

PRECLINICAL PHARMACOKINETICS AND BIOAVAILABILITY STUDIES OF
S-CARBOXYMETHYLCYSTEINE, N-ACETYLCYSTEINE AND (-)-CARBODINE

by

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DEDICATION

This dissertation is dedicated with love and gratitude to my parents, Weibin Shen and Huijun Jiang.

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CHAPTER 1

INTRODUCTION

Bioavailability refers to the rate and extent of absorption of a drug from its dosage form into the systemic circulation. It is usually assessed by the maximum drug plasma concentration (C_{max}), time to reach C_{max} (T_{max}), and the area under the plasma concentration-time curve (AUC). Absolute bioavailability compares the AUC of a drug following non-intravenous administration with the AUC of the same drug following intravenous administration. Relative bioavailability compares the AUCs of a drug when administered via different routes or formulations. Relative bioavailability studies comparing drug absorption from different dosage forms are, in essence, bioequivalence studies. Bioequivalence studies are usually performed to compare the bioavailability of a new formulation with that of a recognized standard formulation.

Bioavailability studies are carried out for both preclinical and clinical evaluation of a drug. Pharmacokinetic parameters generated from bioavailability studies including the rate and extent of drug absorption, distribution, metabolism and elimination are important for screening pharmacologically active intermediates and prodrugs, determining appropriate drug formulation and route of administration, and establishing recommended dosage regimens (e.g., dose, dose interval).

Chapter 2 gives a detailed literature review on drug bioavailability, including

introduction of bioavailability and bioequivalence, principles of drug dissolution and drug absorption, physicochemical and physiological factors that affect bioavailability, formulation effects on bioavailability, models in bioavailability assessment, and experimental design of bioavailability studies (1-3).

The mucoregulating agent S-carboxymethylcysteine has been used for many years in the treatment of chronic obstructive pulmonary diseases (4-6), otitis media with effusion (7), and for nutritional effects as an antioxidant (8-9). Chapter 3 describes the pharmacokinetics and bioavailability of a buccal formulation of S-carboxymethylcysteine in comparison to oral and IV routes of administration.

N-acetylcysteine has been used as a mucolytic agent in the treatment of patients suffering from chronic obstructive pulmonary diseases (10-13) and as an antidote for acetaminophen overdose (14-16). Chapter 4 studies the pharmacokinetics and bioavailability of an oral wafer formulation of N-acetylcysteine in comparison to a marketed capsule formulation.

(-)-Carbodine has activity both in vitro and in vivo against many DNA and RNA viruses such as Venezuelan equine encephalitis virus (17-21). The brain is a primary target organ for this antiviral agent. Chapter 5 sets up an HPLC method for the quantification of (-)-carbodine in mouse plasma, brain, liver and kidney, and determines the basic pharmacokinetic parameters and tissue distribution of (-)-carbodine in mice.

Chapter 6 summarizes the final conclusions.

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CHAPTER 2

LITERATURE REVIEW

1. Introduction

Definition

Bioavailability refers to the rate and extent of absorption of a drug from its dosage form into the systemic circulation. It is usually assessed by the maximum drug plasma concentration (C_{max}), time to reach C_{max} (T_{max}), and the area under the plasma concentration-time curve (AUC).

Absolute bioavailability compares the AUC of a drug following non-intravenous administration with the AUC of the same drug following intravenous administration. Relative bioavailability compares the AUCs of a drug when administered via different routes or formulations. Absolute bioavailability is usually less than one but relative bioavailability can be larger than one. The calculation for bioavailability can be corrected for dose and clearance.

Bioavailability and bioequivalence

Relative bioavailability studies comparing drug absorption from different dosage forms are, in essence, bioequivalence studies. The United States Food and Drug Administration (FDA) has defined bioequivalence as, "the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in

pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study." Bioequivalence studies are usually performed to compare the bioavailability of a new formulation with that of a recognized standard formulation.

Clinical significance of bioavailability and bioequivalence studies

Bioavailability studies provide useful information on the determination of appropriate route of administration and dosage regimen (e.g., dose, dose interval). Bioequivalence studies focus on the development of new drug formulation, which is interchangeable with the standard formulation.

2. Principles of drug dissolution and drug absorption

Dissolution rate theory

The release of a drug from its dosage form is of utmost importance in the process of drug absorption. An active ingredient in a solid dosage form must undergo dissolution before it is available for absorption. In 1897, Noyes and Whitney formulated the Noyes-Whitney equation: $dc/dt = kS (C_s - C_t)$ where dc/dt is the rate of dissolution, k is the dissolution rate constant, S is the surface area of the dissolving solid, C_s is the saturation concentration of drug in the diffusion layer and C_t is the concentration of drug in the bulk dissolution medium. One way to increase the bioavailability of a poorly soluble drug is to increase the surface available thereby reducing the particle size for dissolution.

Drug absorption

Drug absorption includes absorption from skin, muscle, gastrointestinal tract, etc.

Since most drugs are developed for oral administration, our discussion will be limited to gastrointestinal drug absorption. Absorption in the GI tract occurs through the mucosal epithelium, which extends from the oral cavity to the anus. Saliva is secreted in the oral cavity and has a pH of 6-7. Many drugs can be dissolved in saliva and absorbed sublingually or buccally. The stomach pH is about 1-3, where weak acids are unionized and easily absorbed. Basic drugs are solubilized rapidly in the presence of gastric acid, increasing the rate of dissolution. However, both acidic and basic drugs are primarily absorbed in the small intestine. The small intestine has a pH of 6-8 and is primarily responsible for the absorption of most drugs regardless of ionized or unionized forms because of its large surface area. The optimum site for drug absorption after oral administration is the duodenum. The large surface area of the duodenum is due to small projections known as villi and microvilli. The large intestine has less surface area for drug absorption. The rectum can also be used for drug administration.

Passive diffusion is the most common mechanism of drug absorption. According to Fick's law of diffusion, passive diffusion is described by the following equation: $dQ/dt = DAK^*(C_{GI}-C_p)/h$ where dQ/dt is the rate of diffusion, D is the diffusion coefficient, A is the surface area, K is the lipid/water partition coefficient of the drug, h is the thickness of the membrane, and $C_{GI}-C_p$ is the concentration difference of the drug between the GI tract and the plasma. After drugs cross the cell membrane, the clearing system consisting of the blood and lymph removes the drugs from the absorption site. Besides passive diffusion, other mechanisms such as facilitated diffusion and active transport are involved

in moving substances across the membrane. These highly selective carrier-mediated systems are principally used for transporting nutrients and natural substrates across biologic membranes.

First pass metabolism

Before reaching the systemic circulation, an orally administered drug may be subjected to drug metabolism, which is called the first pass effect. Drugs are absorbed by the gut wall and transported via the mesenteric vessels to the hepatic portal vein and then to the liver. First pass metabolism of a drug can occur in the gastrointestinal lumen, gut wall, liver, and lungs. Enzymes located in these sites metabolize the drug before it enters the systemic circulation. Other routes of administration like intravenous, buccal, sublingual and rectal avoid first pass metabolism because drugs are directly absorbed into the systemic circulation.

3. Physicochemical factors that affect bioavailability

Particle size

Since dissolution rate is directly proportional to surface area and small particles have more surface area per unit mass or volume than large particles, it is reasonable to reduce particle size to increase absorption of a poorly soluble drug, thereby increasing its bioavailability. In recent years, particle size reduction studies have been conducted for several compounds such as ferric pyrophosphate, biphenyl dimethyl dicarboxylate (BDD), realgar and delta tocopherol (1-5). The findings suggested that size reduction of regular

particles to nano levels can greatly enhance their bioavailability.

pKa

Since most drug molecules are weak acids or bases, the extent of ionization of these drugs is determined by the molecule's pKa and the environmental pH in which it is dissolved. Modification of environmental pH may affect absorption of certain drugs. For example, the pH of gastric fluids can be increased by administering antacids (e.g., sodium bicarbonate), which may increase the absorption of weak bases or decrease the absorption of weak acids. Alternatively, a more water-soluble salt of an acidic drug may, in turn, increase the absorption because salt forms of drugs usually increase dissolution. Itraconazole is a weak basic antifungal drug. When taken with orange juice or cola, it forms a salt and increases its absorption. Lohitnavy et al. (6) found that the rate and extent of itraconazole's oral absorption were markedly decreased by concurrent use of an antacid suspension. Hence, co-administration of itraconazole and an antacid suspension should be avoided.

Partition coefficient

Lipid/water partition coefficient is an important parameter in the development of new drugs. An increase in absorption may be achieved through minor structural modifications of a parent compound without significantly altering its intrinsic pharmacological properties. Brandl et al. (7) reported that R1626, a lipophilic prodrug of the nucleoside analog R1479, had a higher lipid/water partition coefficient than the parent compound.

Therefore, R1626 easily penetrated across the cell membrane and was rapidly hydrolyzed by the enzymes in the cellular membrane, releasing the anti-HCV agent R1479 into the cytoplasm. This suggested that R1626 could be a potent candidate for the treatment of HCV when administered as the prodrug.

Polymorphism and amorphism

Many drugs such as carbamazepine (8) are capable of existing in more than one crystal form, which is known as polymorphism. Polymorphic compounds usually exhibit different physical-chemical properties, such as solubility, melting point and chemical reactivity, which in turn may affect the biopharmaceutical properties of active ingredients or excipients (compatibility, dissolution rate, bioavailability, pharmacological activity, stability) (9-10). Usually, less stable polymorphic forms have higher solubility and an increased mean absorption rates (11).

Besides polymorphic forms, many compounds may exist in amorphous forms. The amorphous form of a compound is always more soluble than a corresponding crystal form and may exhibit different biopharmaceutical properties. The engineering of pharmaceutical alloys by solubilizing and stabilizing carriers, commonly termed solid dispersions, provide avenues for exploiting the benefits of amorphous systems (12). Atazanavir (ATV) is a low oral bioavailability compound and, clinically, is generally coadministered with ritonavir (RTV), which boosts the oral bioavailability of ATV by inhibiting cytochrome P450 (CYP) 3A and P-glycoprotein (P-gp) via the same pathway.

In order to improve the bioavailability of ATV without co-administration of with RTV, Fukushima K et al. (13) dispersed ATV in sodium lauryl sulfate (SLS) to form an amorphous state, which resulted in a 3.5 to 4.7 fold increase in bioavailability compared with bulk ATV.

Stability

Drug stability in gastrointestinal fluids must be considered since reactions which result in degradation of drug will reduce bioavailability and therapeutic effectiveness. The most important reactions that drugs undergo in the GI tract are acid hydrolysis and enzymatic hydrolysis.

Acid hydrolysis

Penicillin G and omeprazole are examples of drugs that are inactivated by acid hydrolysis in the stomach (14). Enteric coatings and the development of prodrugs or derivatives are strategies that can prevent exposure of these drugs to gastric pH and hence improve their therapeutic effectiveness (11).

Enzymatic hydrolysis

Many drugs, particularly peptides and proteins (e.g. insulin), are subjected to enzymatic hydrolysis in the GI tract when administered orally. Recently, protease inhibitors were investigated as a means to prevent hydrolysis. FK-448 is a potent and specific protease inhibitor, which suppressed the digestion of insulin by pancreatic

enzymes and enhanced its intestinal absorption. The increase in absorption of insulin was related to the inhibition of digestive enzymes, primarily chymotrypsin (15-16).

Some compounds are degraded by bacteria that secrete certain proteases in the GI tract, which contributes to reduced oral bioavailability. Glycosides play a very important role in Chinese traditional medicine. However, the bioavailability of glycosides is low because they are metabolized into aglycons in the presence of intestinal bacteria before they are absorbed (17).

4. Physiological factors that affect bioavailability

GI fluids

The properties and contents of GI fluids will influence drug absorption primarily by affecting dissolution rate. The pH is 1-3 in the stomach, 5-6 in the duodenum, 7-8 in the proximal jejunum and finally reaches 8 in the large intestine. Usually acidic drugs are more rapidly absorbed in acidic conditions and basic drugs are more rapidly absorbed in alkaline conditions. However, both acidic and basic drugs are most rapidly absorbed in the small intestine, due to its large surface area (18).

In addition to the pH considerations, the GI fluids contain a variety of materials that have been shown to have great impact on drug absorption. Of particular interest are bile salts and various enzymes (discussed above). Bile salts, as surfactants, are amphipathic and can self-associate to form micelles, which may improve the bioavailability of poorly water-soluble drugs by enhancing their solubility and dissolution (19).

Gastric emptying

Since most drugs are extensively absorbed from the small intestine, gastric emptying may be a rate-limiting step in the process of absorption. Therefore, those drugs that need to exert their therapeutic effectiveness immediately must be taken before a meal. Gastric emptying is primarily influenced by meal volume, osmotic pressure, and the presence of specific food components. Food, especially those with high fat content, can delay gastric emptying for 3-6 h or more (20), which is good for the absorption of poorly water-soluble drugs with low dissolution rates. In addition, fatty foods stimulate bile secretion, which also improves bioavailability of poorly water-soluble drugs. Chen et al. (21) investigated the effect of food on the pharmacokinetics of rifalazil, a new antibiotic structurally related to rifampin, and found that the systemic exposure to rifalazil based on C_{max} , $AUC(0-T_{last})$, and $AUC(0-\infty)$ was increased progressively as the fat content of the test breakfast was increased from 30% to 60% compared with fasting. This food effect might be a result of delayed gastric emptying by increased dietary fat content. Alhamani et al. (22) also reported that delay in gastric emptying enhances bioavailability of sodium salicylates.

Intestinal transit

Intestinal transit time is as important as gastric emptying time since the small intestine is the primary site of drug absorption. The greater the intestinal motility, the shorter the residence time, which decreases time for drug dissolution and absorption in

the duodenum. As with gastric emptying, there are a couple of factors such as food that will influence intestinal motility and drug absorption. Although food stimulates intestinal transit, it also enhances mixing movements in the intestine, which can increase the rate of drug dissolution. The general consideration made in the previous section still applies. Other factors such as disease states also affect intestinal transit. For example, diarrhea can shorten intestinal transit time, hence reduce drug bioavailability.

Gut wall metabolism

Many drugs are metabolized in the gut wall, especially in intestinal epithelium cells, resulting in reduced bioavailability. Currently, a variety of metabolic enzymes in the gut wall have been identified, including CYP2D6, CYP2C9, CYP2C19, CYP3A4, and CYP3A5. The CYP3A (mainly CYP3A4) family accounts for approximately 80% of the total CYP activity in the gut wall, which is much higher than the ratio in liver (40%) (23). Many clinically important therapeutic drugs are substrates of CYP3A and therefore are subjected to extensive phase I metabolism in the gut wall. Grapefruit juice is known as an intestinal CYP3A4 inhibitor (24, 25) and may improve the bioavailability of many CYP3A4 substrates, such as midazolam, triazolam, diazepam, felodipine (26) and cyclosporine A (27). But the bioavailability of drugs which do not undergo significant intestinal or hepatic metabolism, such as fexofenadine (28), may be reduced when administered with grapefruit juice. Fexofenadine is a weak acid and is ionized in grapefruit juice resulting in a decreased absorption.

