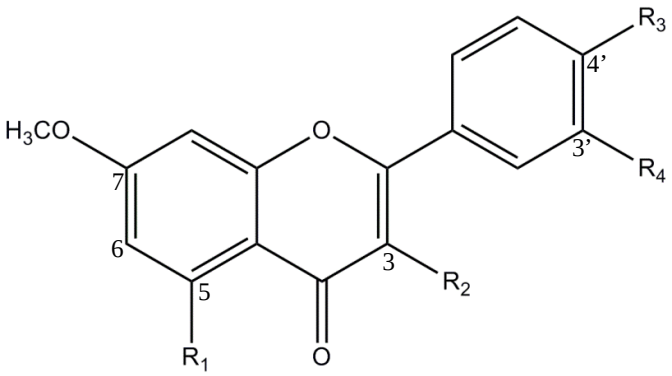
CHAPTER II
LITERATURE REVIEWS

1. KP and its chemical constituents

KP or Krachaidum in Thai belongs to family Zingiberaceae. It has been found in the area of Southeast Asia. It mostly locates in mountain regions of the North and Northeast of Thailand, especially in Loei, Petchaboon, and Phitsanulok. It has been long used as herbal medicine for centuries. Its rhizomes are part of plant that has been used for treatment of diseases. It is an annual crop that grows up to 90 cm tall in March to May. The upper and lower of leaves have dark green and light green colors (Figure 2A). Its stems known as rhizomes are tuber with black to purple inside and brown outside (Figure 2B). Flowers which are the zygomorphic symmetry solitary have pink and purple color with white or purple colors of labetum as presented in Figure 2C (Putiyanan et al., 2008). It contains flavonoids (Sutthanut et al., 2007), phenolic glycosides, and lipids (Azuma et al., 2008). Recently, Chaipech et al. (2011) reported the novel compound in methanolic extract of KP including flav-3-en-3-ol glycosides (kaempferiaosides C and D), and acetophenone glycosides (Kaempferiao sides E and D). However, the flavonoids are the major component especially methoxyflavones including 5-hydroxy-7-methoxyflavone, 5-hydroxy-3,7-dimethoxy flavone, DMF, 3,5,7-trimethoxyflavone, 5-hydroxy-3,7,4'-trimethoxyfla vone, 5-hydroxy-7,4'-dimethoxyflavone, 5-hydroxy-3,7,3',4'-tetramethoxyflavone, TMF, 3,5, 7,4'-tetramethoxyflavone, 5,7,3',4'-tetramethoxyflavone, and PMF, as shown in Figure 3. The major substances are DMF, TMF, and PMF which distribute at high amount in the plant. The flavonoids constituents were identified and quantified by using Gas Chromatography that composed of Flame Ionized Detection, $HP50^{+}$ column crosslink 50% PH ME siloxane length 30 m x diameter 0.32 mm. 0.15 μm film thicknesses, nitrogen as the carrier gas, and chlorpheniramine maleate as the internal standard (Sutthanut et al., 2007). There are many dosage forms that are available in markets including pill, tablet, alcohol extract solution, tea, lozenge, and wine. Moreover, spray, gel, capsule, effervescent tablet, and solid-liquid nanoparticle transdermal drug delivery system were developed in our research team.

Figure 2 A: KP plant, B: KP rhizome, C: KP flower



Substances	R_1	R_2	R_3	R_4
5-OH-7-methoxyflavone	OH	H	H	H
5-OH-3,7-dimethoxyflavone	OH	OCH_3	H	H
DMF	OCH_3	H	H	H
3,5,7-trimethoxyflavone	OCH_3	OCH_3	H	H
5-OH-3,7,4'-trimethoxyflavone	OH	OCH_3	OCH_3	H
5-OH-7,4'-dimethoxyflavone	OH	H	OCH_3	H
5-OH-3,7,3',4'-tetramethoxyflavone	OH	OCH_3	OCH_3	OCH_3
TMF	OCH_3	H	OCH_3	H
3,5,7,4'-tetramethoxyflavone	OCH_3	OCH_3	OCH_3	H
5,7,3',4'-tetramethoxyflavone	OCH_3	H	OCH_3	OCH_3
PMF	OCH_3	OCH_3	OCH_3	OCH_3

Figure 3 Chemical structures of substances in KP

2. Pharmacological activities of KP

KP has claimed to be aphrodisiac, anti-inflammation, hypnotics, inducing the appetite, health promoting, anti-peptic ulcer, and improving the blood circulation. At present, several pharmacological effects of this plant and its major components were proved.

2.1 Anti-inflammation

Sae-wong et al. (2009) reported anti-inflammatory effect by using carrageenan-induced inflammation process in rat and mRNA expression of iNOS and COX-2 assay. Chloroform, hexane, ethanol, ethyl acetate, and water fraction (dose 150 mg/kg) decreased paw edema at 3-5 h. Ethanol fraction and 5-hydroxy-3,7,3',4'-tetramethoxyflavone suppressed mRNA expression of iNOS in dose-dependent manners and they partly affected to mRNA expression of COX-2. 5-hydroxy-3,7,3',4'-tetramethoxyflavone inhibited several inflammatory mediators, i.e., nitric oxide, PGE_2, and TNF-α expressions in RAW264.7 macrophage cells (Tewtrakul and Subhadhirasakul, 2008).

The methanolic (70%) extract of KP showed the inhibition on xanthine oxidase. Moreover, its constituents, especially PMF and 5,7,3'4'-tetramethoxyflavone were the potent compounds (Nakao et al., 2011). The results suggested the anti-hyperuricemia effect of KP.

KP ethanolic extracts inhibited indomethacin-induced, hydrochloric acid/ethanol-induced, and restraint water immersion stress-induced gastric ulcers. It increased amount of gastric mucus content in hydrochloric acid/ethanol-induced gastric ulcers at doses of 60 and 120 mg/kg BW. These results concluded that it partly involved in a preservation of gastric mucus secretion but not inhibited the gastric acid secretion (Rujjanawate et al., 2005).

2.2 Aphrodisiac effect

Sudwan et al. (2006) demonstrated the effect of KP on sexual activity of male rats. They found that all doses of KP (60, 120, and 240 mg/kg BW) did not produce any significant change in courtship behavior, mount frequency, intromission frequency, mount latency (the time interval between the introduction of female into the cage until the first mount), or intromission latency. However, the courtship behavior (time units) in the first 10 min of observation of male rats receiving 240

mg/kg BW was significantly lower than the control group. Chaturapanich et al. (2008) showed that ethanol extract of KP significantly reduced both of mount latency and ejaculation latency (the time taken from the introduction of female into the cage until ejaculation). Whereas, hexane and water extracts had no influence on any sexual behavior parameters. These extracts had no effect on fertility or sperm content. Ethanol extract significantly increased spermatic blood flow without changing the heart rate and the mean arterial blood pressure, suggesting that KP induced a local vasodilation effect. Intravenous route of KP at dose of 10, 20, and 40 mg/kg BW caused increasing in blood flow to the testes in dose-dependent manner. Chaturapanich et al. (2008) concluded that ethanol extract had an aphrodisiac activity probably via a marked increase in blood flow to the testes. The effect of extract on blood vessel was also studied by Wattanapitayakul et al. (2007). They found that ethanol extract of KP at dose of 10 μg/ml significantly increase endothelial nitric oxide production in human umbilical vein endothelial cells. The extract at dose of 1 and 10 μg/ml significantly increased eNOS mRNA. The activation of eNOS gene expressed as early at four h and plateau at 48 h. However, the extract also enhanced eNOS protein expression. Nitric oxide plays a significant role in maintaining normal vascular function, preventing cardiovascular disease, and dilation of blood vessel. Jitjaingam et al. (2005) showed that KP tea did not affect on testicular and prostate gland weights of rats at doses of 60 and 120 mg/kg BW, while it significantly increased weights of seminal vesicle at dose of 120 mg/kg BW more than those of control group. Furthermore, the extract increased cauda epididymal sperm density and diameter of seminiferous tubules higher than those of control group at doses of 60 and 120 mg/kg BW. Recently, Wattanathorn et al. (2012) reported the effect of KP on sexual behaviors. The extract at dose of 200 mg/kg BW can increase frequency of mounting, ejaculation, and intromission but decrease latency of all sexual behaviors in stress aging rats (18 weeks old). Moreover, they found that dopaminergic function in hypothalamus played an important role in improvement of sexual dysfunction of KP.

2.3 Antimicrobial

Kummee et al. (2008) studied antimicrobial activity by using disk diffusion method. Ethanol extract showed no activities against *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermis* TISTR 518, *Enterococcus faecalis* TISTR

459, *Escherichia coli* ATCC 25922, and *Candida albicans* TISTR 5779. It showed activities against dermatophytes including *Trichophyton rubrum* SH-MU-2, *Trichophyton mentagrophytes* SH-MU-3, and *Microsporum gypsum* SH-MU-4 in agar disc dilution method at MIC of 62.5, 125, and 250 µg/ml, respectively. TMF and 5,7,3',4'-tetramethoxyflavone showed anti-plasmodial, anti-fungal, and anti-mycobacterial activity by using microdilution radioisotope technique to *Plasmodium falciparum*, modified formazan assay for *Candida albicans*, and micro alamar blue assay to *Mycobacterium tuberculosis*, respectively (Yenjai et al., 2004). DMF also showed anti-fungal and anti-mycobacterial activity with higher activity than 5,7,3',4'-tetramethoxyflavone as showed in lower IC_{50} (Yenjai et al., 2007). Moreover, KP also showed virucidal activity in dose-dependent manner and directly inactivated dengue virus type 2 particles before virus infection using plaque reduction assay (Phurimsak and Leardkamolkarn, 2005).

2.4 Cardioprotection

Malakul et al. (2011a) reported that KP ethanolic extract was able to inhibit the influx of extracellular Ca^{2+} to the cells and it increased the vasorelaxant activity of acetylcholine in oxidative stress conditions in rat isolated hearts resulting in the vascular relaxation. Furthermore, the authors proved that KP extract reduced the superoxide levels in rat aorta. These finding suggested the cardioprotective properties of KP extract. They also reported that KP ethanolic extract affected on superoxide generation, increased nitrite levels in aorta of diabetic rats, and enhanced the vasorelaxant activity of acetylcholine (Malakul et al., 2011b). These results implied that KP acted as cardioprotective agent both in normal condition and diabetes. Moreover, there was the study regarding the mechanism of vasorelaxation effect of the major compounds, DMF. DMF acted through NO-cGMP and cyclooxygenase pathways. It also enhanced the efflux of K^+ and inhibited Ca^{2+} influx from the extracellular providing the vasodilation (Tep-areenan et al., 2010). This finding proved that KP may be used as antihypertension.

2.5 Anti-allergic

Tewtrakul et al. (2008) reported anti-allergic activity of compounds from KP. 5-hydroxy-3,7,3',4'-tetramethoxyflavone had higher anti-allergic activity

than 5-hydroxy-7-methoxyflavone, and 5-hydroxy-7,4'-dimethoxyflavone with IC_{50} at 8.0, 20.6, and 26.0 μmol/L, respectively.

2.6 Antiobesity

Akase et al. (2011) reported the antiobesity effect of KP powder in obese diabetis mice. The feeding of 1% and 3% of KP powder in mouse foods reduced mouse body weight in dose-dependent manner. In addition, the powder also decrease visceral fat, total cholesterol, triglyceride, insulin, and blood glucose in glucose tolerance test.

2.7 Neuropharmacological activities

The compounds from KP showed anti-cholinesterase activity affecting both AChE and BChE. TMF and DMF showed the most potent compounds. 3,5,7,4'-tetramethoxyflavone, PMF, and 5-hydroxy-7,4'-dimethoxyflavone showed low activities. These results may provide the novel therapeutic agents for Alzheimer's disease (Sawasdee et al., 2009). Wattanathorn et al. (2007) also reported that KP can decrease immobility time in forced swimming test in aged rats with no sedative effects and also produce antidepressant like activity at dose of 100 mg/kg BW after seven days of treatment.

Hawiset et al. (2011) also reported the effect of ethanolic extract of KP on neuropharmacological activities. Rats were administered of 150, 200, and 250 mg/kg BW of KP crude extract for one day, 1 week, and 2 weeks, the immobility time decreased and the swimming time increased in force swimming test suggesting anti-depression activity. Furthermore, all dosage regimens improved spatial learning and memory by increase retention times and decrease escape latency similar to that of Donepezid (cholinesterase inhibitors) in Morris water maze test. Accordingly, KP can be further applied in depression condition and improvement of learning and memory.

2.8 Anticancer

KP also affected on cancer cells including cholangiocarcinoma cells, human bile duct cancer cells (HuCCA-1 and RMCCA-1), HepG2 cells, and human promyelocytic leukemic cells (HL-60). Tiamyuyen and Leardkamolkarn (2005) reported the ability of ethanol and hexane extracts to inhibit growth of HuCCA-1 cells in dose-dependent manner. Both of extracts exhibited cell invasion inhibition and induced nuclear fragmentation that showed the cells apoptosis induction.

Leardkamolkarn et al. (2009) also showed that ethanol extract and TMF changed the cellular morphology, caused HuCCA-1 and RMCCA-1cells death, and reduced rHHGF-induced cell invasion studied by using matrix-coated Transwell plates. TMF exhibited progressive action via caspase-3 mitochondrial enzyme activation enhancing cellular toxicity in a time-dependent manner. KP suppressed HL-60 cell growth and decreased cell viability in dose- and time-dependent manner. Apoptosis induced by KP ethanolic extract was enhanced by treatment with paclitaxel or doxorubicin (Banjerdpongchai et al., 2008). In addition, DMF has an effective chemoprotectant in chemical-induced liver cancer. It inhibited benzo[*a*]pyrene (BaP)-induced DNA binding. BaP is a prototypical environmental polycyclic aromatic hydrocarbons that mainly metabolize by CYP1A1/1B1, and then covalently binds to cellular DNA to start the carcinogenic process. DMF also inhibited BaP-induced CYP1A1 enzyme activity, CYP1A1 protein expression, and CYP1A1 mRNA level (Wen et al., 2005).

