceutics, 2006. **307**(1): p. 77-82.

155. Xiao, Y., et al., *Preparation of silymarin proliposomes and its pharmacokinetics in rats.* Yaoxue Xuebao, 2005. **40**(8): p. 758-763.

156. Schulz, H.U., et al., *The solubility and bioequivalence of silymarin preparations.* Arzneimittelforschung FIELD Full Journal Title:Arzneimittel-Forschung, 1995. **45**(1): p. 61-4.

157. Janiak, B., et al., *Effect of silymarine on contents and functions of some microsomal liver enzymes influenced by carbon tetrachloride or halothane.* Arzneimittel-Forschung, 1973. **23**(9): p. 1322-6.

158. Lorenz, D., et al., *Pharmacokinetic studies with silymarin in human serum and bile.* Methods and Findings in Experimental and Clinical Pharmacology, 1984. **6**(10): p. 655-61.

159. Bachner-Jaschke, I. and H. Koch, *Binding of silymarin substances to plasma proteins and synthetic polymers.* Archiv der Pharmazie (Weinheim, Germany), 1979. **312**(11): p. 954-8.

160. Koch, H.P. and W.A. Ritschel, *Bioavailability of silymarin. I: Volumes of distribution of Silybin, Silydianin, and Silychristin from in vitro data.* Archiv der Pharmazie (Weinheim, Germany), 1981. **314**(6): p. 515-17.

161. Mennicke, W., *What is known about the metabolism and pharmacokinetics of silymarin?* Symp. Pharmacodyn. Silymarin, [Lect.], 1976: p. 98-102.

162. Sonnenbichler, J., J. Mattersberger, and G. Hanser, *Studies on the mechanism of silybin action. III. Resorption of the flavonolignane derivative silybin into rat liver cells.* Hoppe-Seyler's Zeitschrift fuer Physiologische Chemie, 1980. **361**(11): p. 1751-6.

163. Flory, P.J., et al., *Studies on elimination of silymarin in cholecystectomized patients. I. Biliary and renal elimination after a single oral dose.* Planta Medica, 1980. **38**(3): p. 227-37.

164. Sato, T., *New method for measurement of hepatic blood flow in the rat using thermodilution method.* Circulatory shock, 1987. **21**(1): p. 31-7.

165. Tozer, M.R.T., *Clinical pharmacokinetics: concepts and applications: Pages 161-170*. 1995.

Vita

Devendra Shrikant Pade was born in Mumbai, India, on October 20, 1972, the son of Shrikant and Jaya Pade. After completing his High School education at St. Teresa's High School, Mumbai, India in 1988, he entered The Bhavan's College, Mumbai, India for his Higher Secondary education. He continued at The Bhavan's College and graduated with a B.Sc. in Chemistry in 1993. Then he entered the University Department of Chemistry, University of Mumbai, India and graduated with a M.Sc. in Physical Chemistry in 1996. He then worked in USV Limited, a generic pharmaceutical company as an Analytical Research and Development Trainee until 1997 and as an Analytical Research and Development Executive until July 2000. In August 2000, he joined the graduate program of the College of Pharmacy at the University of Texas at Austin. During the course of his graduate program, he was awarded the United States Pharmacopoeia's Pre-Doctoral Fellowship for the year 2001-2003 and 2004-2005. During his graduate career, he presented his research at a number of national scientific meetings. He also worked in collaboration with The Laboratory for Molecular Graphics and Theoretical Modeling of the College of Pharmacy at the University of Texas at Austin and Kinetana Inc. Alberta, Canada. He also held the position of a Teaching Assistant in the College of Pharmacy and the Department of Chemistry at the University of Texas at Austin from 2001-2007.

Permanent address: 19 Plum Hill Rd, East Lyme, CT 06333

This dissertation was typed by the author.