P-glycoprotein

P-glycoproteins (P-gp) are part of a larger family of efflux transporters found in the gut, gonads, kidneys, biliary system, brain and other organs. Since P-glycoproteins block absorption in the gut, they should be considered part of the "first-pass effect". Some drugs like cyclosporin are both substrates and inhibitors of P-gp, other drugs like nifedipine are inhibitors only and some drugs like digoxin are only substrates. In most cases, P-gp and CYP3A4 are induced by many of the same compounds and demonstrate a broad overlap in substrate and inhibitor specificities, suggesting that they act as a concerted barrier to drug absorption (29). Inhibition of P-gp and CYP3A4 (discussed above) will greatly enhance the oral bioavailability of many drugs. Malingre et al. (30) reported that co-administration of cyclosporine, an efficacious inhibitor of P-gp and substrate for CYP 3A4, strongly enhanced the oral bioavailability of docetaxel from 8% to 90%. Some of the excipients such as PEG-300, Cremophor EL, and Tween 80 are also able to inhibit P-gp activity (31).

Blood flow

The GI tract is well perfused by the blood stream, which removes drugs from the absorption sites. The rate-limiting step in the absorption of the compounds that readily penetrate the intestinal membrane may be the rate at which blood perfuses the intestine. In contrast, the absorption of poor permeable drugs will be largely independent of blood flow. Blood flow to the GI tract is temporally increased shortly after a meal and this may

last for several hours. The bioavailability of those drugs with high hepatic extraction ratio may be increased if taken with a meal (32). For example, concomitant food intake can increase the bioavailability of propranolol by 67% because of faster portal vein and hepatic blood flow and less first pass metabolism (33, 34). Disease states also change blood flow, which will be discussed later.

Drug-food interaction

There are a lot of mechanisms that apply to drug-food interactions. Some of them such as the influence of high fat food, the influence of food on gastric emptying, intestinal transit and hepatic blood flow have been discussed above. Other possible mechanisms involved, for example, are adsorption of drug molecules onto food, food-induced secretion of gastrointestinal enzymes, increased GI fluid viscosity and food components competing with the drug for absorption (32).

Drug-drug interactions

Adverse drug interactions present alarming problems for our society and combination therapy must be carefully designed and controlled before use, especially for those drugs with a narrow therapeutic window. The following mechanisms appear to be the primary forms of drug-drug interactions that influence drug absorption and bioavailability (33-35):

- (1) Changes in gastric emptying and intestinal transit
- (2) Changes in enzymatic activities (e.g. CYP)

(3) Induction or inhibition of transporters (e.g. P-gp)

Engelbrecht and Shargel (36) reviewed some drug-drug interactions that affect the bioavailability of the drug, which were listed in table 2.1.

Disease states & physiological disorders

Cardiovascular diseases, liver diseases, diarrhea, kidney diseases, obesity, cancer, surgery, even stress and mood can all affect bioavailability. For example, congestive heart failure is associated with hypoperfusion of the liver and kidneys (37). Many hepatic diseases such as cirrhosis and hepatitis reduce the extent of first pass metabolism and increase oral bioavailability (38-40). Therefore, in chronic liver disease with cirrhosis, dosage reduction is the general rule regardless of the route of elimination of drug. Diarrhea reduces drug bioavailability since there is less time for drug dissolution and absorption because of the rapid intestinal transit time. Renal failure affects bioavailability by altering intestinal and hepatic drug transport and metabolism (41, 42). Animal studies in chronic renal failure have shown a significant decrease (40-85%) in hepatic and intestinal CYP metabolism. High levels of parathyroid hormone, cytokines and uremic toxins have been shown to reduce CYP activity. Phase II reactions and drug transporters such as P-glycoprotein and organic anion transporting polypeptide are also affected.

Sex

Sex-based differences in bioavailability stem from variations between men and women in factors such as body weight, plasma volume, gastric emptying time, plasma

protein levels, hormones, cytochrome P450 activity and drug transporter function. GI motility is influenced by sex hormones, implying that sex-based differences in motility may exist and that transit times in women may vary throughout pregnancy and the menstrual cycle (43, 44). Gastrointestinal enzymes responsible for drug metabolism also vary by sex. For example, gastric alcohol dehydrogenase activity is higher in males than in females (45, 46), resulting in higher blood concentrations of ethanol in females compared to males following an equivalent ingestion. Data on sex differences of CYP and P-gp expression and activity exists but is inconsistent (47, 48). Overall, women have been reported to have a 1.5–1.7-fold greater risk than men of experiencing an adverse drug reaction to medications (49). Detailed pharmacokinetic studies in men and women are particularly important for agents used in the treatment of diseases, such as HIV and cancer, which require lifelong therapy.

Age

The pharmacokinetics of children, particularly infants and old people are tremendously different from that of adults (50, 51). Infants and children have high gastric pH, fast gastric emptying, slow intestinal motility, and low hepatic metabolism. Older people, in general, appear to have decreased gastric emptying and intestinal transit, decreased blood flow, as well as reduced CYP450 activity. It is important to evaluate drug products in the young and elderly patients, not just simply reduce the dose.

Other factors

Other factors such as race (52) and genetic polymorphism (53) can also affect drug bioavailability.

5. Formulation effects on bioavailability

According to the rate at which a dosage form releases the drug into the biological fluids, the oral bioavailability of a drug in different formulations normally decreases in the following order: solutions > suspensions > capsules > tablets. Sustained-release formulations have a prolonged therapeutic effect, but it is hard to rank its oral bioavailability. Numerous factors including stability, lag time, duration of action, taste, convenience to the patients and cost should be considered when developing a new formulation.

6. Models in bioavailability assessment

In vitro models

Cultured intestinal epithelial cell models: T84, HT-29, Caco-2 cells

The human colon adenocarcinoma cell lines T84, HT-29, and Caco-2 have been widely used to study intestinal transport. T84 cells are mainly used for studying tight-junction regulation (54). HT-29 cell lines can secrete mucus and are used to study the role of the mucus layer in drug absorption (55). Caco-2 cells, which are the most widely used with in vitro assays to predict the absorption rate of candidate drug compounds across the intestinal epithelial cell barrier, undergo spontaneous enterocytic differentiation in culture (56). The ability of Caco-2 cells to achieve a higher degree of enterocytic differentiation

than that of HT-29 cells makes this cell line more morphologically and biochemically similar to the small intestinal columnar epithelium and extremely useful for mechanistic studies of drug absorption (57).

Everted intestinal sacs and intestinal rings

In 1954, Wilson and Wiseman described the use of everted intestinal sacs for the study of drug absorption (58). Eversion of the intestinal sacs allows the mucosal surface to be directly exposed to the oxygenated buffer containing drug. In addition, the small serosal volumes yield sufficiently high drug concentrations for chemical analysis. Compared to everted intestinal sacs, everted intestinal rings are simpler and more readily adapted to human studies. However, everted intestinal rings are more widely used as uptake or accumulation models of drug absorption process (59). Both models are also used to assess gut wall drug metabolism (60).

Isolated perfused liver

Isolated and cultured hepatocytes have been widely used to characterize hepatic drug uptake, hepatic metabolism, drug interactions affecting hepatic metabolism and hepatotoxicity (61-63). However, the rapid loss of liver specific functions limits their application. The isolated perfused liver is an ideal model for assessing hepatobiliary disposition of drugs without the influence of many nonhepatic factors that are hard to control in vivo. Meanwhile, it preserves the integrity of hepatic architecture, cell polarity and bile excretion. Kim and Ronald (64) compared the advantages and disadvantages of the isolated perfused liver to other models, which are listed in table 2.2.

In vivo models

Ligated gastrointestinal segments

Specific segments of the GI tract may be isolated and studied with intact blood perfusion. Multiple adjacent ligated loops can be studied in the same animal simultaneously. Levine et al. (65) used single and multiple intestinal loops to study the absorption of quaternary ammonium compounds.

Hepatoportal cannulation

Hepatoportal cannulation is used to distinguish between hepatic and gastrointestinal “first-pass metabolism”. Colter et al. (66) investigated the reduced oral bioavailability of levodopa. Four dogs received ¹⁴C-levodopa in a crossover pattern via hepatoportal catheter, intravenous, and oral administrations. The plasma concentration-time curves following hepatoportal and intravenous administrations were almost identical, indicating that the impaired oral bioavailability of levodopa exclusively occurred within the GI tract and/or gut wall.

Bile duct cannulation

Biliary excretion may result in fecal elimination of drugs or reabsorption of drugs from the GI tract, which is called the enterohepatic circulation. In either case, the oral bioavailability will be affected. Many polar and high molecular weight compounds are subjected to biliary excretion. The excretion profiles of loperamide hydrochloride (67), diltiazem (68), isotretinoin (69) and tiflorex (70) have been studied using bile duct cannulation.

7. Experimental design of bioavailability studies (71-73)

Pilot study

A pilot study in a small number of subjects is recommended before carrying out a full-scale bioavailability study. This study can be used to assess individual variability, determine sampling schedule, optimize dose regimen and provide other information.

Crossover design

Crossover design is extensively used in bioavailability studies. In this type of study, clearance, volume of distribution, and absorption, as determined by physiological variables (e.g. gastric emptying, motility, pH), are assumed to have less interoccasion variability compared to the variability arising from formulation performance. Therefore, differences between two products due to formulation factors can be determined.

According to the number of formulations, a 2×2 or 3×3 crossover study can be performed. For example, the standard two-formulation, two-period, two-sequence crossover design can be used to generate data for comparing two different formulations. Between each treatment, an adequate washout period (e.g. more than 5 half lives of the moieties to be measured) is required.

Some drugs or metabolites have very long half-lives. In this case, crossover studies may not apply and parallel designs are used, but more subjects are needed. For some highly variable drugs, replicated crossover designs may offer some advantages: (1) allow comparisons of within-subject variances for the test and reference products, (2) provide

more information about the intrinsic factors underlying formulation performance, and (3) reduce the number of subjects.

Subjects

Preclinical study

The choice of an animal model depends on previous pharmacological studies, previous experience with related compounds and the purpose of the study design.

Advantages and disadvantages of various animal models are listed below:

(1) Dog

- can ingest various types of dosages forms
- can be dosed repeatedly
- can provide sufficient number of samples for analysis
- similarities in the physiology of GI tract in dogs and man

(2) Monkey

- difficulty to handle
- used when the dog is known to handle a drug class differently from man

(3) Pig

- gastrointestinal, cardiovascular and renal physiologies closely resemble man
- excellent choice but too big to be handled in a lab
- miniature pig is a good choice

(4) Small animals (mouse, rat and hamster)

- poor choices

- cannot ingest most formulations intact
- repeated biological specimens are difficult to obtain
- most used in preliminary studies

(5) Rabbit

- poor choice
- long gastric emptying and difficult to get an empty stomach

Clinical study

The selection of subjects is aimed to minimize variability and permit differences between pharmaceutical products. Therefore, healthy volunteers of 18-55 years old and of weight within the normal range (Body Mass Index = 19-24) are qualified. Subjects should preferably be non-smokers and without alcohol or drug abuse history. Subjects should belong to either sex, but pregnancy and the menstrual cycle should be considered on an individual basis. If the investigated compound has adverse effects, which are unacceptable for healthy volunteers, it may be necessary to use patients.

Study design

Usually, the minimum number of subjects should not be smaller than 12 unless justified. The test conditions (e.g. diet, fluid intake, exercise) should be standardized. Subjects should be fasted at least 12 hours prior to the administration of the investigated compound. Compounds can be orally administered with about 200 ml of water. Before and during each study phase, it is recommended that subjects are allowed water except

for 1 hour before and after drug administration, and are provided standard meals no less than 4 hours after drug administration.

Sampling

For most drugs, 12 to 18 blood samples, including a predose sample as a control, are collected per subject per treatment: 3-4 in the absorption phase, 3-4 around peak concentration and 4-6 in the elimination phase. Usually, the sampling should continue for 3-5 terminal half lives of the drug. If the predose value is higher than 5% of C_{max}, the subject is dropped from the group.

Pharmacokinetic parameters

The following pharmacokinetic parameters should be reported: AUC, C_{max}, T_{max}, t_{1/2}, CL, V_d, K_a, F, etc. For multiple-dose studies, AUC_t, C_{ss-max}, C_{ss-min}, C_{av}, degree of fluctuation $((C_{ss-max} - C_{ss-min})/C_{av})$, and swing $((C_{ss-max} - C_{ss-min})/C_{ss-min})$ should be included.

Statistical analysis

Pharmacokinetic parameters derived from measures of concentration, e.g. AUC, C_{max} should be analyzed using analysis of variance (ANOVA). The method is based upon the 90% confidence interval for the ratio of the population means (Test/Reference), which is equivalent to the corresponding two one-sided tests with the null hypothesis at the 5% significance level. Normally, the 90% confidence interval for AUC-ratio and C_{max}-ratio should lie within an acceptance interval of 0.8-1.25.

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Table 2.1. Overview of drug-drug interactions that affect the bioavailability of the drug from GI tract^a.

Drug interaction	Examples (Precipitant Drugs)	Effect (Object Drugs)
Complexation/chelation	Calcium, magnesium, or aluminum and iron salts Sodium polystyrene sulfonate	Levofloxacin complexes with divalent cations, causing decreased bioavailability Cations in antacids bind to sodium polystyrene sulfonate, causing reduced renal clearance of bicarbonate, resulting in systemic acidosis
Adsorption	Cholestyramine Kaolin Activated charcoal	Decreased bioavailability of digoxin, levothyroxine Decreased bioavailability of digoxin Decreased bioavailability of many drugs
Increased GI motility	Acarbose Laxatives, cathartics	Decreased bioavailability of digoxin Increases GI motility, decreases bioavailability for drugs that are absorbed slowly; decreases bioavailability of drugs from controlled-release products
Decreased GI motility	Anticholinergic agents	Propantheline decreases gastric emptying of acetaminophen, delaying acetaminophen's absorption from small intestine
Alteration of gastric pH	H ₂ -blockers, antacids, and proton pump inhibitors	Increase gastric pH; dissolution of ketoconazole is reduced at basic pH, causing decreased drug absorption and therapeutic failure
Alteration of intestinal flora	Antibiotics	Digoxin has better bioavailability taken after erythromycin; erythromycin administration reduces bacterial inactivation of digoxin Estrogen/progestin birth control requires intestinal flora to facilitate enterohepatic circulation; antibiotics reduce estrogen/progestin levels, resulting in failure of ovulation suppression and menstrual changes
Inhibition of drug metabolism in intestinal cells	Monoamine oxidase inhibitors (e.g., tranlycypromine, phenelzine)	Inhibit metabolism of albuterol and levalbuterol, leading to hypertension

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Table 2.2. Advantages and disadvantages of the isolated perfused liver compared to other models^a.