2.9 Others

Dichloromethane and ethyl acetate extract of KP showed the inhibitory effect on α-glucosidase. 5,7,3'4'-tetramethoxyflavone played the important role as an α-glucosidase inhibitor, then followed by TMF, and PMF. In addition, the extracts had the antimutagenicity properties which the highest effects were found in DMF, 5,3'-dihydroxy-3,7,4'-trimethoxyflavone, 3,5,7-trimethoxyflavone, and 5-hydroxy-7-methoxyflavone (Sripanidkulchai et al., 2004, Azuma et al., 2011).

In addition, KP can inhibit P-glycoprotein function, which plays an efflux of drug, cause drug resistance and decrease oral bioavailability. Ethanol fraction and PMF increased the accumulation of rhodamine 123 and daunorubicin, which are P-glycoprotein substrates (Patanasethanont et al., 2007a). Ethanol and aqueous extracts of KP also inhibited multidrug resistance associated-protein (MRP). The highest activity showed in ethanol extract. DMF and PMF can inhibit MRP function. The extracts and compounds increased the accumulation of anticancer drug, doxorubicin. It suggested that KP extract and its compounds can decrease anticancer drug resistance (Patanasethanont et al., 2007b).

3. Toxicity of KP

Sae-wong et al. (2009) showed that a single oral administration of KP ethanolic extract at dose of 2 g/kg BW did not produce any clinical sign of toxicity including convulsion, hyperactivity, sedation, respiratory depression, and loss of righting reflex. Sudwan et al. (2006) reported a non-significant difference among BW gain of the male rats receiving KP (50% ethanol extraction) at doses of 60, 120, 240 mg/kg BW, and the control group. Complete blood count (CBC) in all treatment groups were not difference from those of the control. Hemoglobin of male rats receiving KP extract at dose of 60 mg/kg BW significantly lower than the control and the highest dose treated group. There were no significant difference in alanine transaminase enzyme (ALT), blood urea nitrogen (BUN), and creatinine (Cr). Aspartate aminotransferase enzyme (AST) of male rats receiving KP at doses of 60 and 240 mg/kg BW were significantly lower than that of the control group. Gross or microscopic findings of kidneys revealed no association with KP treatment, while the male rats receiving KP had vacuolar cell hypertrophy in the liver but not significance. Jitjaingam et al. (2005) also reported the safety of KP tea in rats. They found that it had no effect on kidney function and hematologic system, which indicated by normal level of BUN and Cr and hematological level, respectively. Toxicological study of KP as presented by Chivapat et al. (2004) demonstrated the safety of KP powder in mice with LD_{50} more than 13.3 g/kg BW. Single oral administration of KP at dose of 13.3 g/kg BW, did not show gross and histological lesion changes of the vital organs of mice. For chronic toxicity test, female and male rats were orally administered with 20, 200, 1,000, and 2,000 mg/kg BW of KP powder for six months. The animal's behavior, physical examination, and histological examination did not change even at the highest dose. However, liver weight of rats treated with 2,000 mg/kg BW of KP significantly increased more than the control group. The CBC values in all doses of KP treated groups were in normal range. The electrolyte levels in blood of KP treated groups were in normal levels. Nevertheless, the cholesterol value in female rats that were received 2,000 mg/kg BW of KP showed higher level than control group but not significance. Moreover, Chivapat et al. (2010) reported the safety of KP ethanolic extract when administration of 5, 50, and 500 mg/kg BW/day to male and female rats for six months. At highest dose, male rats had significantly increases in body weight

at week eight until the end of treatment. Male rats treated with 500 mg/kg BW of KP ethanolic extract had lower triglyceride than that of the control group. Glucose and total cholesterol values of female rat received the highest dose of KP ethanolic extract were higher than those of controls groups. However, the CBC, histological of visceral organs, and clinical signs of toxicity in all dosage regimens did not change for six months. In summary, a single oral administration of KP ethanolic extract at dose of 2 g/kg BW was safe. KP powder was safe in rats that treated up to dose of 1 g/kg BW for six months and it also showed high LD_{50} at 13.3 g/kg BW in mice.

4. Pharmacokinetics

4.1 Definition

Pharmacokinetic is the description of the movement of the drug within the body (Figure 4). After a drug is released from its dosage form, the drug is absorbed into the blood circulation and distributed to the tissue. The fraction of drug may be metabolized in tissue to metabolite forms. Finally, the parent drug and/or its metabolite forms are excreted out via feces and/or urine. Therefore, pharmacokinetic associates with the processes of drug absorption, drug distribution to the tissues of the body, and drug elimination from the body by metabolism or excretion or both (Na-Bangchang and Wernsdorfer, 2001).

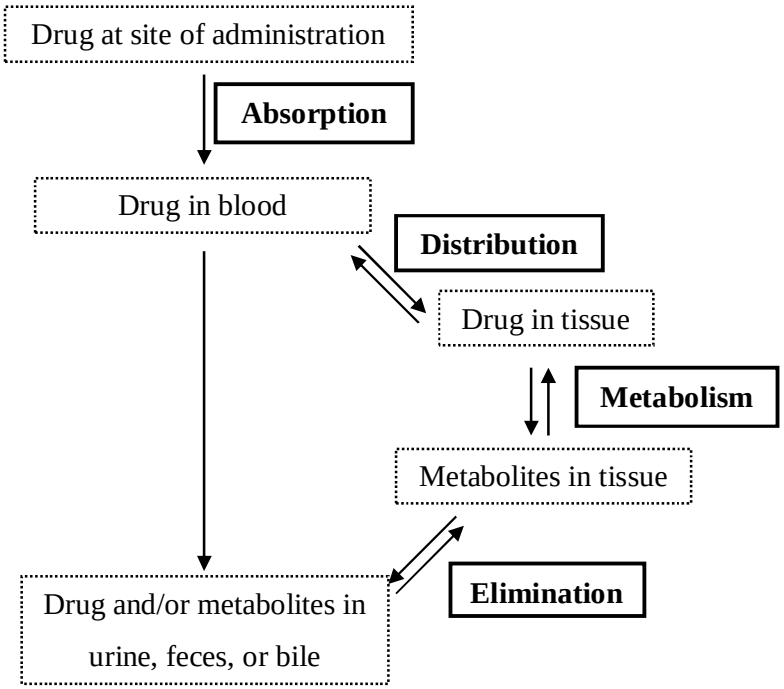


Figure 4 Pharmacokinetic process

The study of pharmacokinetics involves in both experimental and theoretical approaches. The experimental aspect involves the development of biological sampling techniques, analytical methods for measurement of drugs and metabolites, and processes that facilitate data collection and manipulation. The theoretical aspect involves the development of pharmacokinetic models to predict what the body does to the drug after administration. The application of statistics is an integral part of pharmacokinetic studies. Statistical methods are used for pharmacokinetic parameters estimation and data interpretation ultimately for the purpose of designing and predicting optimal dosing regimens for individuals or groups of patients (Shargel et al., 2005).

Pharmacokinetics provides the valuable information for utilization of drugs. There are many benefits of pharmacokinetic data including prediction of plasma, tissue, and urine drug levels with any dosage regimen, calculation of the optimum dosage regimen for each patient individually, evaluation of the possible accumulation of drugs and/or metabolites, correlation of drug concentrations with

pharmacological or toxicological activity, estimation of the rate and the extent of drug absorption, determination of how changes in physiology or disease affect the absorption, distribution, metabolism, and excretion of the drug, and explanation of the drug interaction (Shargel et al., 2005).

Moreover, in order to achieve the pharmacokinetic data, order of reactions and compartment models should be concerned.

4.1.1 Rates and orders of reactions

The pharmacokinetic processes are described in terms of the rate or velocity at which they occur. The order of a reaction refers to the way in which the concentration of drug or reactant influences the rate of a chemical reaction or process. There are zero-order reactions and first-order reactions (Na-Bangchang and Wernsdorfer, 2001).

a) Zero-order reactions

These imply a constant rate of appearance or disappearance of drug into or from the blood circulation, regardless of its concentration. With a few drugs, absorption or elimination is saturated even at low concentration and as a result, their concentrations in blood increase or decrease in a linear fashion over time (Na-Bangchang and Wernsdorfer, 2001).

b) First-order reactions

These reactions imply that the rate of appearance or disappearance of drug into or from the blood is proportion to the concentration. The result is that drug concentrations in blood increase or decrease exponentially with time. Drugs with first-order absorption or elimination have a characteristic half-life which is constant regardless of the amount in the body. Most of drugs perform first-order reactions (Na-Bangchang and Wernsdorfer, 2001).

4.1.2 Compartment models

Pharmacokinetic models may be used to represent drug distribution and elimination in the body. Ideally, a model should mimic closely the physiologic processes in the body. In practice, models seldom consider all the rate processes ongoing in the body and are therefore simplified mathematical expressions. The inability to measure all the rate processes in the body, including the lack of access to biological samples from the interior of the body, limits the sophistication of a

model. Compartment models are classical pharmacokinetic models that simulate the kinetic processes of drug absorption, distribution, and elimination. The models can be divided into two models: one-compartment model and multi-compartment model.

a) One compartment model

This offers the simplest way to describe the process of drug distribution and elimination in the body. The model assumes that the drug can enter or leave the body. The body acts like a single and uniform compartment (Figure 5). The simplest route of drug administration from a modeling perspective is a rapid intravenous injection. The simplest kinetic model that describes drug absorption and elimination in the body is to consider that the drug is administered all at once into a box, or compartment, and that the drug distributes instantaneously and homogenously throughout the compartment. Drug elimination also occurs from the compartment immediately after administration. One-compartment model is popularly to describe the drug candidate in drug development (Shargel et al., 2005).

b) Multi-compartment model

Two-compartment model explains many drugs that demonstrate a plasma concentration-time profile that does not decline as a single exponential process (Figure 6A). The plasma concentration-time profile for a drug that follows a two-compartment model shows that the plasma concentration declines bi-exponential that refers from drug distribution and drug elimination process (Figure 6B). A drug that follows the pharmacokinetics of a two-compartment model dose not equilibrates rapidly throughout the body, as is assumed for one-compartment model. In two-compartment model, the drug distributed into two compartments: the central compartment and the tissue or peripheral compartment (Figure 7). The central compartment represents the blood, extracellular fluid, and highly perfused tissues. The highly perfused tissues are heart, brain, liver, and kidney. The drug distributes rapidly and uniformly in the central compartment. A second compartment, known as the tissue or peripheral compartment, contains tissues in which the drug equilibrates more slowly. The slowly perfuse tissues are bone, tendons, ligaments, teeth, and hair (Shargel et al., 2005). Multi-compartment models do not only compose of two-compartment model, but also three-compartment model is available in certain drugs.

The three-compartment model is complicated, and therefore does not use in drug development.

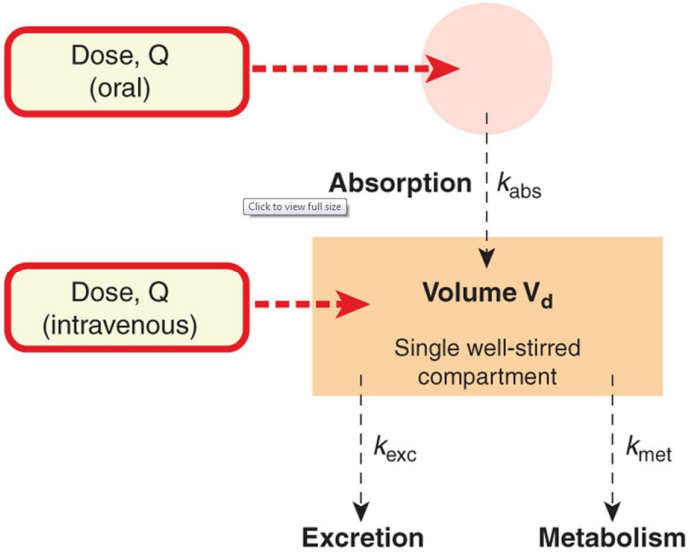


Figure 5 One-compartment model (Rang et al., 2003)

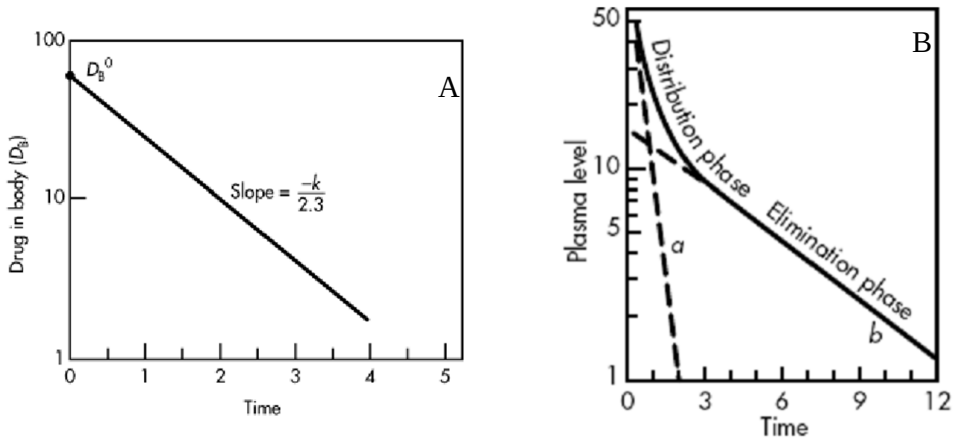


Figure 6 The plasma concentration-time profiles, A: one-compartment, B: two-compartment (Shargel et al., 2005)

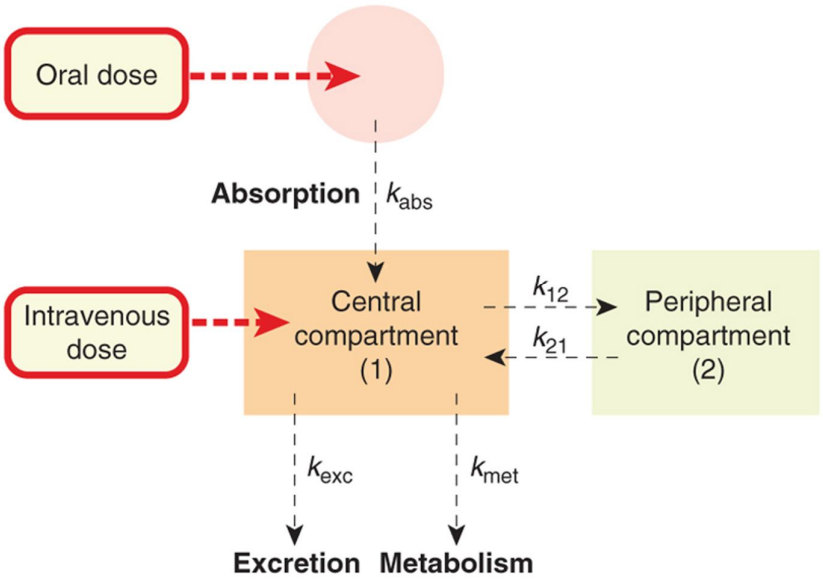


Figure 7 Two-compartment model (Rang et al., 2003)

4.2 Absorption

4.2.1 Definition

Drug absorption is a prerequisite process for all drugs which are administered extravascularly and intended for systemic effect. Most drugs are orally taken; typically, about 75% of a drug is absorbed within 1-3 hours which is mainly at small intestine (Na-Bangchang and Wernsdorfer, 2001).