Feature	Isolated hepatocytes	Cultured hepatocytes	Liver slices	Isolated perfused liver	In vivo
Lobular architecture maintained	-	-	+	+	+
Microcirculation intact	-	-	-	+	+
Neural-hormonal signals intact	-	-	-	-	+
Enzyme systems maintained	+	-	+	+	++
Bile formation	-	-/+ ^b	-	+	+
Viability	Hours	Days	Hours	Hours	Days
Efficient for data collection	+++	+++	++	++	-
Flexibility in experimental design	++	++	+	+	-
Reproducibility	+	+	+	+	+
Convenience	+++	+++	++	++	+
Minimal use of experimental animals	+	++	+	-	-
Representative of physiologic state	-	-/+ ^b	+	++	+++

a. + indicates relative advantages, - indicates relative disadvantages

b. LeCluyse et al., 1994.

CHAPTER 3

PHARMACOKINETICS OF S-CARBOXYMETHYLCYSTEINE: COMPARISON OF BUCCAL VERSUS ORAL FORMULATION

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ABSTRACT

The mucoregulating agent S-carboxymethylcysteine has been used for many years in the treatment of chronic obstructive pulmonary diseases, otitis media with effusion, and for nutritional effects as an antioxidant. The aim of this study is to determine the pharmacokinetics and bioavailability of a buccal formulation of S-carboxymethylcysteine in comparison to oral and IV routes of administration. A 400 mg buccal wafer, a 400 mg oral tablet and a 200 mg IV dose of S-carboxymethylcysteine were administered to each of five beagle dogs in a randomized crossover pattern. Plasma samples were obtained over a period of 7.5 h postdose and measured by precolumn derivatization of S-carboxymethylcysteine using *o*-phthaldialdehyde and subsequent HPLC analysis. All data were analyzed using WinNonlin. The pharmacokinetic parameters generated from each route of administration were compared using ANOVA or paired t-test to detect statistically significant differences. There were no significant differences in $t_{1/2}$ (61-64 min), V_d (405-418 ml/kg) and CL (4.52-4.64 ml/min/kg) among the different routes of administration. In addition, there were no significant differences in Ka (0.028 ± 0.017 vs. 0.034 ± 0.024), Tlag (27 ± 23 vs. 29 ± 16 min) and Tmax (93 ± 50 vs. 90 ± 56 min) between buccal and oral routes of administration. But the absolute bioavailability F and C_{max} of buccal formulation ($83.2 \pm 8.4\%$, 43.4 ± 8.0 mg/L) were significantly higher than those of the oral formulation ($51 \pm 1.4\%$, 29.5 ± 8.0 mg/L). The buccal formulation exhibited a 65% increase in bioavailability as compared to the oral tablet. This increase observed with the buccal formulation resulted, in part, from the decrease in first pass

metabolism.

Key words Pharmacokinetics Bioavailability S-carboxymethylcysteine

Buccal formulation

INTRODUCTION

S-carboxymethylcysteine (S-carboxymethyl-L-cysteine, Carbocysteine, CMC) (Fig. 3.1), a mucoregulating agent, has been used for a long time in the treatment of chronic obstructive pulmonary diseases, such as cystic fibrosis and chronic bronchitis, and otitis media with effusion (1-4). These diseases are characterized by accumulation of thick, viscoelastic mucus secretions and consequent impairment of cough clearance or mucociliary clearance. Clinical and laboratory studies show that CMC has little mucolytic activity. Instead, CMC acts as a mucoregulator, correcting intracellular abnormalities of glycoprotein synthesis and normalizing the secretory functions of the mucosal epithelium (5-7). In addition, decreased inflammation and increased tissue repair are observed by bronchoscopy during CMC treatment. The anti-inflammatory action may be linked to its effect on sialomucin synthesis and subsequent kinin inhibition of the sialoglycoproteins (8-11).

Oxidative stress is another important feature in the pathogenesis of COPD. Oxidative stress has important consequences, including oxidative inactivation of antiproteases and surfactants, mucus hypersecretion, membrane lipid peroxidation, alveolar epithelial injury, remodeling of extracellular matrix, and apoptosis. CMC has also been used as an antioxidant for either therapeutic or nutritional effects (12-13).

Chromatographic methods for the determination of CMC in biological samples have been published (14-15). These methods are either time-consuming or require complex isolation steps. Recently, a rapid and sensitive HPLC method for the quantification of

CMC has been developed, which utilizes *o*-phthaldialdehyde (OPA) as a precolumn derivatizing agent (16-18). This method is used for the determination of CMC in the plasma for this study.

Normally, CMC is orally administered in liquid or solid dosage forms such as syrup, capsule and tablet (23). However, orally administered drugs are inevitably subject to first pass metabolism before they reach systemic circulation, resulting in reduced absolute bioavailability. Buccal formulations are designed for immediate release and absorption of drug through the buccal mucosa, which circumvents first pass metabolism and provides better bioavailability than oral administration. Buccal administration is also applicable for some drugs that are unstable and undergo degradation in the GI tract. Buccal administration is not suitable for drugs that are poorly permeable across the buccal mucosa or for drugs that have an offensive taste.

Previous studies showed that the plasma concentration-time data of CMC fitted a one-compartment model (14). CMC distributes well into lung tissues and respiratory mucus, suggesting local action. Human plasma half-life was estimated to be 1.33 h and the volume of distribution was approximately 60 L (19). So far, no information is available on the extent of first pass metabolism or plasma protein binding. The purpose of this study was to determine the pharmacokinetics and bioavailability of a buccal formulation of S-carboxymethylcysteine in comparison to oral and IV routes of administration.

EXPERIMENTAL

Reagents and chemicals

CMC solution, wafer and tablet were obtained from Kiel Laboratories (Gainesville, GA). The chemical structure of CMC is shown in Fig. 3.1. HPLC-grade methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium hydroxide (NaOH) and sodium acetate (NaAc) were obtained from J.T Baker Inc. (Philipsburg, NJ, USA). Heparin was manufactured by Baxter Healthcare Corporation (Deerfield, IL). OPA, 2-mercaptoethanol and boric acid were obtained from Sigma (St. Louis, MO).

Animals

The use of animals was approved by the University of Georgia Animal Use and Care Committee and was conducted in accordance with the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was performed on 5 healthy beagle dogs (4 female and 1 male) aged 2 years and weighted between 10-13.5 kg. The dogs were housed one animal per cage in the College of Veterinary Medicine of University of Georgia. The living environment of the animals was controlled, with 12 h of light per day, a constant temperature of 70-75 F, daily feeding and tap water. The dogs underwent a physical and basic lab test prior to the study and all dogs were fasted for 12 h before dosing.

Procedure

Each dog acted as its own control, receiving either a 2 ml (100 mg/ml) solution of

CMC as an IV dose, a tablet containing 400 mg of CMC as an oral dose, or a wafer containing 400 mg of CMC as a buccal dose in a randomized crossover pattern. After a one-week washout period, each dog was administered the drug through another route of administration. One week later, the last round of administration was performed on each dog.

On the day of the study, an 18 G catheter (Sherwood Medical Industries, St. Louis, MO) was placed in the cephalic vein for sampling. The catheter was filled with heparinized saline to maintain patency throughout the sampling period. The sampling times were as follows: 0, 5, 10, 20, 30, 45, 60, 90, 120, 180, 270, 360 and 450 min. In the case of oral and buccal administration, three time points (75, 105, and 150 min) were added to get a more reliable estimate of T_{max} and C_{max}. Blood samples (0.5-1 ml) were drawn from the catheter. In the case of IV administration, the drug was administered via another cephalic vein. After centrifuging at 8000 rpm for 5 minutes, the plasma was removed and the samples were stored at -20°C pending assay.

Assay

Preparation of stock and standard solutions

A stock solution of 1.0 mg/ml CMC was prepared in deionized water. Standard solutions of CMC were prepared by serial dilution with deionized water. The final concentrations of the standard solutions were 1000, 500, 250, 100, 50, 25, 10, 5 and 2.5 ug/ml. The stock solution was kept refrigerated when not in use and replaced on a bi-weekly basis. Fresh standard solutions were prepared for each day of analysis.

Preparation of calibration curves

Plasma calibration points were prepared by spiking 100 µl of the biological matrices with 10 µl of each CMC standard solution. The calibration curve of the plasma was in the range of 0.25 – 100 µg/mL with individual calibration points of 100, 50, 25, 10, 5, 2.5, 1, 0.5 and 0.25 µg/mL.

Preparation of samples

Plasma samples were prepared with protein precipitation. One volume of acetonitrile was added to each 100 µl plasma sample and samples were vortexed and centrifuged for 10 min at 13,000 rpm. The pellet was discarded and the supernatant was evaporated until reaching dryness. The residue was reconstituted in 40 µl of borate buffer (0.4 M HBO₃-NaOH, pH 9.5).

Online o-phthaldialdehyde (OPA) precolumn derivatization (20)

OPA reagent solution was made by adding 10 mg of *o*-phthaldialdehyde, 200 µl of MeOH and 10 µl of 2-mercaptoethanol into 2 ml of borate buffer. HPLC injector program was edited as follows: (1) Draw 50 µl from OPA solution; (2) Draw 20 µl from reconstituted samples; (3) Mix 70 µl “in air”, max speed, 6 times; (4) Wait 1 min; (5) Draw 0 µl from ddH₂O (needle wash); and (6) Inject.

Chromatography

The chromatographic analysis was performed using an HPLC system consisting of Agilent 1100 Series components including a quaternary pump, degasser, autosampler, and variable-wavelength UV detector (Palo Alto, CA, USA). Chromatographic

separations were achieved using a Phenomenex SphereClone 5 μ ODS (1) column (4.6 mm \times 250 mm) with a Phenomenex Security Guard C₁₈ guard column (Torrance, CA, USA). The mobile phase used was MeOH-NaAc (pH 6.0)-THF (15:84:1). The flow rate was 1.0 ml/min and the UV wavelength was set at 338 nm. The retention time of OPA-CMC was 8.3 min and each run took 28 min. The linear range of CMC was 0.25-100 μ g/ml ($r^2=0.9998$) and the limit of detection was 0.1 μ g/ml.

Data analysis

The plasma data from all dogs were subjected to WinNonlin non-compartmental analysis. The following parameters were calculated: absolute bioavailability (F), volume of distribution (V_d), half-life ($t_{1/2}$), peak concentration (C_{max}), time to reach peak concentration (T_{max}), area under the concentration-time curve (AUC), and total body clearance (CL). The mean plasma concentration-time data of IV administration of CMC was fitted to a two-compartment model. A one-compartment model with first-order elimination was used to fit the plasma concentration-time data of oral and buccal administration of CMC for the determination of absorption rate constant (K_a) and lag time (T_{lag}). The pharmacokinetic parameters generated for each route of administration were compared by using analysis of variance (ANOVA) or paired t-test to detect statistically significant differences.

RESULTS AND DISCUSSION

Few methods were developed for the determination of CMC. In most cases, amino acids and their derivatives were analyzed by an amino acid analyzer, utilizing an ion-exchange resin column. Those methods were time consuming or couldn't detect low concentrations. Recently, HPLC methods for the determination of CMC have been developed. Among them, OPA precolumn derivatization has high resolution and sensitivity (16-18). OPA in the presence of 2-mercaptoethanol reacts rapidly (within seconds) with primary amino acids to form intensely fluorescent derivatives that could be detected either by FLD or UV detection. Most primary amino acids have a low lower limit of quantification of 0.5 pmol. Only histidine and lysine have a relatively high lower limit of quantification of 2.5 pmol and 3.5 pmol, respectively. The signals of the derivatives are not very stable and attenuate quickly. Thus, OPA precolumn derivatization is extremely suitable for automatic online derivatization (21). The described analytical method used for the separation and quantification of CMC in dog plasma has been shown to be accurate. The chromatograms of a blank dog plasma sample and a beagle dog sample after administration of CMC are shown in Fig. 3.2. The OPA-derivatized CMC eluted at 8.3 min. The lower limit of quantitation was 0.25 ug/ml, which was comparable to previously published assays (0.1-1.0 ug/ml) (17, 18). The relationship between plasma concentration and peak area was found to be linear within the range 0.25-100 ug/ml.

There was no reported work on intravenous studies of CMC to allow bioavailability determination. In our comparative bioavailability study, CMC was administered

intravenously, orally and buccally in a randomized crossover pattern. Each beagle dog was used as its own control. In this type of study, clearance, volume of distribution, and absorption, as determined by physiological variables (e.g. gastric emptying, motility, pH), were assumed to have less interoccasion variability compared to the variability arising from formulation performance. Therefore, differences between two products because of formulation factors could be determined.

The mean plasma concentration-time profiles of CMC following oral administration of a 400 mg tablet, buccal administration of a 400 mg wafer, as well as a 200 mg IV bolus dose are shown in Fig. 3.3. Both wafer and tablet were readily absorbed. The dissolution time for the CMC wafer was less than two minutes when given buccally. CMC was measurable less than 20 min after administration in the majority of animals. The peak concentrations were reached at 1.5 hr and then declined but remained detectable up to 7.5 hours.

The pharmacokinetic parameters generated from WINNONLIN for each route of administration are given in Table 3.1. IV data showed that the volume of distribution of CMC was 405.2 ± 65.5 ml/kg, which was less than total body water (564 ± 25 ml/kg) (24) but much larger than the extracellular water (237 ± 27 ml/kg) (25) of beagle dogs, indicating that CMC was extensively distributed in both extracellular and intracellular fluid in dogs. This was consistent with human data that showed a volume distribution of 60 L (19). The systemic clearance of CMC was 4.57 ± 0.21 ml/min/kg. CMC was eliminated by both renal and hepatic routes with hepatic metabolism accounting for the

majority of the clearance of CMC (23). If we assume that all CMC was removed by hepatic metabolism, the extraction ratio calculated by comparing clearance to hepatic blood flow (185 ± 11 ml/100g liver/min (27) was considered low ($ER < 0.3$).

The half life, V_d and CL of buccal formulation (63.8 ± 6.3 min, 417.5 ± 47.9 ml/kg, 4.52 ± 0.20 ml/min/kg) and oral formulation (61.2 ± 4.0 min, 410.7 ± 51.0 ml/kg, 4.64 ± 0.32 ml/min/kg) formulation were consistent with those of IV administration (61.0 ± 7.8 min, 405.2 ± 65.5 ml/kg, 4.57 ± 0.21 ml/min/kg), indicating that excipients in each formulation did not alter the intrinsic pharmacokinetic behavior of the active compound. In this study, the CMC wafer and tablet were manufactured using excipients such as magnesium stearate (E572), silica, anhydrous colloidal (E551), lactose monohydrate (spray dried), and sodium laurel sulphate. The concentration-time profiles of buccal and oral formulations were similar with close absorption rate constant K_a (0.028 ± 0.017 vs. 0.034 ± 0.024), T_{lag} (27 ± 23 vs. 29 ± 16 min), T_{max} (93 ± 50 vs. 90 ± 56 min) and $t_{1/2}$ (63.8 ± 6.3 vs. 61.2 ± 4.0 min). However, C_{max} and absolute bioavailability F of buccal administration (43.4 ± 8.0 ug/ml, 83.2 ± 8.4 %) were much higher than those of oral administration (29.5 ± 8.0 ug/ml, 51.0 ± 1.4 %). Using ANOVA, no statistically significant differences in $t_{1/2}$, CL and V_d were detected between the different routes of administration. In addition, no statistically significant differences in K_a , T_{lag} and T_{max} were detected between the wafer and capsule formulation. However, F and C_{max} of buccal administration were significantly higher than those of the oral administration.