4.2.2 Mechanism

The uptake of drug across the intestinal membrane can occur via many mechanisms including passive transport, facilitated transport, active transport, and vesicular transport.

a) Passive transport

It is a process which molecule diffuses through paracellular or transcellular routes (Figure 8A and 8B, respectively) according to concentration gradient. The diffusion of molecule originates from a region of higher concentration to a region of lower concentration. Therefore, this transport is not involved in an energy and carrier which affect the non-saturation diffusion. Passive transport is the major way of absorption across the gastrointestinal membrane of most drugs. Several factors can influence the rate of passive diffusion of drugs. These

factors include physicochemical properties of drugs and physiological properties of intestinal membrane: lipophilicity, ionization, molecular weight, configuration, surface area of the membrane, blood perfusion, and protein binding of drugs. The properties of drug including unbound form, almost lipophilicity, unionized form, and small size tend to easily cross the membrane (Na-Bangchang and Wernsdorfer, 2001).

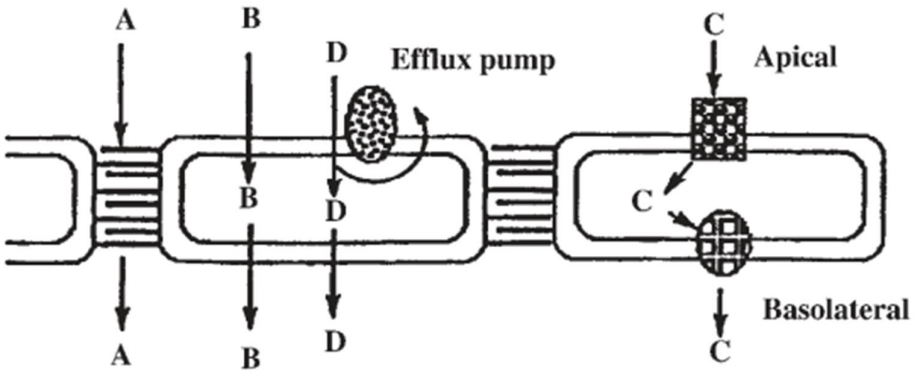


Figure 8 Schematic drawing of mechanism of drug absorption, A: passive diffusion via paracellular route, B: passive diffusion via transcellular route, C: carrier-mediated, D: efflux transport (Borchardt, 2001)

b) Facilitated transport

It does also not need energy for transportation across the membrane but it needs a carrier (Figure 8C). The process is saturable, structurally selective for the drug and shows competition kinetics for drugs of similar structure (Na-Bangchang and Wernsdorfer, 2001).

c) Active transport

It is the mechanism that requires energy and carrier protein (Figure 8C). An energy-consuming process characterizes the transport of drug against a concentration gradient. Active transport requires a carrier molecule which binds the drug to form a carrier-drug complex. This complex shuttles the drug across the membrane and then dissociates the drug on the other side of the membrane. Drugs with similar structure may compete for sites of absorption on the carrier, and moreover, the mechanism may become saturated at high drug concentration due to a limited amount of carrier molecules. This process plays an importance role in the

renal and biliary excretion of drugs and metabolites (Na-Bangchang and Wernsdorfer, 2001).

d) Vesicular transport

It is the process by which the drugs especially the large molecule mass are engulfed by the cell. It can be divided to two types namely pinocytosis and phagocytosis. Pinocytosis refers to the process of engulfment of small molecules or fluids whereas phargocytosis refers to the process of engulfment of larger molecules or macromolecules (Na-Bangchang and Wernsdorfer, 2001).

4.2.3 Blood concentration-time profile

Drug has to be absorbed from the site of administration to blood circulation, and then distributed into the tissues to produce its pharmacological activity. The rate and extent of this absorption process are important to determine the onset and the intensity of drug effect. The rate of absorption influences the rate at which the drug appears in the blood circulation after drug administration. The extent of drug absorption represents the fraction of the administered dose that reaches the blood circulation and is a measure of the drug bioavailability. Rapid drug absorption results in faster onset of drug effect. Larger extent of drug absorption (higher bioavailability) results in higher plasma drug concentration, which produces more intense effect (Shargel et al., 2005).

After administration of oral drug, blood samples are withdrawn and the blood assayed for drug concentration at specific periods of time after administration. This enables a plasma concentration-time curve to be constructed, as shown in Figure 9. At zero time, when the drug is first administered, the drug concentration in plasma will be zero. As the drug passes into gastrointestinal tract, it disintegrates and dissolves. Drug absorption occurs. Initially the drug concentration in plasma rises as the rate of absorption, the drug is being removed by distribution following by elimination. Concentrations continue to rise until a maximum or peak is attained. This represents the highest concentration of drug achieve in the plasma following the administration of a single dose, often termed the Cmax. It is reached when the rate of removal by distribution and elimination. The ascending portion of the plasma concentration-time profile is sometimes called the absorption phase. After the ascending portion, the descending portion occurs. The early descending portion of the

curve can thus reflect the net results of drug absorption, distribution, metabolism, and excretion. In this phase the rate of drug removal from the blood exceeds the rate absorption and therefore the concentration of the drug in plasma declines. This is sometimes called the distribution and elimination phase. Several parameters based on the plasma concentration-time profile that are Cmax, time to maximum concentration (Tmax), and area under the curve (AUC) (Ashford, 2007).

a) Cmax

It represents the highest concentration of the drug achieved in the plasma (Ashford, 2007). A higher Cmax indicates a faster rate or larger extent of drug absorption. For drugs that have a direct relationship between the plasma concentration and the pharmacological response, Cmax is an indication of the intensity of the therapeutic effect (Hedaya, 2007).

b) Tmax

This is the period of time required to achieve the maximum concentration of drug after the administration of a single dose. This parameter relates to the rate of absorption of the drug and uses to assess that rate (Ashford, 2007). A shorter Tmax usually indicates a faster rate of drug absorption (Hedaya, 2007).

c) AUC

This relates to the total amount of drug absorbed into the blood concentration following the administration of a single dose. The plasma concentration-time profile is divided into a number of trapezoids, and the total AUC can be approximated by the sum of the area of these trapezoids. At present, there are available computer programs for calculation of AUC (Hedaya, 2007).

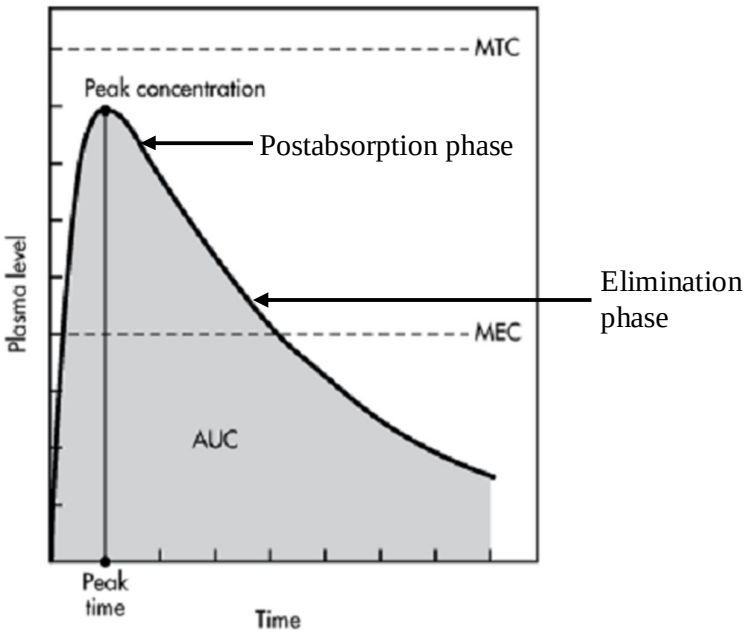


Figure 9 The plasma drug concentration-time profile after oral administration (MEC: minimum effective concentration, MTC: minimum toxic concentration, adapted from Shargel et al., 2005)

4.2.4 Factors affecting drug absorption

There are numerous factors affecting this process, which may come from the physiological conditions or the formulation of the drug (Table 1). The main factors are gastrointestinal motility, splanchnic blood flow, particle size, and formulation (Rang et al., 2003). The most important factors are discussed below.

Table 1 Factors affecting drug absorption

Physicochemical factors	Physiological factors
Hydrophobicity	Surface area at the site of administration
Molecular size	Transit time and motility
Molecular conformation	pH in the lumen and at surface
pKa	Intestinal secretions
Chemical stability	Enzymes
Solubility	Membrane permeability
Complexation	Food and food composition
Particle size	Disease states
Crystal form	Pharmacological effect
Aggregation	Mucus and unstirred water layer
Hydrogen bonding	Water fluxes
Polar surface area	Blood flow
	Bacteria
	Liver uptake and bile excretion

(Ungell and Abrahamsson, 2009)

a) Dosage form

Drug formulation has major effects on absorption. The difference in drug formulations can produce the difference in absorption characteristics. Solution dosage form is usually the most readily absorbed because the drugs have to be in solution in the gastrointestinal fluids before absorption. Capsules may be desired to remain intact for some hours after ingestion in order to delay absorption, or tablets may have a resistant coating to give the same effect. In some cases, a mixture of slow- and fast-release particles is included in a capsule to produce rapid but sustained absorption (Rang et al., 2003).

b) Drug solubility

The chemical structure of drug can influence its dissolution and bioavailability. The physical properties of drug, such as the crystalline state and the particle size, are important in determination of its solubility. Drugs that

have low water solubility are expected to possess bioavailability problems, and methods to improve their solubility should be considered. Minor modification of drug molecule such as salt formation, micronization to decrease the drug particle size and to increase the effective surface area, and complexation with water-soluble materials are tactics that have been used to enhance the water solubility of these drugs (Hedaya, 2007). Complexation is sometimes used to increase drug solubility, particularly of poor water-soluble drugs. One class of complexing agents that is increasingly being employed is the cyclodextrin family. Several drugs for example, miconazole, itraconazole, piroxicam, indomethacin, pilocarpine, naproxen, hydrocortisone, diazepam, and digitoxin have been shown the increasing of their solubility and hence bioavailability increased when they were formed with cyclodextrin (Ashford, 2007).

c) Drug partition coefficient

Lipid-solubility is the major factor determining the ability of a drug to cross various biological membranes throughout the body. Drugs in the gastrointestinal lumen must traverse through the walls of gastrointestinal tract and blood vessels in order to enter the blood stream. Lipid-soluble drugs with favorable partition coefficients are usually well absorbed after oral administration (Na-Bangchang and Wernsdorfer, 2001). Lipophilic drugs cross the biological membrane more readily than hydrophilic drugs.

d) Surface area and particle size

An increase in the total surface area of drug can causes an increase in dissolution rate of the drug. Provided that each particle of drug is intimately wetted by the gastrointestinal fluids, the effective surface area exhibited by the drug will be directly proportional to the particle size of the drug. As a consequence, the smaller the particle size, the greater the effective surface area exhibited by a giving mass of drug and the higher the dissolution rate. Particle size reduction is thus likely to result in increased bioavailability, provided that the absorption of drug is dissolution rate limited (Ashford, 2007).

e) Dissociation constant and gastrointestinal pH

The degree of drug ionization has implications for drug absorption from the gastrointestinal tract. The fractions of drug that exist in the non-ionized and ionized forms are a function of the dissociation constant of the drug and

the pH of the fluid medium at the absorption site. Only the non-ionized form of a drug can cross the biological membrane. The pH range in stomach to colon may vary from 1 to 8; the usual pH ranges in esophagus, stomach, and intestines are crudely 5-6, 2-6, and 5 to 8, respectively. The weak acid drugs are non-ionized in stomach. On the other hand, the weak basic drugs are non-ionized in intestine (Na-Bangchang and Wernsdorfer, 2001).

f) Drug stability and drug interaction

Drug stability at the site of administration can also play a major role in determination of drug bioavailability. The degradation of many acid-labile drugs in the acidic environment of the stomach leads to low systemic bioavailability. Enzymatic hydrolysis of drugs in gastrointestinal tract can also result in variation in the bioavailability. In addition, co-administration of drugs can result in the interference of one drug with the absorption of another. Drug-drug interaction in the gastrointestinal tract may be caused by the formation of complexes that are incapable of crossing the biological membrane, competing for similar mechanisms such as active transport or simply by modifying the stomach-emptying rate or gastrointestinal motility (Hedaya, 2007).

g) Gastrointestinal motility

It has a large effect. The transit time of a drug in the alimentary tract depends on the rate at which it passes along as a result of the physiological movement of the gastrointestinal tract. The rate of gastric emptying is a controlling step in the speed of drug absorption. Drug absorption occurs mainly in the small intestine, and therefore alterations in the rate of gastric emptying will result in parallel alterations in the rate and in some cases, the extent of absorption. Several factors can influence the gastric emptying rate such as the pharmacological properties and type of dosage form of the drug, and various physiological factors. Many disorders (migraine, diabetic neuropathy) cause gastric stasis and slow drug absorption. Drug treatment can also affect motility, either reducing or increasing. Consumption of meals, especially those with high fat content, slows gastric emptying. Moreover, the small intestine is the important site for drug absorption; its transit time is about 3-4 hours. A drug may take about 4 to 8 hours to pass through stomach and

small intestine during the fasting state. During the fed state, the drug may take to 8 to 12 hours (Rang et al., 2003; Na-Bangchang and Wernsdorfer, 2001).

h) First-pass effect

After oral absorption, the drug in the gut lumen may undergo chemical hydrolysis or enzymatic metabolism caused primary by the gut flora. During the absorption process, the drug can be metabolized in the gut wall. The drug absorbed into the portal vein will have to go through the liver before it reaches the systemic circulation. A fraction of absorbed drug can be metabolized during this first pass through the liver before the drug reaches the systemic circulation (Hedaya, 2007).

i) Splanchnic blood flow

It is greatly reduced in hypovolemic states, with resulted in slowing of drug absorption (Rang et al., 2003).

Consequently, drugs that can be absorbable and pass the membrane to blood circulation, should be small size, not protein binding, lipid soluble, non-ionized, and similar pH media (acidic drug in acid media and vice versa for basic drug).

4.2.3 Bioavailability

To pass from the lumen of the small intestine into the systemic circulation, a drug must not only penetrate the intestine mucosa, but also may be inactivated by enzymes in gut wall and liver. The term ‘bioavailability’ is used to indicate the proportion of drug that passes into the systemic circulation after oral administration (Rang et al., 2003). By definition, when a medication is intravenously administered, its bioavailability is 100%. Whereas, a medication that is extravenously administered, its bioavailability decrease due to incomplete absorption and first pass metabolism. Accordingly, the bioavailability depends on rate of absorption and the first-pass metabolism. The AUC is a common measurement of the extent of bioavailability for a drug given by a particular route, the formula shows below.

$$\text{Bioavailability} = \frac{\text{AUC of extravenous route / Dose of extravenous route}}{\text{AUC of intravenous route / Dose of intravenous route}}$$

After extravenously administration of drugs, bioavailability may vary from a value of 0 (no drug absorption) to 1 (complete drug absorption) or express as a percent by multiply with 100 (0-100 %).

4.3 Distribution

Distribution is the process of reversible transfer of drug from one to another site within the body. Drug is distributed throughout the entire body to various sites including the sites of action, as well as the sites of storage in plasma or in the tissue, and the sites of elimination (metabolism and excretion). Only the unbound (unassociated with plasma or tissue proteins) and non-ionized and lipid-soluble forms of drug readily traverse the biological membranes and so distribute out from the site of absorption. Delivery of the unbound and non-ionized fraction of drugs is through blood stream and body fluids, which act as carriers for the distribution (Na-Bangchang and Wernsdorfer, 2001).

Distribution of drugs to and from blood and tissues occurs at various rates and to various extents. Factors that determine the distribution pattern of a drug with time include the physicochemical characteristics of the drug, binding of drug to plasma, tissue proteins or other macromolecules, and blood perfusion to tissues. Binding of drugs to both of plasma and tissue proteins or other macromolecules are generally a reversible process. Magnitude of vasculature and blood supply also plays a major role (Na-Bangchang and Wernsdorfer, 2001).

4.3.1 Factors affecting drug distribution

Drug distribution can be rate-limited and extent-limited by the permeability of biological membranes, blood perfusion to tissues or organs, drug binding affinity to tissue components, and protein binding of drug.

a) Membrane permeability

In order to enter an organ, a drug must penetrate all membranes which separate the organ from the site of drug administration. Lipid-soluble drugs have a tendency of passing rapidly through most of the biological membranes, while polar drugs (water-soluble) are subject to permeability rate-limited. Highly lipophilic drugs readily cross gut wall, capillary wall, and blood-brain barrier (Na-Bangchang and Wernsdorfer, 2001).

b) Blood perfusion and drug binding affinity

Time to reach distribution equilibrium in a tissue depends on blood perfusion to that particular tissue. Tissue receiving a high blood flow will rapidly equilibrate with the drug whereas those that are poorly perfused will reach drug equilibrium more slowly. A perfusion-rate limitation prevails when the tissue membranes present essentially no barrier to drug distribution. The rate of blood perfusion of the tissue and the affinity of the drug to the tissue determine the pattern of drug distribution in any tissues. The tissue concentration of drug with low affinity equilibrates rapidly with the plasma drug concentration and declines rapidly as the drug concentration in plasma is dropping. Drugs with high tissue affinity on the other hand, equilibrate slowly with the plasma drug concentration and are likely to accumulate or concentrate in the tissue (Na-Bangchang and Wernsdorfer, 2001).

c) Protein binding of drug

Drugs generally interact with plasma or tissue protein. Drugs highly bound to plasma proteins are contained in plasma. Drug-protein binding is mostly a reversible process. If the binding is an irreversible process, it impacts to the toxicity. The major proteins which interact with drugs are albumin, α-1-acid glycoprotein, and lipoproteins (Na-Bangchang and Wernsdorfer, 2001).

Volume of distribution (Vd) is a parameter providing the physiologic distribution of a drug. However, Vd dose not correspond to a physiologic body fluid compartment. It is the ideal value. It is known in term of apparent Vd. Vd is an available volume in both the general circulation and the tissues of distribution of the body which would be required to contain the administered dose that is evenly distributed at the concentration measured in plasma or blood. Drugs having low Vd are likely to be located within the plasma by virtue of their high affinity to plasma proteins. In contrast, drugs with a large Vd are those distributing throughout the body fluids. The Vd of a drug may be estimated if the total dose given and the plasma drug concentration after equilibrium are known (Na-Bangchang and Wernsdorfer, 2001):

$$Vd = \frac{\text{Amount (mg) of drug in the body}}{\text{Plasma drug concentration (mg/l) after equilibrium}}$$

4.4 Metabolism

When drug is administered into body, it is absorbed into blood circulation, distributed into target organ, metabolized, and excreted out of the body, these are pharmacokinetic process. Metabolism process can cause more polarity of drug which is easier to be excreted out of the body. In addition, this process may activate pro-drug to be active molecule to achieve therapeutic effect. On the other hand this process may convert drug to toxic molecule (Correia, 2004).

Metabolism
Drugs (lipophilic and less polar) ⟶ Metabolites (hydrophilic and more polar)

Factors affecting drug metabolism include age, gender, genetic, species differences, diet, disease, co-administered substances, tissue differences, auto-induction, protein binding, and route of administration.

4.4.1 Type of metabolism

Metabolism of xenobiotics or drug can be divided to phase I and phase II reactions. For some compounds, phase I reactions may occur before phase II reactions, and in some cases phase II products undergo further phase I metabolism.

a) Phase I reactions

The reactions usually convert the parent drug to more polar metabolite by introducing or unmasking a functional group (-OH, -NH_2, -SH). Often these metabolites are inactive, although in some instances activity are only modified. If phase I metabolites are sufficiently polar, they may be readily excreted. However, many phase I products are not eliminated rapidly and undergo a subsequent reaction in which an endogenous substrate such as glucuronic acid, sulfuric acid, acetic acid, or an amino acid combines with the newly incorporated functional group to form a highly polar conjugate. Such conjugation or synthetic reactions are phase II metabolism (Correia, 2004). The possible pathways for metabolism and elimination are concluded in Figure 10. Phase I reactions have several reactions (Table 2), including oxidative reactions (*N*-dealkylation, *O*-dealkylation, aliphatic hydroxylla

tion, aromatic hydroxylation, *N*-oxidation, *S*-oxidation, and deamination), and hydrolysis reactions. These reactions may facilitate the removal of drugs from the body, either directly, or via conjugation by phase II reactions. Table 3 lists the major classes of phase I metabolism enzymes involved in drug metabolism. Cytochrome P450 enzymes (CYP450) are a superfamily of haem-thiolate containing enzymes which play a major role in the metabolism of many drugs and other xenobiotics (Eddershaw and Dickins, 2004). The name of CYP450 is derived from the spectral properties of this hemoprotein. In its reduced (ferrous) form, it binds carbon monoxide to give a complex that absorbs light maximally at 450 nm (Correia, 2004). In order to classify the CYP450, the amino acid sequence of each isoform is utilized. Isoforms with greater than 40% sequences homology are assigned to the same family (e.g. CYP1, CYP2, CYP3, etc.). Isoforms with greater than 55% sequences homology are assigned to the same subfamily (e.g. CYP1A, CYP1B, etc.). The CYP450 are identified by a number denoting the family, a letter denoting the subfamily, and a number identifying the specific member of the subfamily. For example, CYP1A2 is the second member of subfamily A of family 1 (Montellano, 1999). The metabolism via CYP450 is the oxidation reaction. The reaction requires CYP450 enzyme, CYP450 reductase, NADPH, and molecular oxygen. A simplified scheme of oxidative cycle is presented in Figure 11. Briefly, oxidized (Fe^{3+}) CYP450 combines with a drug substrate to form a binary complex (step①). NADPH donates an electron to the flavoprotein reductase, which in turn reduces the oxidized CYP450-drug complex (step②). A second electron is introduced from NADPH via the same flavoprotein reductase, which serves the reduce molecular oxygen and to form an "activated oxygen"-CYP450-substrate complex (step③). This complex in turn transfers activated oxygen to the drug substrate to form the oxidized product (step④) (Correia, 2004).

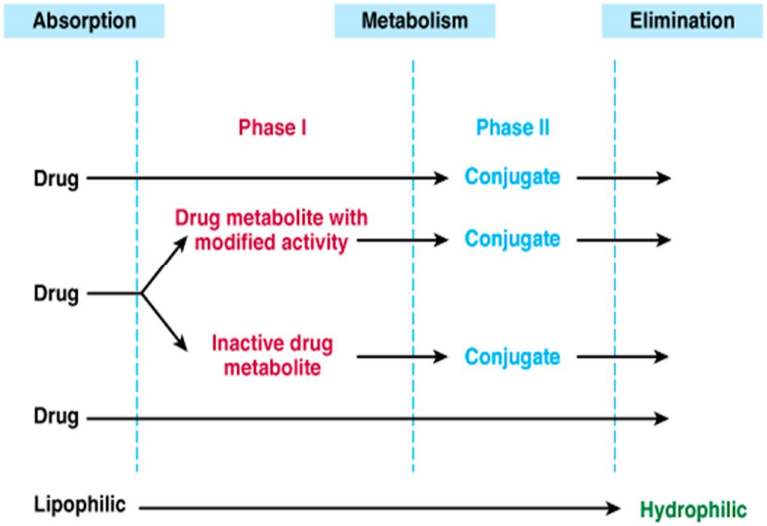


Figure 10 Scheme of drug metabolism (Correia, 2004)

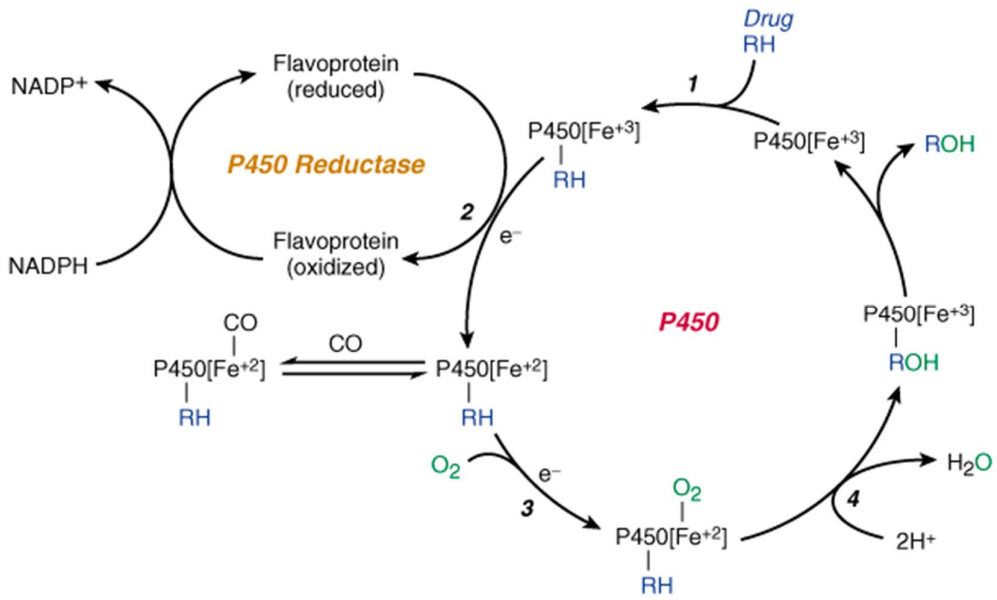
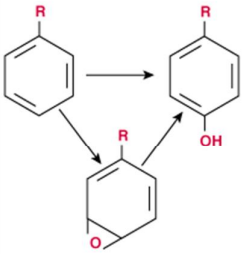
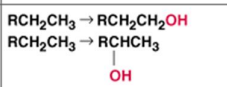
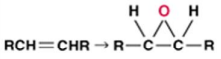
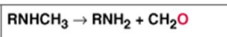
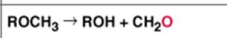
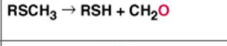
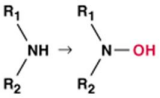
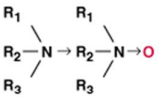
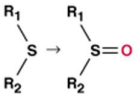
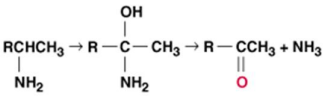


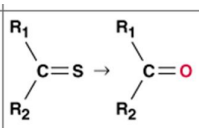
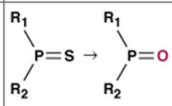
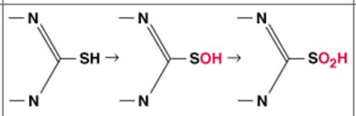
Figure 11 Cytochrome P450 cycle in drug oxidation (RH, parent drug; ROH, oxidized metabolite; e^-, electron.) (Correia, 2009)

Table 2 Major reactions involved in phase I metabolism

Reaction Class	Structural Change	Drug Substrates
Oxidations		
Cytochrome P450-dependent oxidations:		
Aromatic hydroxylations		Acetanilide, propranolol, phenobarbital, phenytoin, phenylbutazone, amphetamine, warfarin, 17α-ethinyl estradiol, naphthalene, benzpyrene
Aliphatic hydroxylations		Amobarbital, pentobarbital, secobarbital, chlorpropamide, ibuprofen, meprobamate, glutethimide, phenylbutazone, digitoxin
Epoxidation		Aldrin
Oxidative dealkylation		
N-Dealkylation		Morphine, ethylmorphine, benzphetamine, aminopyrine, caffeine, theophylline
O-Dealkylation		Codeine, *p*-nitroanisole
S-Dealkylation		6-Methylthiopurine, methitural
N-Oxidation		
Primary amines		Aniline, chlorphentermine
Secondary amines		2-Acetylaminofluorene, acetaminophen
Tertiary amines		Nicotine, methaqualone
***S*-Oxidation**		Thioridazine, cimetidine, chlorpromazine
Deamination		Amphetamine, diazepam

(Correia, 2009)

Table 2 Major reactions involved in phase I metabolism (Cont.)