Both of the mean plasma concentration-time profiles of oral and buccal

administrations of CMC were consistent with a one-compartment model, which confirmed previously reported results (14). However, the mean plasma concentration time-course for the IV administration was a two-compartment model. A shallow distribution phase with a distribution $t_{1/2}$ of 18.3 min was observed when dosed intravenously. The fitted mean plasma concentration-time profiles using one-compartment and two-compartment models are shown in Fig. 3.4.

The most probable metabolic pathway for S- carboxymethylcysteine is sulphoxidation. The biotransformation of S- carboxymethylcysteine in different species (rat, dog, monkey and man) has been investigated and showed that dicarboxymethyl sulphide was their common metabolite. Dicarboxymethyl sulphide was further metabolized to inorganic sulphate in the monkey (22). However, no information was available regarding its first pass metabolism. In our study, the oral formulation exhibited a 51% absolute bioavailability. Oral bioavailability may be affected by many factors, such as incomplete dissolution and absorption of drugs, drug stability in GI tract, drug-food or drug-drug metabolism, gastric emptying, intestinal transit, gut wall metabolism, efflux gut wall transporters (P-glycoprotein) and hepatic first pass metabolism. In this case, incomplete absorption and first pass metabolism might explain the reduced oral bioavailability of CMC. The buccal formulation exhibited an 83% absolute bioavailability, which was 65% higher than that of the oral tablet. This increase was due, in part, to the avoidance of first pass metabolism. In addition, buccal administration of CMC prevented degradation in the GI tract.

In summary, the buccal formulation greatly improved the bioavailability. In addition, it is suitable for the patients who have trouble in swallowing solid dosage forms and therefore may improve patient compliance.

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Table 3.1. Pharmacokinetic parameters (mean \pm SD) generated from WinNonlin analysis of plasma data collected after IV, oral and buccal administration of S-carboxymethylcysteine.

	IV	Oral	Buccal
HL (min)	61.0 \pm 7.8	61.2 \pm 4.0	63.8 \pm 6.3
AUC _{0$\rightarrow$$\infty$} (min*ug/ml)	3754.4 \pm 615.9	3834.0 \pm 723.9	6174.8 \pm 506.0
Cmax (ug/ml)	101.2 \pm 23.7 ^a	29.5 \pm 8.0	43.4 \pm 8.0
Tmax (min)	-	90 \pm 56	93 \pm 50
Tlag (min)	-	29 \pm 16	27 \pm 23
Vd (ml/kg)	405.2 \pm 65.5	410.7 \pm 51.0	417.5 \pm 47.9
CL (ml/min/kg)	4.57 \pm 0.21	4.64 \pm 0.32	4.52 \pm 0.20
F (%)	-	51.0 \pm 1.4	83.2 \pm 8.4
Ka (min ⁻¹)	-	0.034 \pm 0.024	0.028 \pm 0.017

^a In the case of IV administration, Cmax was represented by the extrapolated C0.

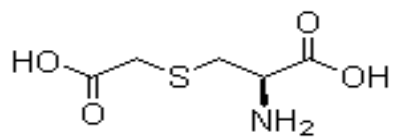


Fig. 3.1. Chemical structure of S-carboxymethylcysteine.

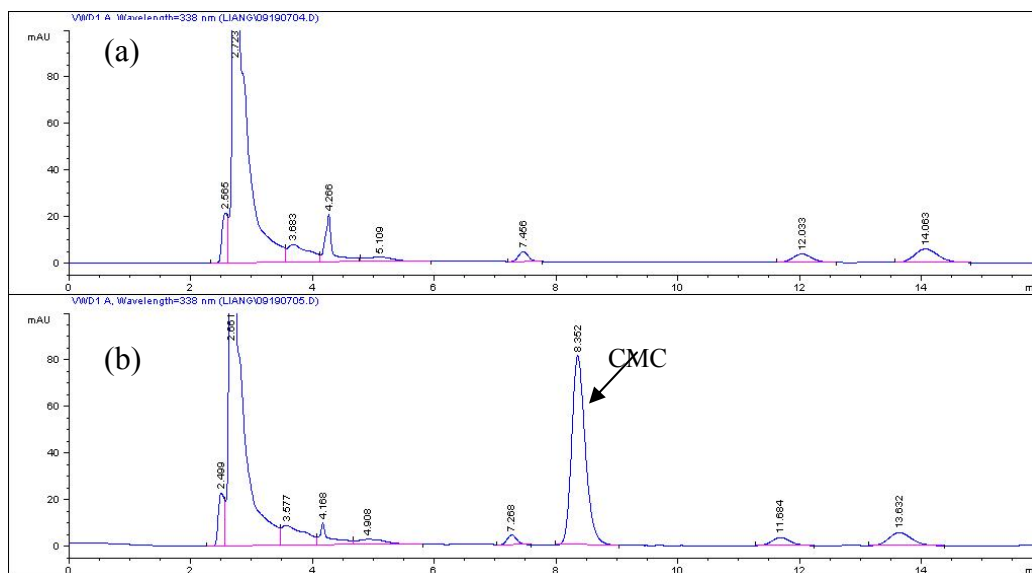


Fig. 3.2. Chromatograms of (a) a blank dog plasma sample and (b) a representative real plasma sample (25 ug/ml) after administration of S-carboxymethylcysteine.

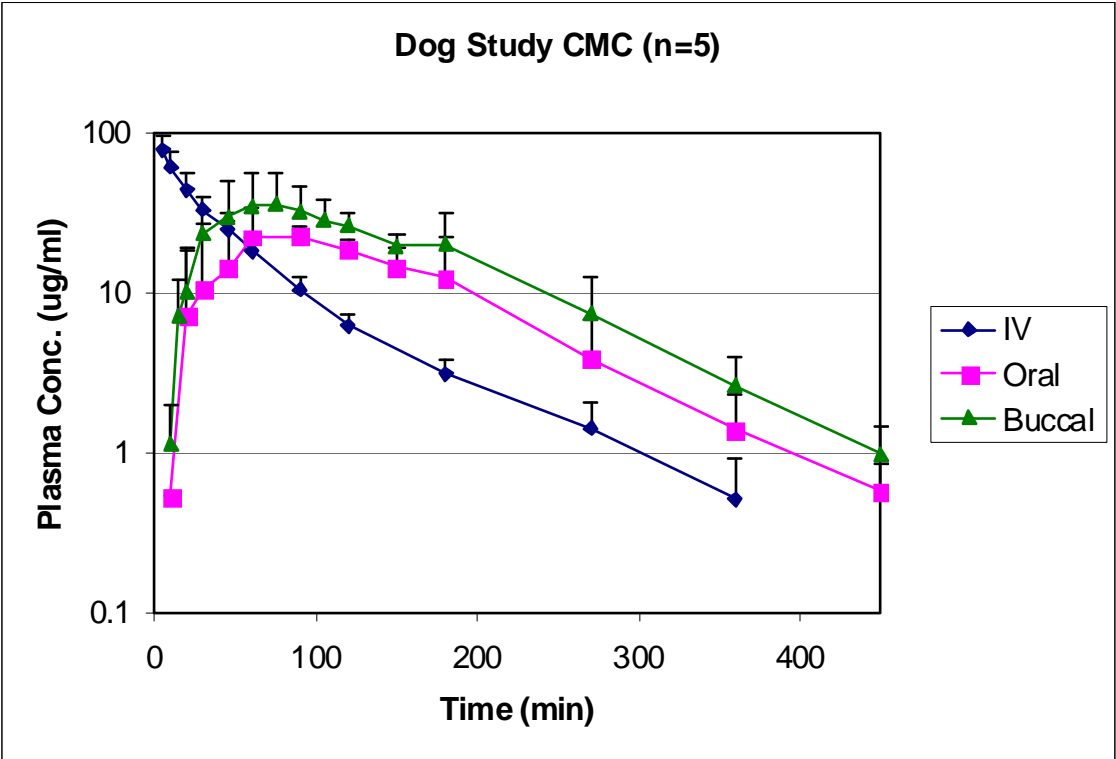


Fig. 3.3. Concentrations (mean plus standard deviation) versus time profiles after IV, oral and buccal administration of S-carboxymethylcysteine.

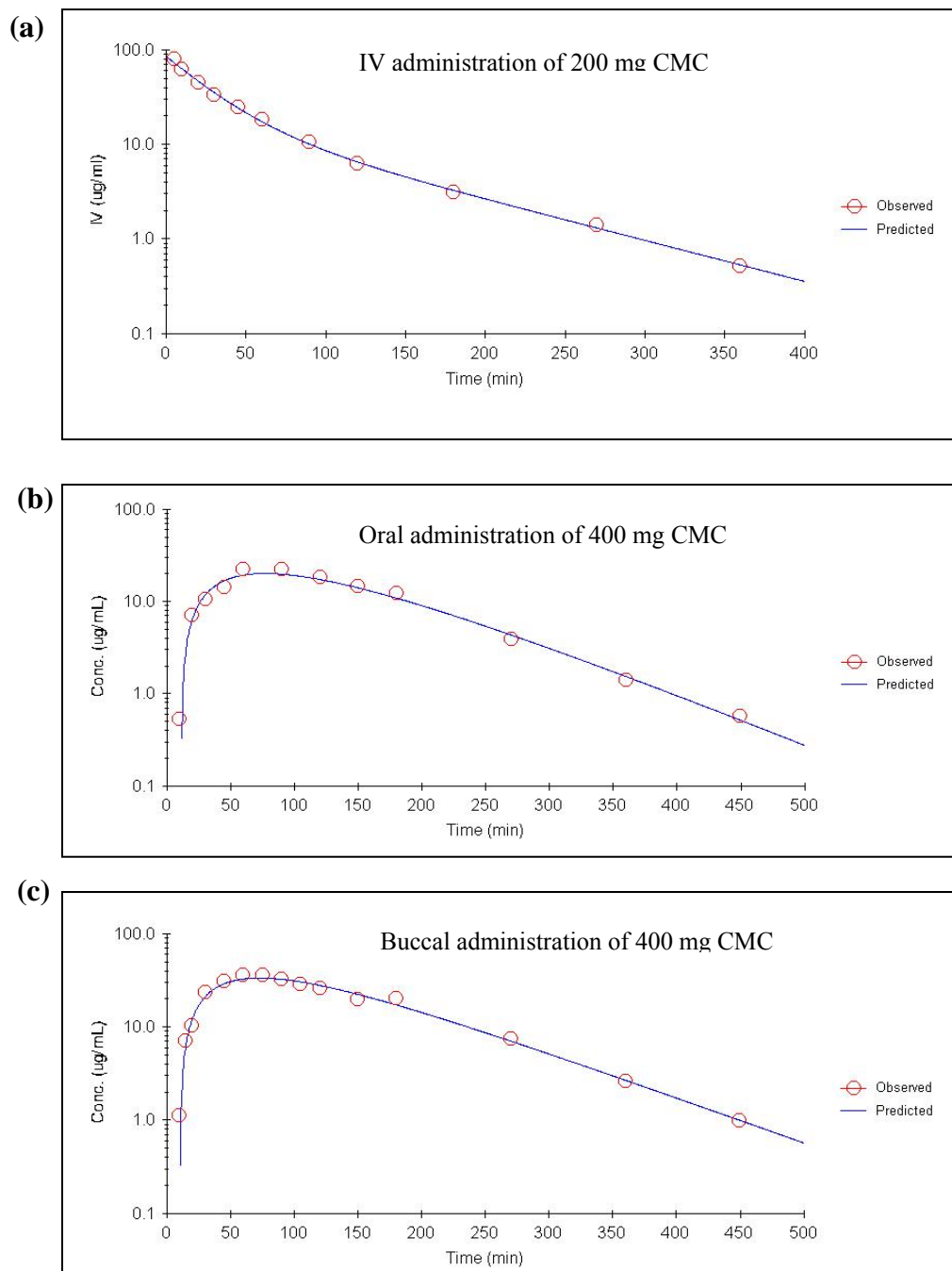


Fig. 3.4. The mean plasma concentration-time profile after (a) IV administration of S-carboxymethylcysteine was fitted to a two-compartment model. The mean plasma concentration-time profiles after (b) oral and (c) buccal administration of S-carboxymethylcysteine were fitted to a one-compartment model.

CHAPTER 4

PHARMACOKINETICS OF N-ACETYLCYSTEINE: COMPARISON OF A WAFER VERSUS A MARKETED CAPSULE

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ABSTRACT

N-acetylcysteine (NAC) has been used as a mucolytic agent in the treatment of patients suffering from chronic obstructive pulmonary diseases and as an antidote for acetaminophen overdose. The aim of this study is to determine the pharmacokinetics and bioavailability of an oral wafer formulation of NAC in comparison to a marketed capsule formulation. Oral doses of NAC in two different dosage forms, as a 400 mg wafer and as a 400 mg capsule, as well as a 200 mg IV dose, were given to five beagle dogs. Plasma samples were collected over a period of 12 h postdose and measured by precolumn derivatization of NAC using 1-Fluoro-2, 4, -dinitrobenzene and subsequent HPLC analysis. All data were analyzed using WinNonlin. The plasma data was fitted to a two-compartment model. The PK parameters for each administration were compared using ANOVA or paired t-test to detect statistically significant differences. There were no significant differences in $t_{1/2}$ (240-257 min), CL (4.99-5.11 ml/min/kg) and V_d (1762-1855 ml/kg) among different types of administration. In addition, there were no significant differences in K_a (0.043 ± 0.011 vs. $0.033 \pm 0.013 \text{ min}^{-1}$), C_{\max} (27.2 ± 5.0 vs. $27.2 \pm 7.6 \text{ ug/ml}$), T_{\max} (63 ± 16 vs. $87 \pm 34 \text{ min}$) and absolute bioavailability (43.2 ± 3.7 % vs. 53.8 ± 10.0 %) between wafer and capsule formulation. The relative bioavailability of NAC wafer to the merchandized NAC capsule was 83 ± 16 %.

Key words Bioavailability N-acetylcysteine Pharmacokinetics

INTRODUCTION

N-acetylcysteine (N-acetyl-L-cysteine, acetylcysteine, NAC, Fig. 4.1.) is a mucolytic agent that has been used over five decades for the treatment of chronic obstructive pulmonary diseases, such as cystic fibrosis and chronic bronchitis (1-4). These diseases are characterized by accumulation of thick, viscoelastic mucus secretions and consequent impairment of cough clearance or mucociliary clearance. The mucolytic effect results from NAC's sulfhydryl group interacting with disulfide bonds in mucoprotein, with the mucus being broken into smaller and less viscous units. In addition, NAC directly acts as an antioxidant or a "scavenger" of oxygen radicals, regulating the redox status in the cells, hence influencing inflammation pathways (5-7).