Desulfuration		Thiopental
		Parathion
Dechlorination	$CCl_4 \rightarrow [CCl_3^{\cdot}] \rightarrow CHCl_3$	Carbon tetrachloride
Cytochrome P450-independent oxidations:		
Flavin monooxygenase (Ziegler's enzyme)	$R_3N \longrightarrow R_3N^+ \rightarrow O^- \xrightarrow{H^+} R_3N^+OH$	Chlorpromazine, amitriptyline, benzphetamine
	$RCH_2N(H)-CH_2R \rightarrow RCH_2-N(OH)-CH_2R \rightarrow RCH=N(O^-)-CH_2R$	Desipramine, nortriptyline
		Methimazole, propylthiouracil
Amine oxidases	$RCH_2NH_2 \rightarrow RCHO + NH_3$	Phenylethylamine, epinephrine
Dehydrogenations	$RCH_2OH \rightarrow RCHO$	Ethanol
Reductions	$RN=NR_1 \rightarrow RNH-NHR_1 \rightarrow RNH_2 + R_1NH_2$	
Azo reductions		Prontosil, tartrazine
Nitro reductions	$RNO_2 \rightarrow RNO \rightarrow RNHOH \rightarrow RNH_2$	Nitrobenzene, chloramphenicol, clonazepam, dantrolene
Carbonyl reductions	$RCR' \rightarrow RCHR'$ (O, OH)	Metyrapone, methadone, naloxone
Hydrolyses		
Esters	$R_1COOR_2 \rightarrow R_1COOH + R_2OH$	Procaine, succinylcholine, aspirin, clofibrate, methylphenidate
Amides	$RCONHR_1 \rightarrow RCOOH + R_1NH_2$	Procainamide, lidocaine, indomethacin

(Correia, 2009)

Table 3 Reaction types catalyzed by phase I enzymes

Enzyme	Reaction
CYP450	oxidation
Monoamine oxidase	oxidation
Flavin-containing monoxygenase	oxidation
Alcohol dehydrogenase	oxidation
Aldehyde dehydrogenase	oxidation, reduction
Xanthine oxidase	oxidation
Epoxide hydroxylase	hydrolysis
Carboxylesterase	hydrolysis
Peptidase	hydrolysis
Carbonyl reductase	reduction

(Eddershaw and Dickins, 2004)

b) Phase II reactions

Phase II metabolism usually changes phase I metabolites or parent drugs to be more polar and readily excreted via urine or bile, it should be noted that such metabolism may also give rise to reactive, potentially toxic metabolites which may bind covalently to tissue macromolecules (Manchee et al., 2004). It involves conjugation reactions (glucuronidation, sulfation, acetylation, methylation, and glutathione conjugation) (Gonzalea and Tukey, 2008) (Table 4). Glucuronidation and sulfate conjugation are very common phase II reactions that result in water-soluble metabolites being rapidly excreted in bile (for some high-molecular-weight glucuronides) and/or urine (Shargel et al., 2005).

Table 4 Phase II enzymes and their reactions with functional groups

Enzyme	Reaction	Functional group
UDP-glucuronosyltransferase	Glucuronidation	-OH, -COOH, -NH,-NOH, $-NH_2$, -SH, ring N
UDP-glycosyltransferase	Glycosidation	-OH, -COOH, -SH
Sulfotransferase	Sulfation	-OH, -NH, -NOH, $-NH_2$
Methyltransferase	Methylation	-OH, $-NH_2$
Acetyltransferase	Acetylation	-OH, $-NH_2$, $-SO_2NH_2$,
Amino acid conjugation		-COOH
Glutathione-*S*-transferase	Glutathione conjugation	epoxide, organic halide
Fatty acid conjugation		-OH

(Manchee et al., 2004)

4.4.2 Major sites of metabolism

Metabolism can mainly occur in liver. Many drugs are absorbed from small intestine and transported via portal vein to liver where they undergo extensive metabolism. Moreover, extra-hepatic metabolism can be found at mouth, stomach, small intestine, large intestine, kidney, lung, plasma, blood cells, placenta, skin, and brain (Younggil, 2002). The intestines contain intestinal microorganisms that are capable of many metabolism reactions. In addition, drugs may be metabolized by gastric acid, by digestive enzymes, or by enzymes in the wall of intestines. At subcellular level, drug-metabolizing enzymes may be located in the endoplasmic reticulum, mitochondria, cytosol, lysosomes, or even the nuclear envelop or plasma membrane. The enzymes are located in the lipophilic membranes of the endoplasmic reticulum of the liver and the other tissues (Figure 12). When these lamellar membranes are isolated by homogenization and fractionation of the cell, they re-form into vesicles called microsomes. Microsomes retain most of the morphologic and functional characteristics of the intact membranes, including the rough and smooth surface features of the rough (ribosome-studded) and smooth (no ribosomes) endoplasmic reticulum. Whereas the rough microsomes tend to be dedicated to protein synthesis, the smooth microsomes are relatively rich in enzymes responsible

for oxidative drug metabolism. In particular, they contain the important class of CYP450 enzymes (Correia, 2004).

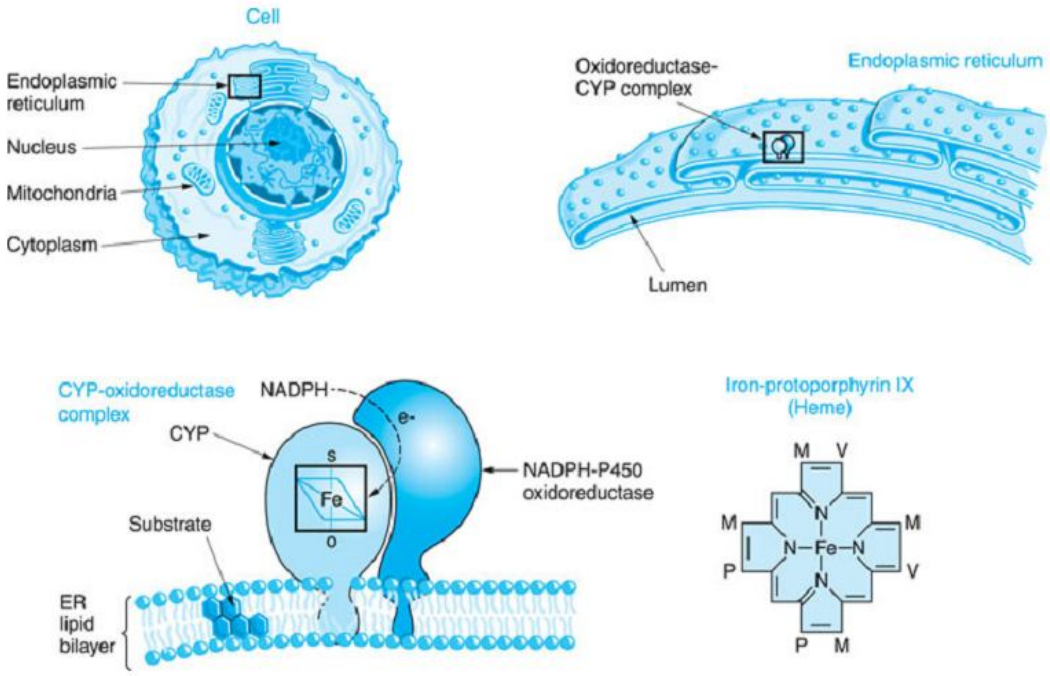


Figure 12 Location of CYP450 in the cell (Gonzalez and Tukey, 2008)

4.4.3 Enzyme induction/enzyme inhibition

Differences in the rate of metabolism of a drug can be due to drug interactions. Most common, this occurs when two drugs are co-administered and are metabolized by the same enzyme. Since most of these drug-drug interactions are due to CYP450, it becomes important to determine the identity of the CYP450 that metabolizes a particular drug and to avoid co-administering drugs that are metabolized by the same enzyme. Some drugs are CYP450 inducers that can induce not only their own metabolism, but also induce metabolism of other co-administered drugs. Some drugs can also inhibit CYP450 independently of being substrates for a CYP450 (Gonzalez and Tukey, 2008).

a) Enzyme induction

Some of chemical substances, dissimilar drug substrates, or environmental pollutants on repeated administration induce CYP450 by

enhancing the rate of its synthesis or reducing its rate of degradation. Induction results in an acceleration of substrate metabolism and usually decreases in the pharmacologic action of the inducer and also of co-administered drugs. Nevertheless, in the case of drugs metabolically transformed to reactive metabolites, enzyme induction may exacerbate metabolite-mediated toxicity (Correia, 2004). Herbal products such as St. John's wort can increase hepatic levels of CYP3A4, therefore increasing the metabolism of many orally administered drugs (Gonzalez and Tukey, 2008).

b) Enzyme inhibition

Certain drug substrates may inhibit CYP450 enzyme activity through binding with CYP450 heme-iron. Some substances irreversibly inhibit CYP450 via covalent interaction of a metabolically generated reactive intermediate that may react with the CYP450 apoprotein or heme moiety or even cause the heme to fragment and irreversibly modify the apoprotein (Correia, 2004). Major compound in grapefruit juice: naringin, and furanocoumarins, are potent inhibitors of CYP3A4, and therefore some drug inserts recommend not taking medicine with grapefruit juice because it could increase the bioavailability of a drug (Gonzalez and Tukey, 2008).

4.4.4 Enzyme kinetic

The process of biotransformation or metabolism is the enzymatic conversion of a drug to a metabolite. In the body, the metabolic enzyme concentration is constant at a given site, and the drug (substrate) concentration may vary. When the drug concentration is low relative to the enzyme concentration, there are abundant enzymes to catalyze the reaction, and the rate of metabolism is a first-order process. Saturation of the enzyme occurs when the drug concentration is high, all the enzyme molecules become complexes with drug, and the reaction rate is at a maximum rate; the rate process then becomes a zero-order process. The maximum reaction rate is known as Vmax, and the substrate or drug concentration at which the reaction occurs at half the maximum rate corresponds to a composite parameter Km. These two parameters determine the profile of a simple enzyme reaction rate at various drug concentrations. The relationship of these parameters is described by the Michaelis-Menten equation (Figure 13). Enzyme kinetic generally considers that 1 mole of drug interact with 1 mole of enzyme to form an enzyme-drug intermediate.

The enzyme-drug intermediate further reacts to yield a reaction product or a drug metabolite (Figure 14). The rate process for drug metabolism is described by the Michaelis-Menten equation, which assumes that the rate of an enzymatic reaction is dependent on the concentrations of both the enzyme and the drug. An energetically favored drug-enzyme intermediate is initially formed, followed by the formation of the product and regeneration of the enzyme. The Vmax corresponds to the rate when the entire available enzyme is in the form of the drug-enzyme (ED) intermediate. At Vmax, the drug concentration is in excess, and the forward reaction, k_3[ED], is dependent on the availability of more free enzyme molecules. The Km is a useful parameter that reveals the concentration of the substrate at which the reaction occurs at half V max. In general, for a drug with a large Km, a higher concentration will be necessary before saturation is reached (Shargel et al., 2005).

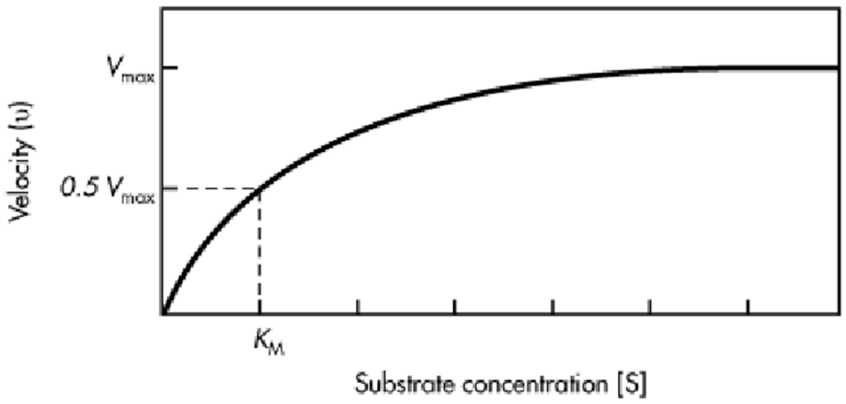


Figure 13 Michaelis-Menten enzyme kinetics (Shargel et al., 2005)

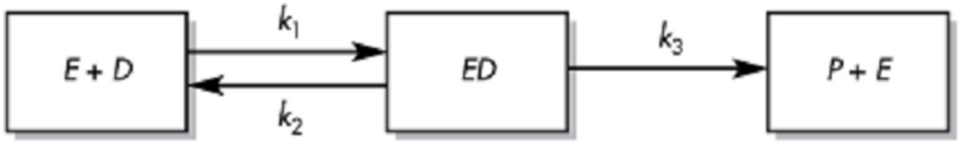


Figure 14 Enzymatic reaction, [D]: drug, [E]: enzyme, [ED]: enzyme-drug intermediate, [P]: product or metabolite, k_1, k_2, and k_3: first-order rate constant (Shargel et al., 2005)

Lineweaver-Burk equation, which is the reciprocal of the Michaelis-Menten equation, is used to obtain the estimation of Vmax and Km and to distinguish between various types of enzyme inhibition. The Lineweaver-Burk equation is:

$$\frac{1}{v} = \frac{K_M}{V_{max}} \frac{1}{[D]} + \frac{1}{V_{max}}$$

Wherease, [D] represents drug concentration.