NAC has also been used as an antidote for acetaminophen (paracetamol) poisoning for over thirty years (8-10). The acetaminophen-induced liver toxicity results from the accumulation of an acetaminophen metabolite N-acetyl-p-benzoquinoneimine (NAPQI) that depletes the hepatocytes of glutathione (GSH) and causes hepatocellular damage. NAC is effectively deacetylated to cysteine and cystine by hepatocytes, thus stimulating the synthesis of GSH and compensates for the depletion of GSH caused by acetaminophen overdose (11-12). Recently, NAC has been shown to have potential effects as an anticarcinogen (13-14) and anti-HIV agent (15-16).

Chromatographic methods for the determination of NAC in biological samples have been published (17-20). Most of them were developed by the quantitative chemical derivatization of NAC with a variety of electrophilic agents, resulting in stable adducts.

In our study, 1-Fluoro-2, 4, -dinitrobenzene (DNFB) was used to derivatize NAC for subsequent HPLC analysis.

Previous human studies (21-24) showed that the pharmacokinetics of NAC fitted a two-compartment model. NAC was rapidly absorbed and reached peak plasma concentration within 1-2 h after oral administration. The oral bioavailability was less than 10%, indicating an extensive first pass metabolism. NAC was present in high concentrations in the airways, but its distribution in kidney, liver, brain, adrenal gland, spleen and muscle were also noted. The volume of distribution at steady state ranged from 0.33-0.47 L/kg, which was consistent with distribution mainly to extracellular body water. After IV administration, plasma protein binding was 50% at 4 h and 20% at 12 h postdose. NAC was deacetylated in the gut wall, liver and lungs, and the major metabolites were cysteine, cystine and sulphate. Only approximately 30% of NAC was cleared by kidney and the elimination half-life was 5.5-6.5 h.

Different formulations of NAC have different oral bioavailabilities. In general, a solution has the highest oral bioavailability, followed by suspension, capsule and tablet. Currently, NAC capsules, tablets and powders are commercially available. Our study compared the pharmacokinetics and oral bioavailability of a new wafer formulation to those of a marketed capsule (GNC, Pittsburgh, PA).

EXPERIMENTAL

Reagents and chemicals

NAC solution and wafer were obtained from Kiel Laboratories (Gainesville, GA). NAC capsule was purchased from GNC (Pittsburgh, PA). The chemical structure of NAC is shown in Fig. 4.1. Dithiothreitol (DTT) and HPLC-grade methanol (MeOH) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Heparin was manufactured by Baxter Healthcare Corporation (Deerfield, IL). Sodium bicarbonate (NaHCO_3), diethyl ether, hydrochloric acid (HCl), disodium EDTA and trisodium citrate were obtained from J.T Baker Inc. (Philipsburg, NJ, USA). 1-Fluoro-2, 4-dinitrobenzene (DNFB) was obtained from Sigma (St. Louis, MO).

Animals

The use of animals was approved by the University of Georgia Animal Use and Care Committee and was conducted in accordance with the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was performed on 5 healthy beagle dogs (4 female and 1 male) aged 2 years and weighted between 10-13.5 kg. The dogs were housed one animal per cage in the College of Veterinary Medicine of University of Georgia. The living environment of the animals was controlled, with 12 h of light per day, a constant temperature of 70-75 F, daily feeding and tap water. The dogs underwent a physical and basic lab test prior to the study and all dogs were fasted for 12 h before dosing.

Procedure

Each dog acted as its own control, receiving either a 2 ml (100 mg/ml) solution of NAC as an IV dose, a wafer containing 400 mg of NAC or a capsule containing 400 mg of NAC as an oral dose. After a one-week washout period, each dog was administered the compound with another dosage form. One week later, the last round of administration was performed on each dog.

On the day of the study, an 18 G catheter was placed in the cephalic vein for sampling. The catheter was filled with heparinized saline to maintain patency throughout the sampling period. The sampling times were as follows: 0, 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480 and 720 min. Blood samples (0.5-1 ml) were drawn from the catheter. In the case of IV administration, the drug was administered via another cephalic vein. After centrifuging at 8000 rpm for 5 min, the plasma was removed and then stored at -20°C pending assay.

Assay

Preparation of stock and standard solutions

A stock solution of 1.0 mg/ml NAC was prepared in deionized water. Standard solutions of NAC were prepared by serial dilution with deionized water. The final concentrations of the standard solutions were 1000, 500, 250, 100, 50, 25, 10, 5 and 2.5 µg/ml. The stock solution was kept refrigerated when not in use and replaced on a bi-weekly basis. Fresh standard solutions were prepared for each day of analysis.

Preparation of calibration curves

Plasma calibration points were prepared by spiking 100 μl of the biological matrices with 10 μl of each NAC standard solution. The calibration curve of the plasma was in the range of 0.25 – 100 $\mu\text{g/mL}$ with individual calibration points of 100, 50, 25, 10, 5, 2.5, 1, 0.5 and 0.25 $\mu\text{g/mL}$.

Preparation of samples

Fifty μl of plasma was diluted with distilled water to 100 μl and 50 μl of 0.5 % DTT was added and vortexed for 10 s. After keeping the tubes in a water bath at 37 °C for 30 min, 100 μl of 0.5 M NaHCO_3 and 12.5 μl of 5% DNFB (in methanol) were added to each tube and vortexed for 30 s. Then, these tubes were packed in aluminous foils and incubated at 60 °C for another 30 min. After cooling the samples to room temperature, 700 μl of diethyl ether was added and the samples were vortexed for 30 s and centrifuged for 5 min at 5000 rpm. Supernatants were discarded and 100 μl of 6 M HCl and 700 μl of diethyl ether were added. All the samples were vortexed for 60 s and centrifuged for 5 min at 5000 rpm. Supernatants were transferred into separate clean tubes and evaporated until dryness at a low temperature (< 40 °C). The residue was reconstituted in 100 μl of 50 mM trisodium citrate and 1 mM disodium EDTA (pH 7.0) and injected to the HPLC column.

Chromatography

The chromatographic analyses were performed using an HPLC system consisting of Agilent 1100 Series components including a quaternary pump, degasser, autosampler, and variable-wavelength UV detector (Palo Alto, CA, USA). Chromatographic separations were achieved using a Phenomenex SphereClone 5 μ ODS (1) column (4.6 mm \times 250 mm) with a Phenomenex Security Guard C₁₈ guard column (Torrance, CA, USA). The mobile phase consisted of MeOH-trisodium citrate buffer (50 mM trisodium citrate and 1mM sodium EDTA, pH 7.0) (1:3, v/v). The flow rate was 1.0 ml/min and the UV wavelength was set at 365 nm. The retention time of DNFB-NAC was 15 min and each run took 25 min. The linear range of NAC was 0.25-100 μ g/ml ($r^2=0.996$) and the limit of detection was 0.1 μ g/ml.

Data analysis

The plasma data from all dogs were subjected to WinNonlin non-compartmental analysis. The following parameters were calculated: absolute and relative bioavailability, volume of distribution (V_d), half-life ($t_{1/2}$), peak concentration (C_{max}), time to reach peak concentration (T_{max}), area under the concentration-time curve (AUC), and total body clearance (CL). A two-compartment model with first-order elimination was used to fit the NAC plasma data. The absorption rate constant (K_a) and lag time (T_{lag}) were determined. The pharmacokinetic parameters generated for each administration were compared by using analysis of variance (ANOVA) or paired t-test to detect statistically significant differences.

RESULTS AND DISCUSSION

Recently NAC has enjoyed a renaissance, but the diversity of pharmacological applications of NAC is mainly due to its sulfhydryl group, which is nucleophilic and has redox activity (11). The described analytical method used for the separation and quantification of NAC in dog plasma was validated according to the FDA guidelines. Compared to traditional analytical methods (e.g. amino acids analyzer), DNFB derivatization of NAC has better resolution and higher sensitivity. The chromatograms of a blank dog plasma sample and a dog plasma sample after administration of NAC are shown in Fig. 4.2. NAC is able to undergo thiol-disulfide exchange reactions with other thiol redox compounds, such as cysteine and glutathione. It's extensively bound to peptides and proteins in plasma. In our assay for the determination of total NAC concentration in plasma, DTT was used to release NAC from the oxidized forms before DNFB precolumn derivatization. The DNFB-derivatized NAC eluted at 15 min. The lower limit of quantitation was 0.25 ug/ml, which was even lower than previously published assays (3-5.5 ug/ml) (20). The relationship between plasma concentration and peak area was found to be linear within the range 0.25-100 ug/ml.

The mean plasma concentration-time profiles of NAC following oral administration of a 400 mg wafer and a 400 mg capsule, as well as a 200 mg IV bolus dose, are shown in Fig. 4.3. Both wafer and capsule formulations were readily absorbed from the gastrointestinal tract and NAC was measurable less than 20 min after administration in

the majority of animals. There are various reasons for the delay of absorption, which is known as the lag time. For example, the physicochemical characteristics of a drug, formulation factors, and gastric emptying can all affect the onset of absorption. In our study, the lag time for the wafer and capsule formulations were similar (24 ± 12 vs. 32 ± 14 min), as well as the absorption rate constant K_a (0.043 ± 0.011 vs. $0.033 \pm 0.013 \text{ min}^{-1}$), indicating similar rates of absorption. Concentrations peaked at 1-1.5 hr and then declined in a biexponential pattern and remained detectable for up to 12 hours.

The pharmacokinetic parameters generated from WINNONLIN for each administration are given in Table 4.1. PK parameters determined by non-compartment and compartment analysis were not significantly different. The mean plasma concentration-time profiles of all administrations were fitted to a two-compartment model, which were consistent with previously reported results (21). The fitted plasma concentration-time profiles using two-compartment model are shown in Fig. 4.4. IV data showed that the volume of distribution of NAC was $1.78 \pm 0.34 \text{ L/kg}$, which was much higher than the total body water ($0.56 \pm 0.03 \text{ L/kg}$) (26) of dogs, indicating that NAC was extensively distributed in total body fluid and had significant tissue binding in dogs. The systemic clearance of NAC was $5.11 \pm 0.54 \text{ ml/min/kg}$. The glomerular filtration rate (GFR) of dogs is $2.9 \pm 0.3 \text{ ml/min/kg}$ (27). Therefore, the drug was probably metabolized or excreted by active tubular secretion (ATS). Since the hepatic blood flow of dogs is $185 \pm 11 \text{ ml/100g liver/min}$ (28), the metabolic extraction ratio of NAC (< 0.3) would be low

even assuming that NAC was eliminated exclusively by liver. The absolute oral bioavailability of wafer and capsule formulation were $43.2 \pm 3.7 \%$ and $53.8 \pm 10.0 \%$, respectively, which were much higher than in humans ($F < 10\%$). This, in part, was due to the species differences in the metabolism of NAC. The kidney and liver are the main organs responsible for the biotransformation of NAC to cysteine. The activities of NAC-deacetylating enzyme (acylase) in various tissues of different species (rat, rabbit, dog, monkey, and man) have been investigated (25). Acylase activity was highest in the kidney in all species studied. Enzyme activity in the liver was 10–22 % of that in the kidney in the rat, rabbit, monkey, and man, but almost no hepatic activity was observed in the dog. This low hepatic activity indicated that the 43.2% to 53.8% absolute oral bioavailability of NAC in beagle dogs was not due to the first pass metabolism in liver but rather due to the incomplete GI tract absorption or metabolism in the gut wall.

The mean concentration-time profiles of wafer and capsule formulations were similar and almost superimposable as evidenced by their close C_{max} (27.2 ± 5.0 vs. 27.2 ± 7.6 ug/ml), T_{max} (63 ± 16 vs. 87 ± 34 min), AUC (2835.8 ± 610.4 vs. 3559.4 ± 942.9 min*ug/ml), and $t_{1/2}$ (256.8 ± 38.6 vs. 243.6 ± 15.8 min). In addition, the $t_{1/2}$, Vd and CL of the wafer (256.8 ± 38.6 min, 1.86 ± 0.38 ml/kg, 4.99 ± 0.5 ml/min/kg) and capsule (243.6 ± 15.8 min, 1.76 ± 0.25 ml/kg, 5.01 ± 0.59 ml/min/kg) formulations were consistent with those observed after IV administration (239.8 ± 27.6 min, 1.78 ± 0.34 ml/kg, 5.11 ± 0.54 ml/min/kg), indicating that the excipients in each formulation did not

alter the inherent pharmacokinetic behavior of the active ingredient. In our study, the NAC wafers were manufactured using excipients such as magnesium stearate (E572), silica, anhydrous colloidal (E551), lactose monohydrate (spray dried), and sodium laurel sulphate. The relative bioavailability of NAC wafer to the marketed NAC capsule was $83 \pm 16 \%$, which corresponded to the general order of the oral bioavailability of a drug in different formulations: solutions > suspensions > capsules > tablets/wafers. This is due to the different dissolution rates of the drug products.

ANOVA and paired *t*-test were applied to compare the statistical differences of the pharmacokinetic parameters. Using ANOVA, no statistically significant differences in $t_{1/2}$, CL and V_d were detected among the different formulations. In addition, no statistically significant differences in K_a , C_{max} , T_{max} , and F were detected between wafer and capsule formulation.

To summarize, preclinical studies of NAC and subsequent statistical analysis clearly indicates that there is no significant difference between the wafer formulation and the marketed capsule formulation.

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Table. 4.1. Pharmacokinetic parameters (mean \pm SD) generated from WinNonlin analysis of plasma data collected after IV, oral wafer and oral capsule administration of N-acetylcysteine.

	IV	Wafer	Capsule
HL (min)	239.8 \pm 27.6	256.8 \pm 38.6	243.6 \pm 15.8
AUC _{0$\rightarrow$$\infty$} (min*ug/ml)	3304.6 \pm 709.3	2835.8 \pm 610.4	3559.4 \pm 942.9
Cmax (ug/ml)	88.2 \pm 19.2 ^a	27.2 \pm 5.0	27.2 \pm 7.6
Tmax (min)	-	63 \pm 16	87 \pm 34
Tlag (min)	-	24 \pm 12	32 \pm 14
Vd (L/kg)	1.78 \pm 0.34	1.86 \pm 0.38	1.76 \pm 0.25
CL (ml/min/kg)	5.11 \pm 0.54	4.99 \pm 0.5	5.01 \pm 0.59
F (%)	-	43.2 \pm 3.7	53.8 \pm 10.0
Ka (min ⁻¹)	-	0.043 \pm 0.011	0.033 \pm 0.013

^a In the case of IV administration, Cmax was represented by the extrapolated C0.

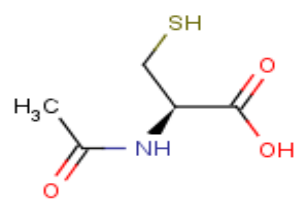


Fig. 4.1. Chemical structure of N-acetylcysteine.