The type of enzyme inhibition is usually classified by enzyme kinetic studies and observing changes in the Km and Vmax.

In the case of competitive enzyme inhibition, the inhibitor and drug-substrate compete for the same active center on the enzyme. The drug and the inhibitor may have similar chemical structures. An increase in the drug concentration may displace the inhibitor from the enzyme and partially or fully reverse the inhibition. Competitive enzyme inhibition is usually observed by a change in the Km, but the Vmax remains the same (Figure 15).

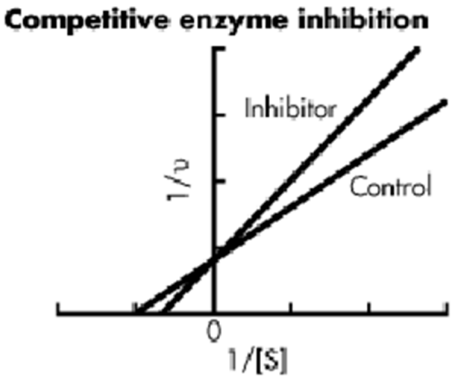


Figure 15 Lineweaver-Burk plot as competitive enzyme inhibition (Shargel et al., 2005)

In noncompetitive enzyme inhibition, the inhibitor may inhibit the enzyme by combining at a site on the enzyme that is different from the active site (an allosteric site). In this case, enzyme inhibition depends only on the inhibitor

concentration. In noncompetitive enzyme inhibition, Km is not altered, but Vmax is lower (Figure 16). Noncompetitive enzyme inhibition cannot be reversed by increasing the drug concentration, because the inhibitor will interact strongly with the enzyme and will not be displaced by the drug.

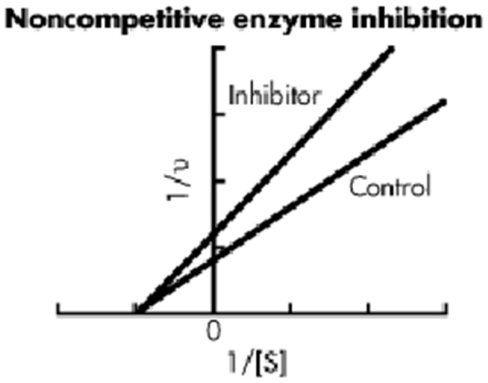


Figure 16 Lineweaver-Burk plot as noncompetitive enzyme inhibition (Shargel et al., 2005)

Other types of enzyme inhibition, such as mixed enzyme inhibition and enzyme uncompetitive inhibition, have been described by observing changes in Km and Vmax (Figure 17).

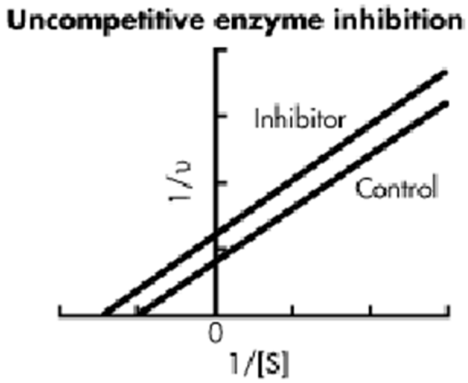


Figure 17 Lineweaver-Burk plot as uncompetitive enzyme inhibition (Shargel et al., 2005).

4.4.5 Drug metabolism studies

Drug metabolism is a definite determinant of the pharmacokinetic behavior of drug. *In vitro* metabolism studies are one piece of the puzzle to understand the pharmacokinetic characteristics of a given compound, to optimize pharmacokinetic parameters and to select the most drugs like compounds that will progress into development. Relatively recent prospect of obtaining equivalent data from *in vitro* and *in vivo* studies has provided the pharmaceutical industry with an incentive to validate *in vitro* models with respect to increase throughput and/or to replace animal studies where appropriate. An early assessment using animal *in vitro* and *in vivo* data together with human *in vitro* data allows a qualitative prediction whether humans will act in similar ways as did the animal models. *In vivo* metabolism studies play a role later in development in both, animals and humans. Use of transgenic animals facilitates understanding the role of drug metabolizing enzymes in the organism. Nevertheless, animal studies cannot entirely replace clinical studies in predicting all responses in human, but for ethical reasons, the risk to human volunteers participating in early clinical studies should be minimized. This is supported by a variety of *in vitro* metabolism studies. Moreover, computational models are set up to predict the *in vitro* studies which help to speed-up the selection and optimization processes although applications on the metabolism of drug are still limited. *In vitro* metabolism studies are recognized as an important tool for predicting drug-drug interactions and variability in exposure due to pharmacogenetic differences in the population. Besides studies on enzymes inhibition and induction information has to be generated on the enzymes involved in the metabolism of a drug particularly for drugs which are subject to high metabolic clearance in the liver (Dudda and Kurzel, 2006).

The *in vitro* preparations most commonly used for studying drug metabolism are liver microsomes, a preparation known as the S9 fraction, overexpressed recombinant metabolizing enzymes, and intact hepatocytes. Each of these *in vitro* systems have their specific values and limitations regarding availability of tissues costs, completeness of enzymatic capability, ease of handling, sensitivity to population differences. A comparison between the various systems to study the metabolism of a drug *in vitro* is given in Table 5. Whereas complicated studies

require the technology to be availability in-house preparation e.g. of slices or performing an organ perfusion study, other can be performed with cells or fractions commercially available or easily prepared. A typical preparation scheme for preparation of S9 and microsomes fractions by differential centrifugation is given in Figure 18 (Dudda and Kurzel, 2006).

Table 5 Comparison of *in vitro* studies for drug metabolism study

***In vitro* system**	**Pros**	**Cons**
Perfused organs	Phase I and II present, whole metabolic profile observed, best correlation to *in vivo*	expensive, *ex vivo* animal trial, complex methodology, high technical effort, batch variability, more complicated than enzyme-only system, quality control, limited use for multiple compounds
Slices	Phase I and II present, whole metabolic profile observed, good correlation to *in vivo*	expensive, *ex vivo* animal trial, diffusion controlled, complex methodology, high technology effort, batch variability, more complicated than enzyme-only system, quality control, limited use for multiple compounds
Cells in primary culture (e.g. hepatocytes)	Phase I and II present, whole metabolic profile observed, induction modeled, population pools for cryopreserved hepatocytes possible, good correlation to *in vivo*	expensive, batch variability, quality control, complex methodology, high technology effort, limited use for multiple compounds

(Dudda and Kurzel, 2006)

Table 5 Comparison of *in vitro* studies for drug metabolism study (Cont.)

***In vitro* system**	**Pros**	**Cons**
S9 fraction	easy to use, cheap, phase I and II present, whole metabolic profile observed	addition of cofactors (complex mixtures), lower enzyme activity than microsomes/supersomes, induction not modeled
Microsomes	easy to use, cheap, population pools	addition of cofactors (single mixtures), only membrane-bound metabolizing enzymes such as CYPs, FMOs, and UGTs partial metabolic profile, induction not modeled
Cytosol	easy to use, cheap	addition of cofactors (simple mixtures), only membrane-bound metabolizing enzymes such as alcohol dehydrogenases, sulfotransferase, glutathione S transferase, N-acetyl transferase partial metabolic profile, induction not modeled
Supersomes	easy to use, moderately cheap, no addition of co-factors, single enzyme only	Currently only CYPs, FMOs, UGTs, GSTs, and SULTs, single enzyme only accuracy of kinetics?, induction not modeled

(Dudda and Kurzel, 2006)

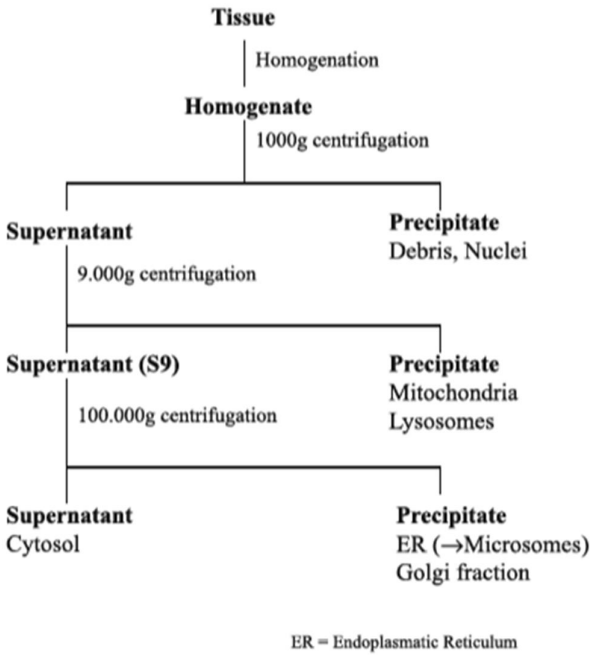


Figure 18 Preparation of S9 and microsomes fraction (Dudda and Kurzel, 2006)

a) Liver microsomes

Liver microsomes are the simplest and most useful tools for determining metabolic stability during the early stages of drug discovery. They can be prepared from all animal species, contain high activities of all CYP450 and variety of other phase I and phase II metabolism enzymes. Microsomes are commercially available, and are stable for up to five years at -80^{o}C (Ducharme and Dudley, 2006).

b) S9 fraction

This consists of both microsomal and cytosolic fractions including a wide range of phase I and phase II enzymes, a broader range than microsomes, although at lower activity. A standard cocktail of cofactors need to be added for these activities to be expressed. Enzyme induction does not occur (Ducharme and Dudley, 2006).

c) Hepatocytes

Hepatocytes contain all liver enzymes and cofactors and preserve intact cell-cell communication systems. Availability of human liver tissue is often a problem, which is likely to improve with the development of cryopreservation methods. Cryopreserved hepatocytes now retain almost all their drug metabolizing capacities and can be used to assess rates and routes of metabolism, as well as modeling enzyme induction. Overall metabolic stability can be assessed by incubating compounds with human hepatocytes in suspension, but the characterization of metabolic routes may require longer incubation times and the use of monolayer cell cultures. The latter should be regarded as qualitative rather than quantitative studies, as the expression of most drug metabolizing enzymes declines in monolayer cultures. An alternative system giving the presentation of liver-specific functions involves hepatocyte monolayers overlaid on extracellular matrix, which dramatically improves cell viability and allows the formation of functional bile canalicular networks. Despite being labour intensive, this system allows the measurement of permeability and active transport in addition to the production of metabolites, and is becoming widely used in drug discovery (Ducharme and Dudley, 2006).

d) Computer-based study

The computational (*In silico*) prediction of drug metabolism may also be a prospect for the future, since the structures of several CYP450 have been determined, including those of CYP2A6, CYP2C9, and CYP3A4. These structures may be used to predict metabolism of a drug candidate by fitting the compound to the enzyme's active site and determining oxidation potentials of sites on the molecule. However, the structures, determined by X-ray analysis of crystals of enzyme-substrate complexes, are static, whereas enzymes are flexible, and this vital distinction may be limiting. With refinement of structures and more powerful modeling software, *in silico* drug metabolism may be a reality in the future (Gonzalez and Tukey, 2008).

4.5 Excretion

Excretion is the process of removal of the drug from the body (Na-Bangchang and Wernsdorfer, 2001). Drugs are eliminated from the body either

unchanged by the process of excretion or converted to metabolites. Kidney is the most important organ for excreting drugs and their metabolites.

4.5.1 Routes of excretion

Drug excretion can occur almost anywhere in the body. The excretion can be found in kidney as the urine, liver as the bile or feces, sweater gland as the sweat, breast as the milk, and lung as the volatile substances.

a) Renal excretion

Kidney is the main excretion organ for the removal of metabolic waste products and parent drugs or metabolites as well. The parent drugs and/or metabolites that are non-volatile, water soluble, low molecular weight, or slowly metabolism by the liver are excreted by renal excretion. They pass through the kidney to the bladder and ultimately into the urine (Shargel et al., 2005). Renal excretion of drugs and metabolites in the urine involves three distinct processes including glomerular filtration, active tubular secretion, and passive tubular reabsorption (Buxton, 2006). The glomerular filtration is a unidirectional process that occurs for most small molecules (MW < 500), including undissociated (non-ionized) and dissociated (ionized) drugs. Most drugs, unless highly bound to plasma protein (large molecules), cross the glomerular filter freely. The major driving force for glomerular filtration is the hydrostatic pressure within the glomerular capillaries. The active tubular secretion is an active transport processes. The transports are capacity limited and may be saturated. Many drugs, especially weak acids and weak bases, are actively secreted into the renal tubule, and thus more rapidly excreted. The passive tubular reabsorption occurs after the drug is filtered through the glomerulus and can be an active or a passive process. The reabsorption of drugs that are acids or weak bases is influenced by the pH of the fluid in the renal tubule or urine (ranging from 4.5 to 8.0) and the pKa of the drug. Undissociated or non-ionized drugs (lipid-soluble drugs) are passively reabsorbed by diffusion across the tubule back to the body so are not efficiently excreted in the urine. The pH of urine depends on diet, pathophysiology, and drug intakes (Rang et al., 2003, Shargel et al., 2005). Therefore, the excretion via kidney can conclude follow this equation: renal excretion = glomerular filtration + active tubular secretion - passive tubular reabsorption.

b) Biliary and fecal excretion

Drugs or metabolites in the feces are principally unabsorbed. Transporters analogous to those in the kidney also are present in hepatocytes, and those actively secrete drugs and metabolites into bile. P-glycoprotein transports amphipathic lipid-soluble drugs, whereas MRP-2 is mainly involved in the secretion of conjugated metabolites of drugs (e.g., glutathione conjugation, glucuronides, and some sulphates). (Buxton, 2006)

c) Other routes

Excretion by other routes such as saliva depends mainly on diffusion of non-ionized lipid-soluble form of drugs through the epithelial cells of the gland and depends on the pH (Buxton, 2006). In addition, the other routes for excretion are bile, sweat, milk, or other body fluids. Volatile drugs, such as gaseous anesthetics or drugs with high volatility are excreted via the lungs into expired air (Shargel et al., 2005).

Clearance (Cl) is the parameter proving the excretion rate. It may be defined as the volume of fluid cleared of drug from the body per unit of time. The unit for clearance is milliliters per minute (ml/min) or liters per hour (L/h) (Shargel et al., 2005). Cl can be calculated from volume of distribution (Vd) multiplying with elimination rate constant (Ke) (Cl = Vd x Ke). Cl parameter depends on plasma protein binding (unbound fraction will be only clearance), maximum elimination capacity of organ, and organ blood flow.

5. Liquid Chromatography – Mass Spectrometer (LC-MS)

Nowadays, mass spectrometry has proved to be one of the most effective techniques in biomedical research, particularly for the analysis of complex mixtures in biological samples (Prasain et al., 2004). It provides a highly specific method for determining or confirming the identity or structure of drugs and raw materials. Mass spectrometry in conjugation with liquid chromatography provides a method for characterizing impurities in drugs and formulation excipients and also highly sensitive and specific methods for determining drugs and their metabolites in biological fluids and tissues. LC-MS components are illustrated in Figure 19. The instrument composes of HPLC which is sample inlet, ionization source, mass analyzer, ion

detector, and computer integration. The sample should be carried out an extraction step. In latter step, the mixture will be carried out to separate in chromatography. A liquid mobile phase is pumped under pressure through a stainless steel column containing particles of stationary phase with a diameter of 3-10 μm. The analyst is loaded onto the head of the column via a loop valve and separation of mixture occurs according to the relative lengths of time spent by its components in the stationary phase. It should be noted that all components in a mixture spend more or less the same time in the mobile phase in order to exit the column. After that, each component will be carried to identify its structure by mass spectrometer. A mass spectrometer works by generating charged molecules or molecular fragments either in a high vacuum or immediately prior to the sample entering the high vacuum region. The ionized molecules have to be generated in the gas phase. Once the molecules are charged and in the gas phase, they can be manipulated by the application of either electric or magnetic fields to enable the determination of their molecular weights (Watson, 2005).

Ionization sources convert state of sample to molecule ions which are the source of mass analyzer. Chemical ionization (CI), Fast atom bombardment (FAB), Electrospray ionization (ESI), and Atmospheric pressure chemical ionization (APCI) are methods of ionization for LC-MS. CI uses methane, isobutene, and ammonia which are called reagent gases. The reagent gases are ionized by electron beam and further transfer their protons to sample resulting in $[M+H]^+$. FAB usually uses for non-volatile molecules and/or large molecule such as peptides. It can produce $[M+H]^+$ or $[M+H]^-$. The sample in liquid matrix is bombarded under vacuum containing an inert gas e.g. xenon and argon. ESI produces ions of sample in liquid phase by electrospray. Solvents and mobile phases should be organic solvents namely, acetonitrile, ethyl acetate, and methanol because the buffer solutions especially those containing alkali metals may decrease sensitivity by competing for ionization. The ionization of APCI occurs in gas phase at atmosphere pressure. The sample is heated containing nebulizer gas. Sample is ionized by electron which is supplied by a discharge source or ^{63}Ni beta emitter. ESI and APCI are the method of choice in MS in drug development which they can produce ions almost 100% (Biddlecombe et al., 2004).

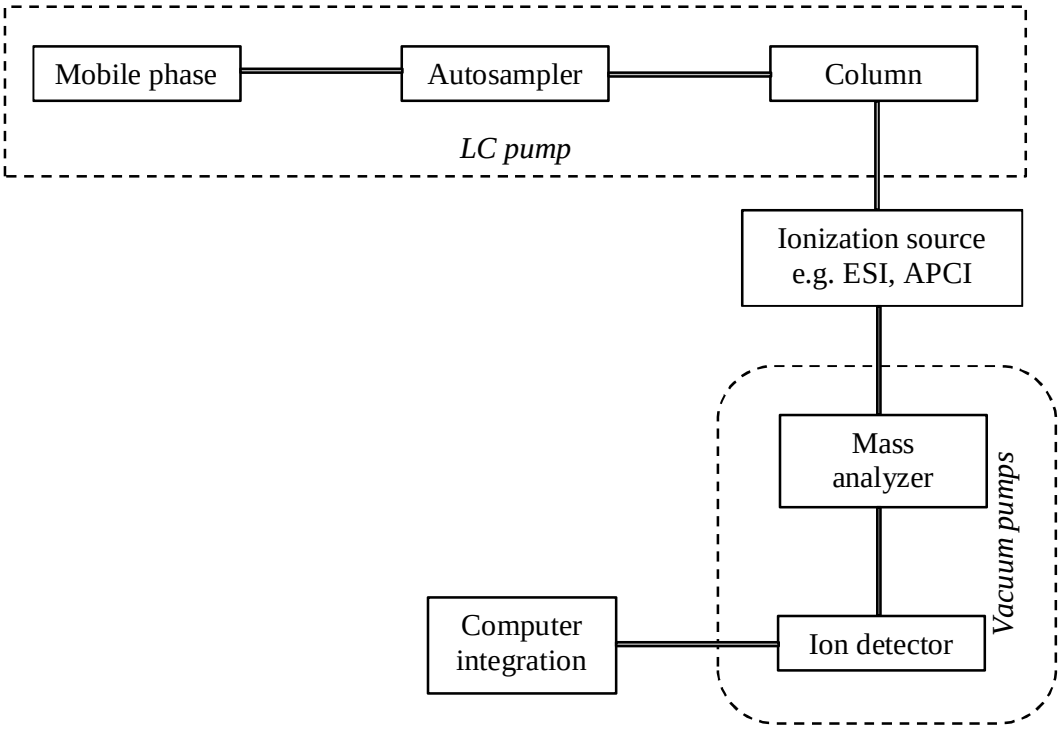


Figure 19 Composition of LC-MC (Adapted from Ramanathan and Lelacheur, 2009)

Mass analyzers measure mass to charge ratio (m/z) of ion. There are several types including quadrupoles MS, ion trap MS, time of flight (TOF) analyzers, and tandem MS. Quadrupoles MS compose of four parallel rods generating difference voltages. The certain m/z passes through to the ion detector. Ion trap MS analyzes m/z by trapping ions and then the trapped ions are placed in unstable orbits to detect m/z. TOF combines with ion source, a flight tube, and a detector. It detects mass based on the difference velocities which depend on mass. The smaller molecules have the higher velocity. When the samples go into the flight tube at different velocities, the various mass ions are separated and particular ions enter to a detector. Tandem MS or MS-MS contains two mass spectral analyzers which are separated by collision gas cells. The sample is ionized and first detected by MS1 and then the selected ion is fragmented by collision process (collision-induced dissociation, CID) with high-pressure gas e.g. helium or nitrogen before analysis m/z by MS2 (Biddlecombe et al., 2004).

6. SMEDDS

SMEDDS is one of the techniques for enhancement of drug solubility and oral absorption of poorly-soluble drugs. It is an isotropic solution of oil, surfactant, and one or more hydrophilic solvents or co-surfactants which spontaneously forms the fine oil-in-water microemulsion on mild agitation in the presence of water or gastrointestinal fluid with gastrointestinal motility after oral administration. Oils from natural sources for example, castor oil, coconut oil, sesame oil, soy bean oil, olive oil, and sunflower oil are widely used in SMEDDS. The poorly water-soluble drug is solubilized in oil phase. Surfactant is the amphiphilic molecule composing of hydrophilic head and hydrophobic tail. The surfactants with high HLB (8-18) such as Cremophor EL, Cremophor RH 40, Tween 20, and Tween 80 are preferred for the formation of oil-in-water microemulsion. Surfactants cause the dispersing of poorly water-soluble drug in gastrointestinal fluids resulting in the spontaneous formation of small droplet of microemulsion. The SMEDDS with a small droplet size of less than 50 μm cause the reduction of the interfacial energy, increase the surface area for pancreatic lipase to hydrolyze oil, and promote a rapid release of the drug. In addition, one of components in SMEDDS may include co-surfactant which is the short chain alcohol such as propylene glycol, ethanol, polyethylene glycol, and glycerol. The co-surfactants adjust the HLB value of formulation, increase the fluidity of the interface, and damage liquid crystalline or gel structure which is the barrier of microemulsion formation (Gursoy and Benita, 2004, Patel et al., 2010, Sachan et al., 2010).

There is unknown mechanism of SMEDDS to increase oral absorption. However, some mechanism has been predicted such as the presence of the drug in a dissolved form and the small droplet size in gastrointestinal fluid producing a large interfacial surface area for drug absorption bringing about the increase of oral bioavailability. The SMEDDS also increases the membrane fluidity to facilitate transcellular absorption, opens tight junction to allow paracellular transport, and inhibits P-glycoproteins and/or CYP450 enzymes to increase intracellular concentration and residence time by surfactants. Moreover, oils have the benefit to increase the amount lipophilic drug transport through the intestinal lymphatic system (Patel et al., 2010, Sachan et al., 2010). Surfactants penetrate into intestinal cell

membranes and disturb lipid bilayers resulting in the increased oral absorption (Gursoy and Benita, 2004).

SMEDDS can be applied by filling in hard or soft gelatin capsules for oral administration. It is the lipid-based drug delivery systems which is easy to prepare and stable (Patel et al., 2010, Sachan et al., 2010).

Pseudo-ternary phase diagram is used to identify the self-microemulsifying regions and optimize the concentration of surfactants, co-surfactants, and oils. The diagram composes of oil, surfactant and/or co-surfactant, and water at each corner of the diagram represents 100% of that particular vehicle (Figure 20). Pseudo-ternary phase diagram is constructed by using water titration method or saline titration method. The components which show the broadest self-emulsifying regions with using small amount of surfactant are selected (Patel et al., 2010, Sachan et al., 2010).

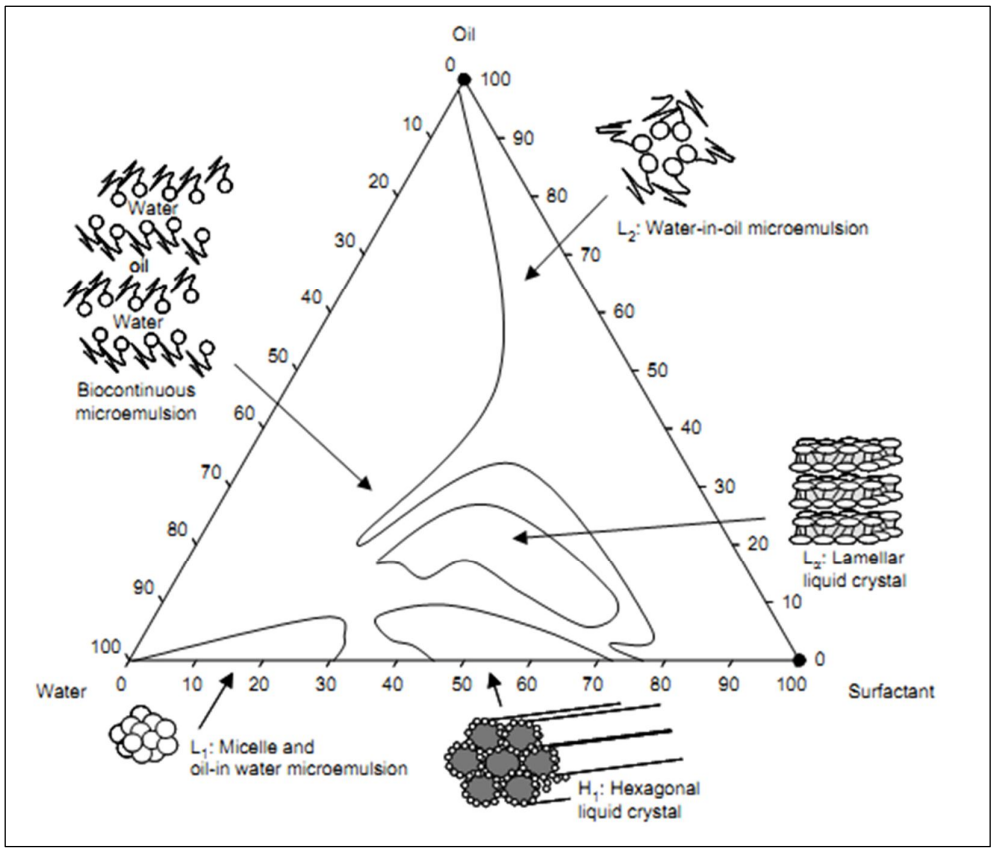


Figure 20 Example of pseudo ternary phase diagram (Cannon and Long, 2008)

Recently, there are many SMEDDS products that have been developed and success to improve drug solubility and oral absorption such as paclitaxel, acyclovir, simvastatin, vinpocetine, glyburide, and valsartan (Yang et al., 2004, Patel and Sawant, 2007, Meng and Zheng, 2007, Chen et al., 2008, Bachhav and Patravale, 2009, Dixit et al., 2010). In addition, the natural products have been prepared as SMEDDS such as *Pueraria lobata* isoflavone, curcumin, and silymarin (Cui et al., 2005, Wu et al, 2006, Cui et al., 2009). These substances were approved that can enhance drug solubility and improve oral bioavailability.

7. CD

The drug candidates that are poor water solubility remain to be one of the major challenges to the formulation scientists. Complexation with CD is one of the most well studied traditional solubilization techniques. The increased applications of CD, cyclic carbonates known to form complexes with hydrophobic drugs, and the successful approval of CD-containing products in the recent years have resulted in renewed interest in this technology (Tong and Wen, 2008).

CDs are cyclic oligosaccharides consisting of a variable number of D-glucose residues attached by α-(1, 4) linkages. There are three forms including α-, β-, and γ-CD which consist of six, seven, and eight D-glucose units, respectively. α-CD had the smallest cavity at the internal cavity size of 5 Å whereas β-CD and γ-CD have the larger cavity at the internal diameter of 6 and 8 Å, respectively. The structure has the cavity that is relatively hydrophobic because of CH_2 groups and the special arrangement of functional groups in the cavity, therefore making the cavity relatively non-polar. While, the outer size of CD expresses the hydrophilic. Drug molecule that contains an appropriate size can be included in the interior cavity by hydrophobic interactions, not involving covalent bond (Sinko, 1993).