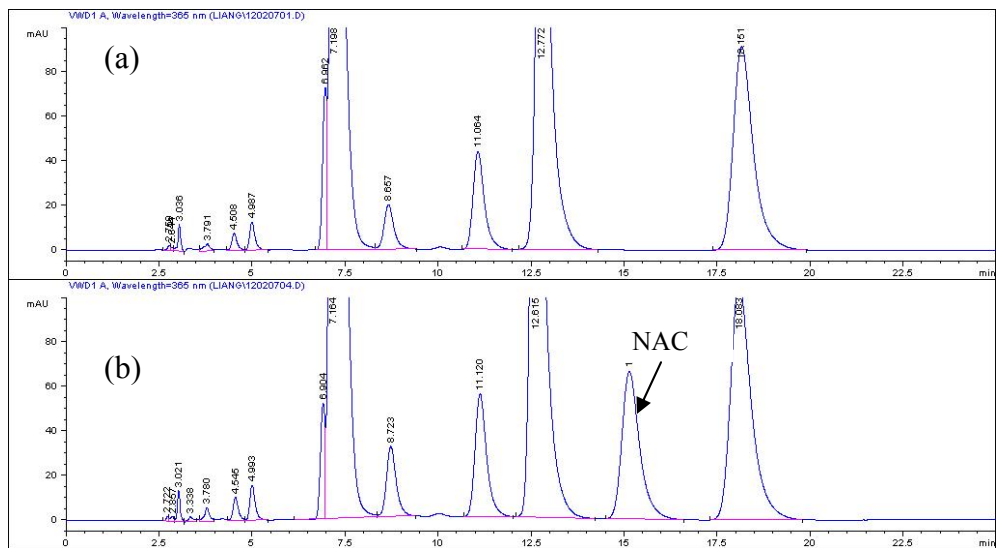


Fig. 4.2. Chromatograms of (a) a blank dog plasma sample and (b) a representative real plasma sample (25 ug/ml) after administration of N-acetylcysteine.

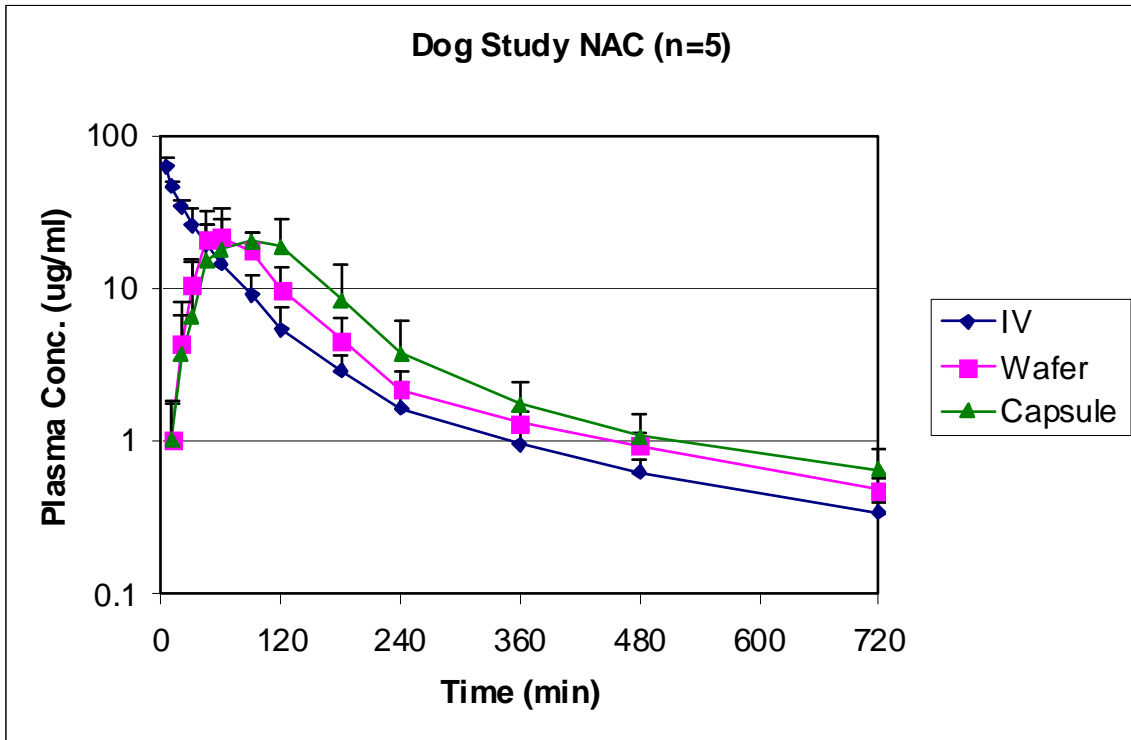


Fig. 4.3. Concentrations (mean plus standard deviation) versus time profiles after IV, oral wafer and oral capsule administration of N-acetylcysteine.

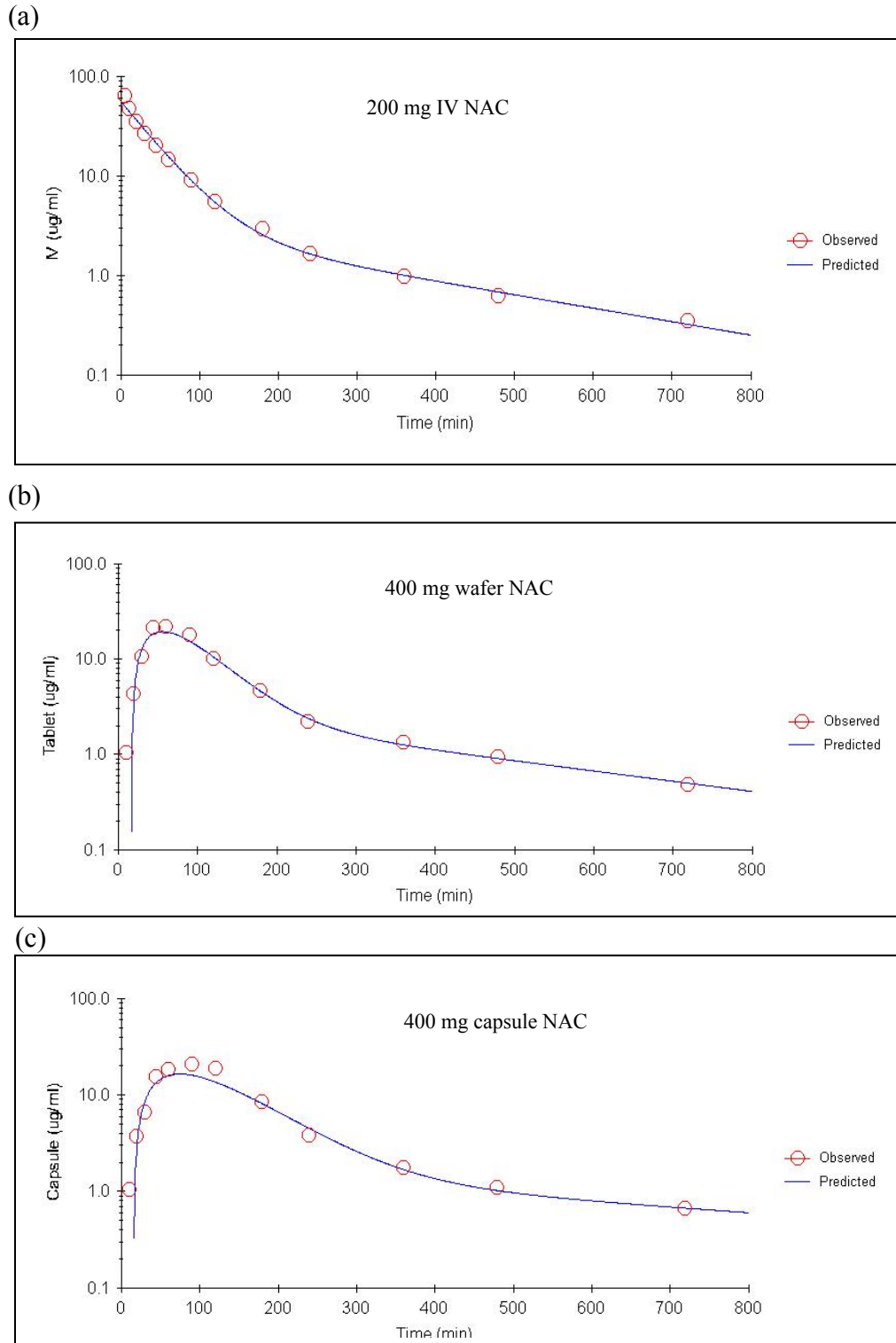


Fig. 4.4. The mean plasma concentration-time profiles after (a) IV, (b) oral wafer and (c) oral capsule administration of N-acetylcysteine were fitted to a two-compartment model.

CHAPTER 5

PHARMACOKINETICS OF THE ANTIVIRAL AGENT (-)-CARBODINE IN MICE

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ABSTRACT

(-)-Carbodine has activity both in vitro and in vivo against DNA and RNA viruses such as Venezuelan equine encephalitis virus. Thus, the brain is a primary target organ for this antiviral agent. The purpose of this study is to determine the basic pharmacokinetic parameters and tissue distribution of (-)-carbodine in mice. An HPLC method for the quantification of (-)-carbodine in mouse plasma, brain, liver and kidney was developed and validated. Thirty mice received either a 200 mg/kg IV dose or a 500 mg/kg oral dose of (-)-carbodine. Samples were obtained at 5, 15, 30, 45, 60, 90, 120, and 150 minutes. Pharmacokinetic parameters were determined using WinNonlin. The metabolism of (-)-carbodine was studied and its major metabolite was characterized by LC-MS and LC-MS/MS. Acceptable intra-day and inter-day assay precision (<13%) and accuracy (<19%) were observed over a linear range of 0.5-50 ug/ml in plasma or 1.5-150 ug/g in brain, liver and kidney ($R^2=0.997-0.999$). The recoveries of (-)-carbodine ranged from 65-83%. The $t_{1/2}$, V_d and CL of (-)-carbodine were 24 min, 690 ml/kg, and 40 ml/min/kg, respectively. AUC for brain (199 mg*min/L), liver (878 mg*min/L) and kidney (4668 mg*min/L) indicated significant uptake of (-)-carbodine by the kidney. (-)-Carbodine does not have extensive penetration across the blood-brain barrier as evidenced by the low AUC observed in the brain. Half-lives in tissues were longer (38-73 min) as compared to the plasma half-life (24 min). The oral bioavailability of (-)-carbodine is 1.2 %. This low value was due to extensive first pass metabolism in the liver. Cytidine deaminase was identified as the metabolic enzyme responsible for the

transformation of (-)-carbodine to its uridine analog (-)-carbouridine. A prodrug will need to be developed to improve the oral bioavailability of (-)-carbodine and increase its penetration across the blood-brain barrier.

Key words (-)-carbodine bioavailability tissue distribution metabolism

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) causes Venezuelan equine encephalitis or encephalomyelitis (VEE) in all equine species. The virus can also infect healthy adult humans. People with weakened immune systems and the young and the elderly can become severely ill or die from this disease. VEEV has a wide geographic distribution in South America including Venezuela, Mexico, and Florida and infection of VEEV may give rise to sequela like mental retardation, amnesia, abortion, epilepsy and hydroanencephaly in humans and animals (1). The brain is the primary tissue in which VEEV replicates. Following a period of inactivity from 1973-1991, VEE reemerged during the past decade in South America and Mexico. Adaptive mutations, particularly mutations in the E2 envelope glycoprotein gene to efficient replication in equines are major determinants of emergence and the ability of VEEV to spread geographically (2, 3). Currently, no licensed therapeutics exists for the treatment of VEEV in man. The threats from the natural outbreak of VEEV as well as the potential use of VEE as a biological weapon indicate the importance of developing agents against VEEV (4, 5).

The carbocyclic analog of cytidine (carbodine) is a broad-spectrum antiviral agent against DNA and RNA viruses, such as influenza viruses (6, 7), yellow fever virus (8), measles virus (9), and Venezuelan equine encephalitis virus (10). The main mode of antiviral action by carbodine is through the inhibition of CTP synthetase that converts UTP to CTP, which results in a depletion of CTP pools (11). Shannon et al. (12) have demonstrated that carbodine is phosphorylated intracellularly to its 5'-triphosphate (C-

CTP), which inhibits CTP synthetase by a feedback mechanism. Reduced levels of CTP might be a rate limiting factor for the synthesis of new virus particles.

However, since CTP synthetase is also needed for host cell RNA and DNA synthesis, it acts as a target enzyme for both antiviral and cytotoxic activities of carbodine, which limits the application of carbodine. In order to increase the antiviral selectivity of carbodine, Julander et al. (10) have investigated the activity of (-)- and (+)-enantiomers as well as racemic carbodine against TC-83 VEEV strain both in Vero cell culture and infected C3H/HeN mouse model. The (-)-carbodine was found to have activity while the (+)-carbodine showed no activity in Vero cell culture and the racemic mixture showed intermediate activity. In addition, when infected C3H/HeN mice were treated with (-)-carbodine, significant improvement in antiviral selectivity index and various disease parameters were noted. It was anticipated that treatment with (-)-carbodine might greatly reduce the cytotoxicity observed after treatment with racemic carbodine. However, a detailed molecular mechanism needs to be characterized.

The purpose of this study is to develop an HPLC method for the quantification of (-)-carbodine in mouse plasma, brain, liver and kidney and determine the basic pharmacokinetic parameters, tissue distribution and metabolic pathway of (-)-carbodine in mice.

EXPERIMENTAL

Chemicals and Reagents

(-)-Carbodine was synthesized in Dr. Chu's lab (College of Pharmacy, UGA). HPLC-grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Sodium phosphate dibasic and triethylamine were obtained from J.T Baker Inc. (Philipsburg, NJ, USA). Formic acid ammonium salt was purchased from Sigma Chemical CO. (St. Louis, MO, USA). Isoflurane (USP) was manufactured by Abbott Laboratories (North Chicago, IL, USA). Tetrahydrouridine was obtained from CALBIOCHEM (La Jolla, CA, USA).

Instrumentation

HPLC system

The chromatographic analyses were performed using an HPLC system consisting of Agilent 1100 Series components including a quaternary pump, degasser, autosampler, and variable-wavelength UV detector (Palo Alto, CA, USA). Chromatographic separations were achieved using a Phenomenex SphereClone 5u ODS (1) column (4.6 mm×250 mm) with a Phenomenex Security Guard C₁₈ guard column (Torrance, CA, USA). The mobile phase used was 0.1% triethylamine in 0.05 M sodium phosphate buffer (adjusted to pH 6.0 using phosphoric acid). The mobile phase flow rate was 1.0 ml/min and the detection wavelength was optimized and set at 285 nm.

LC-MS/MS system

The above Agilent LC system was connected to a Quattro microTM API tandem mass spectrometer equipped with an electrospray source (Waters, USA). The same column

and guard column were used to achieve chromatographic separations. The mobile phase used was 0.1 % triethylamine in 0.01 M formic acid ammonium (adjusted to pH 6.0 using formic acid). The mobile phase flow rate was 1.0 ml/min and the analytes were infused directly into the mass spectrometer. The resulting optimized MS/MS detection parameters are shown in the following table.