Most of the CD derivatives presently in applications are synthesized from β-CD due to the appropriate internal cavity diameter. The structure of β-CD is shown in Figure 21. The β-CD, the host, admits the guest molecule to form the inclusion complex as shown in Figure 22. CDs are fairly stable in alkaline media, whereas they are hydrolytically cleaved by strong acids to give linear oligosaccharides. The

presence of guest molecules has been showed to decelerate the ring-opening rate of β-CD (Tong and Wen, 2008). There are many benefits of the complexation of drug and CD including enhancing drug solubility, increase oral bioavailability, enhancing drug stability, convert liquids and oils to free-flowing powders, decrease the evaporation and stabilize flavors, reducing odors and tastes, prevent the haemolysis as well as avoid the admixture incompatibilities (Davis and Brewster, 2004). CDs have been successfully used to solubilize insoluble drugs. Concerning bioavailability, both the rate and extent of absorption are typically enhanced. Not only is the blood concentration higher, but the area under the curve is also larger. It is generally believed that the mechanism of bioavailability enhancement by CD complexation is through solubility and dissolution rate improvement. However, it should be noted that CDs might also alter the lipid barrier of absorption site, which may contribute to the enhanced drug absorption. This effect of CDs on the lipid barrier can be attributed to the ability of CDs to form complexes with membrane components namely, cholesterol, phospholipids, and proteins (Tong and Wen, 2008).

There are many commercial drug-CD complexes namely, nimesulide (Nimedex®, Mesulid®), omeplazole (Omebeta®), piroxicam (Brexin®), benexate (Ulgut®, Lonmiel®), tiaprofenic acid (Surgamyl®), hydrocortisone (Dexocort®), and itraconazole (Sporonox®) (Davis and Brewster, 2004).

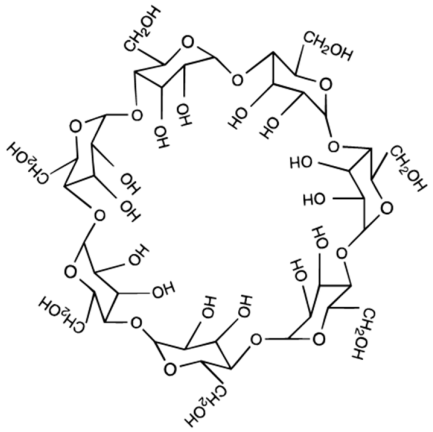


Figure 21 Structure of β-CD (Cornors, 1997)

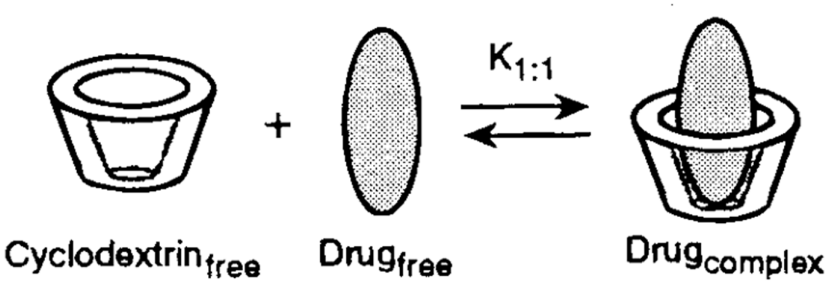


Figure 22 Reaction of CD-drug formation (Stella and Rajewski, 1997)

There are many studies revealing nephrotoxicity of β-CD when it is used in parenteral route. The reason of nephrotoxic is unknown however; the scientists have suggested that kidney tubule cells uptake β-CD causing disturbance the intracellular function. Besides, β-CD may extract lipid membrane components of kidney tubule cells. Consequently, the modification of β-CD derivatives has been developed to prevent the nephrotoxicity. Hydroxypropyl-β-CD (HP-β-CD) is the derivative which is an amorphous and non-crystalline nature. It has been applied with its benefits for example, it is safe in parenteral route and it is greater water soluble than that of β-CD. Therefore, the utilization of HP-β-CD is increasing (Stella and Rajewski, 1997).

The preparation of complexes can be performed to various methods such as coprecipitation, neutralization, kneading, freeze-drying, spray-drying and coevaporation, and grinding methods. The coprecipitation and kneading method are not useful for water-insoluble drugs unless organic solvents can be used. The major problem with using organic solvents is that most organic solvents will compete for inclusion in the CD cavity, and therefore inhibit complex formation. The neutralization method takes advantage of acidic or basic functional groups and is rather useful for insoluble drugs. Nonetheless, the drugs are stable in acidic or basic conditions. The absence of water in the grinding method makes it suitable for drugs that are unstable in water (Tong and Wen, 2008).

In order to characterize the complexation, Differential Scanning Calorimetry (DSC), X-ray powder diffraction (XRD), Fourier transforms infrared spectroscopy (FT-IR), and X-ray crystallography are applied (Tong and Wen, 2008). DSC is used

to confirm whether inclusion complex or physical mixture which is prepared by mixing drug and CD in a mortar. If the complex is formed, its thermogram should be different to that of the physical mixture, which will show the combination of CD and drug. In the complex thermogram, the melting peak of the drug is usually absent; it is considered that drug is completely dispersed in the cavity of CD, as shown in Figure 23.

For XRD analysis, the drug displays sharp crystalline peak, which is the characteristic of an organic molecule with cystallinity. CD shows an amorphous structure lacking sharp peak. Therefore, the XRD spectrum of inclusion complex is similar to XRD spectrum of single CD. While, the XRD spectrum of physical mixture composes of the spectrum of the drug and CD (Figure 24).

Besides DSC and XRD, FT-IR is the common instruments for characterization the inclusion complex. IR spectrum of inclusion complex is similar to that of CD whereas IR spectrum of physical mixture composes of the spectrum of the drug and CD (Figure 25).

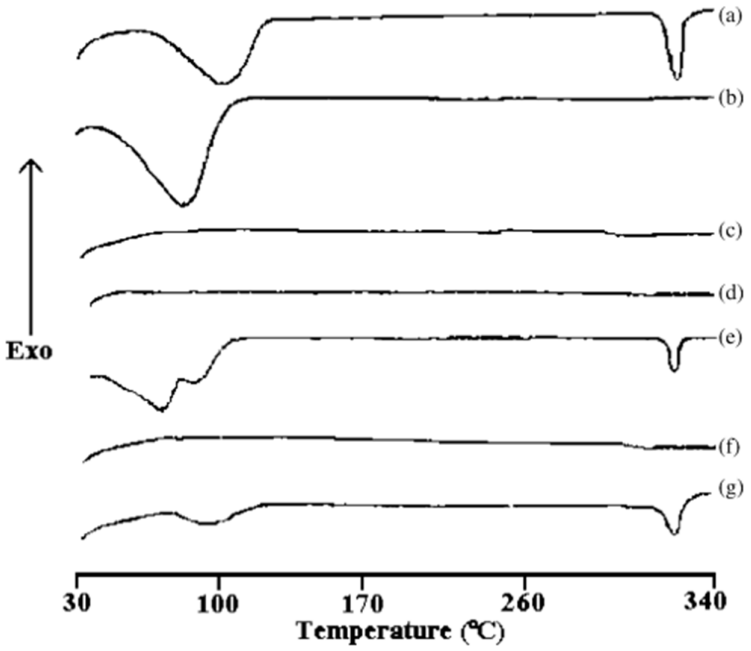


Figure 23 DSC curves: (a) quercetrin, (b) β-CD, (c) HP-β-CD, (d) inclusion complex between β-CD and quercetrin, (e) physical mixture between β-CD and quercetrin, (f) inclusion complex between HP-β-CD and quercetrin, and (g) physical mixture between HP-β-CD and quercetrin (Parlhad and Rajenfrakumar, 2004)

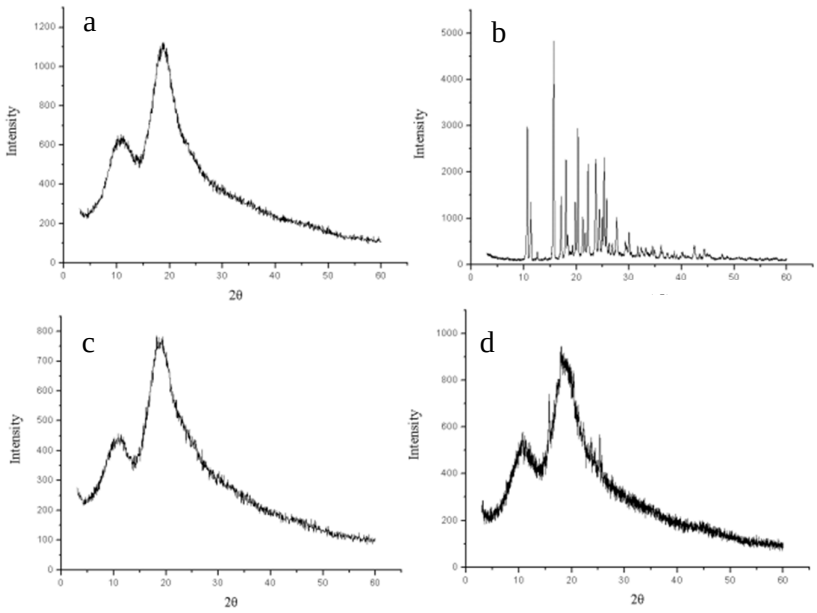


Figure 24 XRD spectrum: (a) HP-β-CD, (b) naringenin, (c) inclusion complex between HP-β-CD and naringenin, and (d) physical mixture of HP-β-CD and naringenin (Wen et al., 2010)

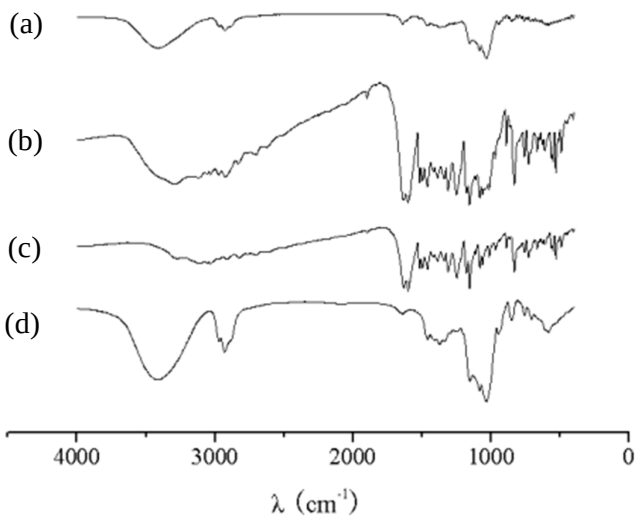


Figure 25 FT-IR spectrum: (a) inclusion complex between HP-β-CD and naringenin, (b) physical mixture of HP-β-CD and naringenin, (c) naringenin, and (d) HP-β-CD (Wen et al., 2010)

8. Caco-2 cells

Caco-2 cell culture technique is one of the models for assessing drug absorption. Caco-2 cells are human colon carcinoma cell lines. The cells undergo enterocyte like differentiation in culture and spontaneously differentiate into polarized columnar cells that are representative of the small intestine, with developed microvilli, many of the transport systems namely, amino acid, small peptide, bile acid, glucose, and vitamin B12 transporters, P-glycoprotein efflux system, and polarized distribution of brush border enzymes such as alkaline phosphatase, sucrase, and amino peptidases. Caco-2 cells attribute many mechanisms of drug transport including passive transport both of paracellular and transcellular, carrier-mediated transport, transcytosis, active transport, and also P-glycoprotein efflux. In addition, CYP450 enzymes and some of conjugation enzymes are found in the cells. Caco-2 cells are grown on porous membrane as shown in Figure 26, usually for a period of 15-21 days in culture medium. The cells form confluent monolayers, leading to increasing tightness of the cell layer following. The tight junctions result in the greater transepithelial electrical resistance (TEER) values which these values use to evaluate the monolayer of the cells. TEER values should be in the range of 300 – 750 $\Omega.cm^2$ which assure the formation of proper monolayers with efficient tight junction (Artursson et al., 2001; Ashford, 2007; Habucky, 1995; Ungell and Artursson, 2009).

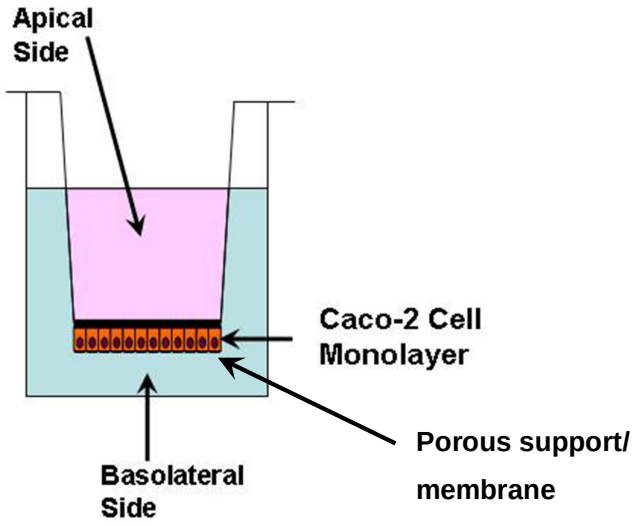


Figure 26 Caco-2 cell culture (http://www.apredica.com/caco2.php, 2010)

As the characteristics of Caco-2 cells are similar to small intestine, therefore the cells are useful tools for understanding the mechanism of absorption of drugs not only passive transport but also active transport. Moreover, this technique decreases the number of animals, require only small amounts of compound for transport studies, and can be used as a rapid screening tool for evaluation the drug absorption. Nevertheless, there are many disadvantages. From a practical viewpoint, maintaining the cell line requires a designated aseptic area, training in aseptic techniques, and continuous maintenance of the cell line as the monolayer throughout the transport process. Caco-2 cells spend a large number of times to growth and ready to study absorption. From a physiological standpoint, the Caco-2 cells do not produce the mucus as observed in the small intestine, and the tight junction characterizes more like the colon than that of small intestine. The disappearance of drug on the apical side of the membrane is not reflected that the 100% of drug can cross the membrane to basolateral side but the drug may bind to the membrane. This will need investigation, or the drug may have a stability issue. Therefore, the drug that is studied by using Caco-2 cell technique has to be non-binding to the membrane and resist enzymes secreted by the cells. Moreover, the permeable support membrane and

the thickness of the unstirred water layer have been concerned (Ashford, 2007; Habucky, 1995).