Capillary	Cone	Extractor	Source	Desolvation	Collision
energy	energy	energy	Temp	Temp	energy
(kV)	(V)	(V)	(°C)	(°C)	(V)
3.50	30.0	3.0	130	375	16.0

Small animal benchtop anesthesia system

This system was obtained from SBH Scientific (Windsor, Ontario Canada).

Animals

NIH Swiss female mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). Animal studies were approved by the University of Georgia Animal Care and Use Committee, and conducted in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were maintained on Purina Lab Rodent Chow 5001 and water *ad libitum* and maintained at light/dark cycle from 7:00 am to 19:00 pm at an AAALAC approved facility on campus. Animals were conditioned for a week before they were utilized in the experiments.

Assay

Preparation of stock and standard solutions

Stock solution of 1.0 mg/ml (-)-carbodine was prepared in deionized water. Standard solutions of (-)-carbodine were prepared by serial dilution with deionized water. The final concentrations of the standard solutions were 500, 250, 100, 50, 25, 10 and 5 ug/ml. Quality control standards with concentrations of 400, 80, 16 and 5 ug/ml were also made with the same method. Stock solution was kept refrigerated when not in use and replaced on a bi-weekly basis. Fresh standard solutions were prepared for each day of analysis or validation.

Calibration curves

Blank plasma, brain, liver and kidney were collected from untreated anesthetized mice. The brain, liver and kidney were minced and homogenized with two volumes of ice-cold acetonitrile (w/v) using an Ultra-Turbax T8 tissue grinder (IKA Labortechnik, Germany). Plasma, brain, liver and kidney calibration points were prepared by spiking 100 µl of the biological matrices with 10 µl of each (-)-carbodine standard solution.

The calibration curve of the plasma was in the range of 0.5 – 50 µg/mL with individual calibration points of 0.5, 1, 2.5, 5, 10, 25, and 50 µg/mL. The calibration curve of the brain, liver and kidney homogenates were in the range of 1.5 – 150 µg/g with individual calibration points of 1.5, 3, 7.5, 15, 30, 75 and 150 µg/g.

Sample preparation

Plasma, brain, liver and kidney samples were prepared with protein precipitation by ice-cold acetonitrile. After spiking, samples were vortexed briefly and one volume of each matrix of ice-cold acetonitrile was added. To prevent the metabolism of (-)-carbodine in liver and kidney homogenates, liver and kidney samples were processed on ice. Samples were vortexed and centrifuged for 10 min at 13,000 rpm. The pellet was discarded and the supernatant was evaporated until reaching dryness. The residue was reconstituted in 100 μ l of deionized water. An injection volume of 20 μ l was used for all samples.

Method validation

Calibration curves were acquired by plotting peak area against the analyte concentration. Precision and accuracy were determined for the 3 QC points (40, 8, 1.6 μ g/ml plasma or 120, 24, 4.8 μ g/g tissue) and the LOQ (0.5 μ g/ml plasma or 1.5 μ g/g tissue). Five replicates of each QC point and LOQ were analyzed every day to determine the intra-day accuracy and precision. This process was repeated three times over 3 days in order to determine the inter-day accuracy and precision. Absolute recovery was determined by dividing the response of the analyte in the biological matrices by the response of the standard solution in deionized water. Freeze and thaw stability was determined over 3 cycles. Autosampler stability was determined by preparing samples and injecting them at different time intervals over 24 hours.

Animal study

The use of animals was approved by the UGA Animal Use and Care Committee. Animals were fasted 12 hours before dosing. Thirty female NIH Swiss mice weighted 20-25 g received an oral dose of (-)-carbodine at 500 mg/kg. Three mice were sacrificed at each time point. Blood, brain, liver and kidney samples were harvested at 2, 5, 10, 20, 30, 45, 60, 75, 90 and 120 min. Blood samples were then centrifuged at 8000 rpm for 10 min to collect plasma. Liver and kidney tissues were homogenized immediately in two volumes of ice-cold acetonitrile (w/v) to prevent loss from metabolism. Plasma, brain and processed liver and kidney samples were stored at -20°C until further analysis.

Twenty-four female NIH Swiss mice weighed 20-25 g were anesthetized with isoflurane (1.25-1.75% v/v) and dosed with 200 mg/kg (-)-carbodine by tail vein injection. Mice are sacrificed at different time points and blood and tissue samples were collected at 5, 15, 30, 45, 60, 90, 120 and 150 min into clean heparinized tubes. Blood samples were then centrifuged at 8000 rpm for 10 min to collect plasma. Brain, liver and kidney tissues were processed as discussed above. All samples were stored at -20°C until further analysis.

Data analysis

All the data generated from animal studies were subjected to WinNonlin non-compartmental analysis. The following parameters were calculated: absolute bioavailability (F), volume of distribution (V_d), half-life ($t_{1/2}$), peak concentration (C_{max}),

time to reach peak concentration (T_{max}), area under the concentration-time curve (AUC), and total body clearance (CL).

Characterization of (-)-carbodine's major metabolite

A plasma sample taken 30 min after intravenous bolus administration at 200 mg/kg was fully scanned by LC-MS. Molecular-ion 242 and 243 were selected and analyzed by LC-MS/MS. Pure (-)-carbodine and some candidates of its major metabolite were also qualified by LC-MS and LC-MS/MS. Their primary and secondary MS graphs were compared to those of the plasma sample.

Characterization of (-)-carbodine's major metabolic enzyme

Tetrahydrouridine (0, 1, 10, 100 and 1000 ug/ml) was added to kidney homogenates spiked with 50 ug/ml (-)-carbodine. All samples were vortexed and processed after 10 min incubation at room temperature. The concentration of (-)-carbodine remaining in each sample was analyzed by HPLC.

RESULTS AND DISCUSSION

Venezuelan equine encephalitis or encephalomyelitis is a mosquito-borne viral disease characterized by symptoms such as severe headache, chills, fever, myalgia, retro-orbital pain, nausea and vomiting. The disease may progress to convulsions, paralysis, coma and death. Currently, investigational attenuated virus vaccine and inactivated vaccine are available and recommended (13, 14), Sharma A et al. (15) also reported the inactivation of VEEV by 1, 5-iodonaphthylazide (INA), but no specific medications are approved for the treatment of VEE (16, 17). (-)-Carbodine was found to have high

activity against VEEV with low toxicity (10). The chemical structure of (-)-carbodine is shown in Fig. 5.1.

So far, no publication has described the determination of (-)-carbodine in biological matrices. In our study, an HPLC method was developed for the quantification of (-)-carbodine. Fig. 5.2 a-d shows chromatograms of each extracted blank matrix and extracted matrix spiked with 5 ug/ml (-)-carbodine. The calibration curve for each day of validation and analysis shows a linear response ($R^2=0.997-0.999$) in all biological matrices through a range of 0.5-50 ug/ml (plasma) or 1.5-150 ug/g (tissue) (Table 5.1).

The extraction efficiencies for (-)-carbodine from the various matrices are expressed in terms of absolute recovery. The absolute recoveries are determined by comparing the peak areas of spiked matrices to the corresponding peak areas of the untreated standard solutions. (-)-Carbodine recoveries from plasma, brain, liver and kidney range from 65-83%. The absolute recoveries for each individual matrix are displayed in Table 5.2.

Precision and accuracy measurements are acquired for 3 QC points and the L.O.Q for the compound. Precision is reported as percent relative standard deviation (RSD) and accuracy is reported as percent error. Values for the intra-day (n=5) precision and accuracy range from 0.74-12.71 % and 1.13-18.65 %, respectively. Inter-day (n=15) precision and accuracy range from 2.64-12.99 % and 2.38-17.11 %, respectively. The intra-day and inter-day precision and accuracy data for each individual matrix are shown in Table 5.3.

Stability testing is performed for (-)-carbodine at the concentration level of 5 ug/ml. Spiked samples are found stable over 3 freeze/thaw cycles in all matrices except in liver and kidney homogenates (Table 5.4). The liver and kidney samples have to be processed immediately after their removal from the body. Prepared samples from all biological matrices are stable in the autosampler at room temperature for up to 24 hours (Table 5.5).

The mean plasma and tissue concentration-time profiles after IV administration of 200 mg/kg of (-)-carbodine are shown in Fig. 5.3. The pharmacokinetic parameters generated from WINNONLIN non-compartmental analysis for plasma and tissues after IV administration are given in Tables 5.6 and 5.7. The plasma concentration declines monoexponentially, indicating a one-compartment model. The plasma half-life, volume of distribution, and total body clearance are 24 min, 1.4 L/kg, and 40 ml/min/kg, respectively. The large V_d indicates that (-)-carbodine is extensively distributed into total body water and suggests significant tissue binding. The value of clearance indicates that this compound is rapidly cleared from the body with an extraction ratio in the liver of approximately 0.7.

The concentration-time profiles of all tissues decline in a biexponential fashion, indicating a two-compartment model. Peak tissue concentrations are observed at the first time point of 5 min in all tissues. The concentration-time profile of the kidney initially follows a similar pattern with that of plasma with concentrations of (-)-carbodine approaching those in the plasma. However, concentrations in the kidney exceed the concentrations in plasma after 45 minutes and then decline with a longer $t_{1/2}$ of 38

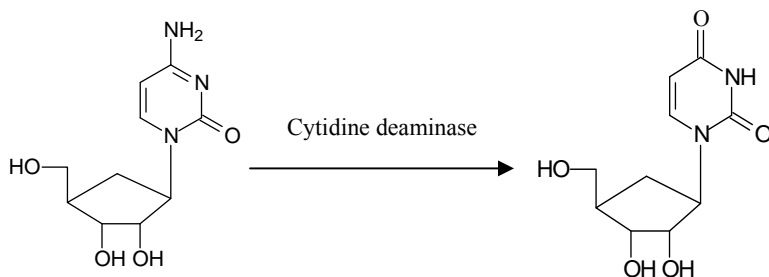
minutes. The concentration of (-)-carbodine in liver declines quickly initially but also exhibits a longer terminal $t_{1/2}$ (73 minutes) as compared to plasma. The brain has the lowest concentrations and had an elimination half-life of 49 minutes. While the brain concentrations were low they were still above the EC_{50} (0.2 ug/ml) throughout the experiment.

(-)-Carbodine is eliminated slower in tissues with terminal elimination half-lives of 38-73 min as compared to the plasma half-life, indicating that (-)-carbodine may be sequestered in tissues (especially the kidney). AUC and relative exposure (RE) for brain (199 mg*min/L, 0.04), liver (878 mg*min/L, 0.18) and kidney (4668 mg*min/L, 0.95) indicate low penetration of (-)-carbodine across the blood-brain barrier but significant uptake by the kidney. Active influx transporters may be involved in transporting (-)-carbodine into kidney. Concentrations of (-)-carbodine in tissues and plasma were compared at 15, 45 and 90 min in Table 5.8 and Fig. 5.4 to explain its disposition. The C_{tissue}/C_{plasma} ratios are relatively constant between 15 and 45 min but exhibit a 4-6 fold increase at 90 min, indicating sequestration of (-)-carbodine in all tissues.

The mean concentration-time profile of oral administration of 500 mg/kg of (-)-carbodine is shown in Fig. 5.5. (-)-Carbodine is quickly absorbed from the gastrointestinal tract and the peak concentration in plasma reaches 2.54 ug/ml at 30 min after administration. After that, the plasma concentrations of (-)-carbodine decline quickly and are not detectable after 2 hr. Tissue concentrations of (-)-carbodine in brain, liver and kidney are not detectable with oral dosing of 500 mg/kg of (-)-carbodine. The

pharmacokinetic parameters generated from WINNONLIN non-compartmental analysis for oral administration of (-)-carbodine are given in Table 5.6. The $t_{1/2}$, Vd, and CL are 28 min, 1.5 L/kg, and 37 ml/min/kg, respectively, which are consistent with the IV data. The absolute bioavailability of (-)-carbodine is only 1.2 %, indicating extensive first pass metabolism.

(-)-Carbodine is found to be extremely unstable in mouse kidney homogenate at room temperature. In vivo studies also show a new peak in the chromatograms of all biological matrices after 200 mg/kg intravenous bolus administration of (-)-carbodine (Fig. 5.6 a-d). This metabolite is found to be an inactive uridine analog and has a molecular weight of 242. Its structure was characterized by LC-MS and LC-MS/MS (Fig. 5.7) and is shown in Fig. 5.8. *In vitro* competitive inhibition studies using tetrahydrouridine as the competitive inhibitor indicates that cytidine deaminase is the metabolic enzyme that transforms (-)-carbodine to its uridine analog (Fig. 5.9). Tetrahydrouridine is a unique inhibitor of cytidine deaminase. Both tetrahydrouridine and (-)-carbodine are incubated with mouse kidney homogenate. As tetrahydrouridine concentration increases, the concentration of (-)-carbodine also increases, indicating that (-)-carbodine is one of the substrates of cytidine deaminase. The following scheme appears to be the most probable metabolic pathway in mice for (-)-carbodine:



Shannon et al. (12) reported that carbodine was metabolized to carbodine 5'-triphosphate (C-CTP) in mouse leukemia L1210 cells. However, in our study, (-)-carbodine is not directly metabolized to C-CTP. The metabolic pathway can be broken down into several steps: (-)-carbodine (C-cytidine) \rightarrow C-uridine \rightarrow C-UTP \rightarrow C-CTP. In the last step, C-UTP competes with UTP for CTP synthetase, resulting in the depletion of CTP pool.

In conclusion, (-)-carbodine undergoes extensive first pass metabolism in the liver and has limited penetration across the blood-brain barrier in mice. After 500 mg/kg oral administration, most of the drug is metabolized and (-)-carbodine is not detectable in tissues. After 200 mg/kg IV dose, the brain concentration of (-)-carbodine is above EC_{50} throughout the experiment, which is consistent with a previously published pharmacodynamic study (10) which indicated in vivo effectiveness of (-)-carbodine in VEEV infected mice. A prodrug which would increase oral bioavailability as well as increase penetration into the brain would decrease (-)-carbodine's total body exposure and increase the therapeutic index of the compound.

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Table 5.1. Linear regression equations generated from validation data from untreated mouse plasma, brain, liver and kidney (n=3).

Matrix	Slope	Intercept	R ²
Plasma	21.58±0.87	-1.87±2.123	0.999±0.001
Brain	22.35±0.87	-2.89±0.71	0.997±0.001
Liver	21.18±1.31	-0.88±2.57	0.998±0.002
Kidney	22.84±0.87	-0.55±0.28	0.999±0.001

Table 5.2. Percent absolute recoveries of (-)-carbodine from untreated mouse plasma, brain, liver and kidney. (n=20)

Concentration	Plasma	Brain	Liver	Kidney
40 ug/ml (120 ug/g)	81.5±1.9	79.9±2.3	67.6±3.6	67.8±2.7
8 ug/ml (24 ug/g)	80.6±2.0	69.0±1.4	64.5±5.3	73.4±1.5
1.6 ug/ml (4.8 ug/g)	82.8±2.8	73.1±1.7	72.4±1.5	77.6±3.7
0.5 ug/ml (1.5 ug/g)	78.1±2.1	75.4±7.2	64.7±4.7	72.3±2.6

Table 5.3. Intra-day (n=5) and Inter-day (n=15) precision (%R.S.D) and accuracy (%Error) of the method in mouse plasma, brain, liver and kidney. T.C denotes theoretical concentration and E.C denotes experimental concentration.

T.C	Day 1			Day 2			Day 3			Inter-day		
	E.C	Error	R.S.D.	E.C	Error	R.S.D.	E.C	Error	R.S.D.	E.C	Error	R.S.D.
<i>Plasma (ug/ml)</i>												
40	40.33	2.98	3.56	37.10	7.25	2.30	38.50	3.76	2.66	38.59	4.79	4.44
8	7.87	1.63	2.49	7.25	9.39	2.42	8.20	2.95	2.22	7.77	4.84	5.69
1.6	1.52	4.92	2.99	1.53	4.62	3.02	1.54	3.47	2.20	1.53	4.34	2.64
0.5	0.52	4.69	5.34	0.60	18.38	1.90	0.53	6.43	3.83	0.55	9.83	6.99
<i>Brain (ug/g)</i>												
120	117.5	3.33	4.41	122.9	3.11	2.89	120.4	2.08	2.80	120.3	2.84	3.68
24	21.57	10.11	1.11	20.80	13.34	1.99	21.00	12.48	2.24	21.12	12.02	2.34
4.8	4.89	2.97	3.12	4.34	9.49	2.22	4.53	5.60	0.74	4.59	6.02	5.53
1.5	1.77	18.25	2.43	1.78	18.65	8.03	1.72	14.44	4.61	1.76	17.11	5.40
<i>Liver (ug/g)</i>												
120	120.9	3.38	3.96	120.2	1.21	1.38	121.5	2.54	2.91	120.9	2.38	2.77
24	23.49	2.12	2.62	25.04	4.34	2.51	22.66	5.58	2.24	23.73	4.03	4.88
4.8	4.52	5.89	4.20	4.43	7.83	3.67	4.58	4.70	4.17	4.50	6.15	3.98
1.5	1.52	10.41	12.71	1.43	9.86	11.89	1.767	17.86	3.57	1.57	12.73	12.99
<i>Kidney (ug/g)</i>												
120	113.0	5.81	1.53	113.3	5.61	4.04	114.5	5.25	4.99	113.6	5.57	3.55
24	23.73	1.13	6.13	24.00	1.78	2.05	24.86	4.74	4.31	24.33	3.63	4.57
4.8	5.12	6.62	4.95	4.92	3.85	4.72	4.80	2.66	3.80	4.95	4.38	5.02
1.5	1.57	5.03	5.09	1.57	4.65	3.38	1.62	8.18	4.26	1.59	5.96	4.31

Table 5.4. Results of freeze/thaw stability of (-)-carbodine in mouse plasma, brain, liver and kidney, represented by area \pm S.D. (n=5) of each day and % R.S.D of the area between days.

	Plasma	Brain	Liver	Kidney
Day 1	247.4 \pm 4.2	250.5 \pm 7.1	253.8 \pm 2.1	241.2 \pm 2.2
Day 2	243.6 \pm 4.4	250.0 \pm 6.7	231.9 \pm 0.7	-
Day 3	241.8 \pm 3.4	246.1 \pm 5.1	223.6 \pm 5.2	-
Day 4	240.5 \pm 0.4	243.2 \pm 2.6	219.3 \pm 5.5	-
%R.S.D.	2.0	2.3	6.2	-

Table 5.5. Results of autosampler stability of (-)-carbodine in mouse plasma, brain, liver and kidney, represented by area \pm S.D. (n=5) of each day and % R.S.D of the area between hours.

	Plasma	Brain	Liver	Kidney
0h	256.0 \pm 4.8	257.6 \pm 4.6	253.8 \pm 2.1	241.2 \pm 2.2
12h	237.8 \pm 15.3	247.9 \pm 3.3	217.7 \pm 0.9	202.9 \pm 4.4
24h	237.5 \pm 8.0	237.2 \pm 4.4	228.7 \pm 1.2	215.7 \pm 17.3
%R.S.D.	5.3	3.9	6.9	8.7

Table 5.6. Pharmacokinetic parameters of (-)-carbodine.

	Oral	IV
$t_{1/2}$ (min)	28	24
AUC (min*ug/ml)	149	4912
C_{max} (ug/ml)	2.54	-
T_{max} (min)	30	-
V_d (L/kg)	1.5	1.4
Cl_T (ml/min/kg)	37	40
F (%)	1.2	

Table 5.7. Tissue distribution after IV bolus administration of 200 mg/kg (-)-carbodine.

	Brian	Liver	Kidney
$t_{1/2}$ (min)	49	73	38
Cmax (ug/g)	5.7	80.9	260.5
AUC (min*ug/ml)	199	878	4668
RE*	0.04	0.18	0.95

*RE=AUC_{tissue}/AUC_{plasma}

Table 5.8. Tissue/Plasma concentration ratios at 15, 45, and 90 min after 200 mg/kg IV administration of (-)-carbodine.

	15 min	45 min	90 min
$C_{\text{Brain}}/C_{\text{Plasma}}$	0.04	0.06	0.35
$C_{\text{Liver}}/C_{\text{Plasma}}$	0.10	0.11	0.42
$C_{\text{Kidney}}/C_{\text{Plasma}}$	0.65	0.55	3.30

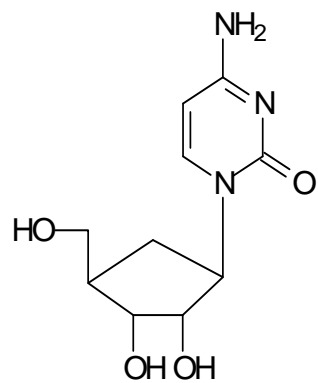
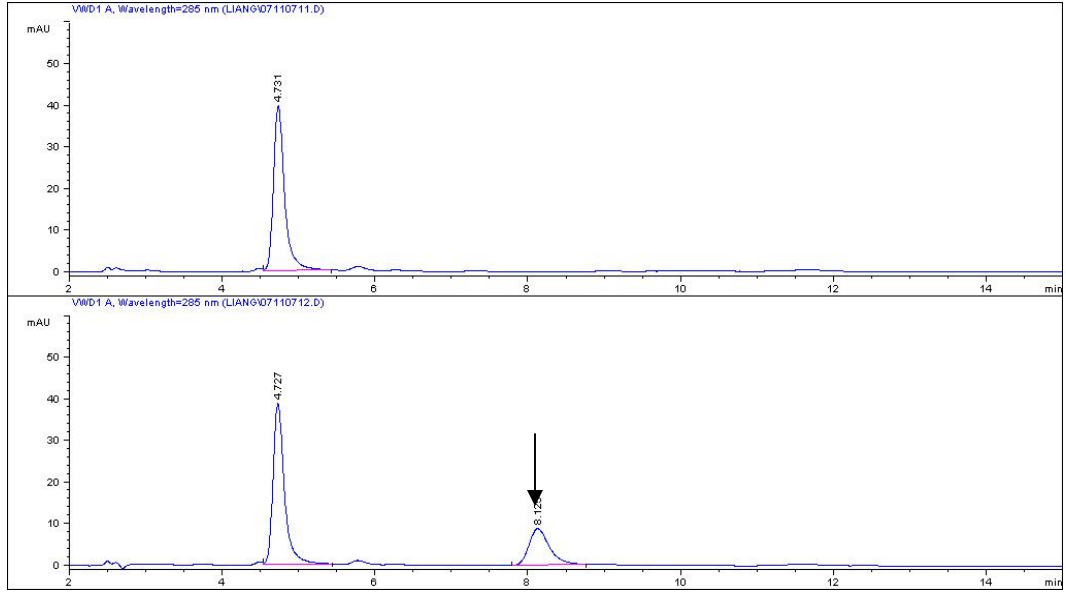
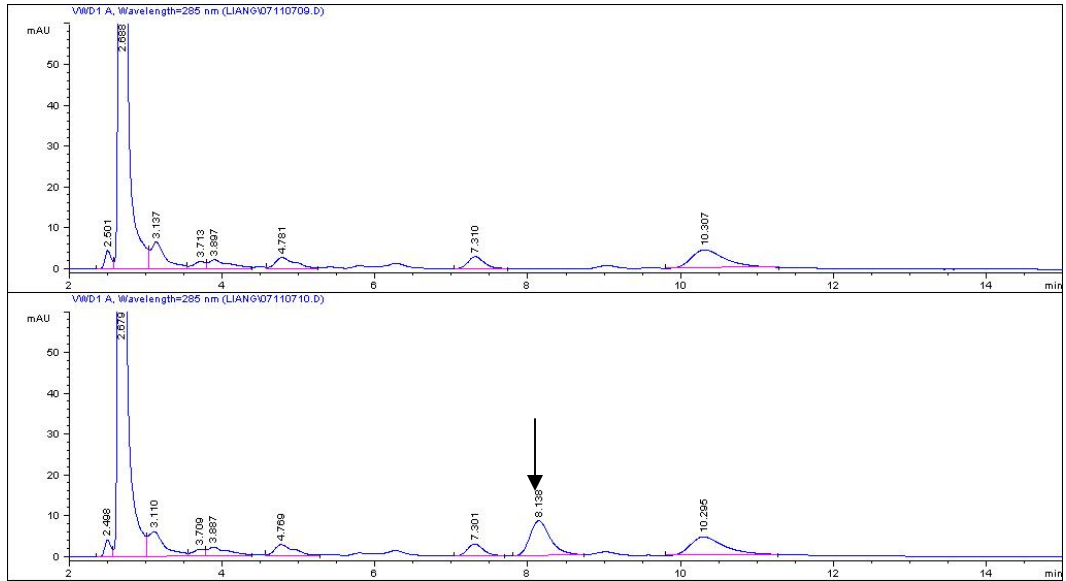


Fig. 5.1. Chemical structure of (-)-carbodine.

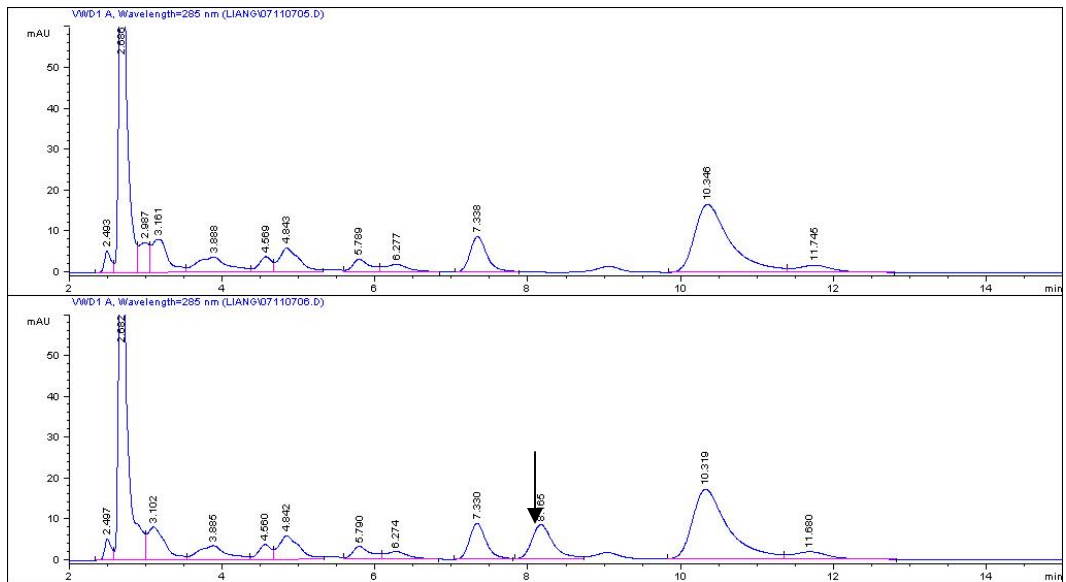
(a)



(b)



(c)



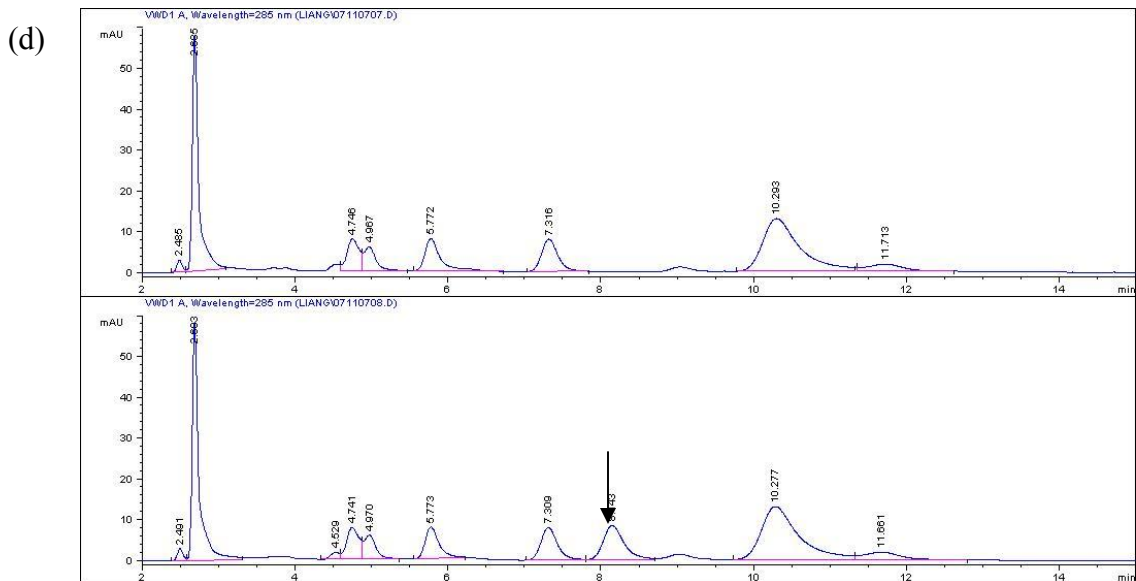


Fig. 5.2. (a) Chromatographs of mouse plasma spiked with 5µg/ml (-)-carbodine (bottom) and blank mouse plasma (top). (b) Chromatographs of mouse brain homogenate spiked with 5µg/ml (-)-carbodine (bottom) and blank mouse brain homogenate (top). (c) Chromatographs of mouse liver homogenate spiked with 5µg/ml (-)-carbodine (bottom) and blank mouse liver homogenate (top). (d) Chromatographs of mouse kidney homogenate spiked with 5µg/ml (-)-carbodine (bottom) and blank mouse kidney homogenate (top). Arrows indicate peaks of (-)-carbodine.

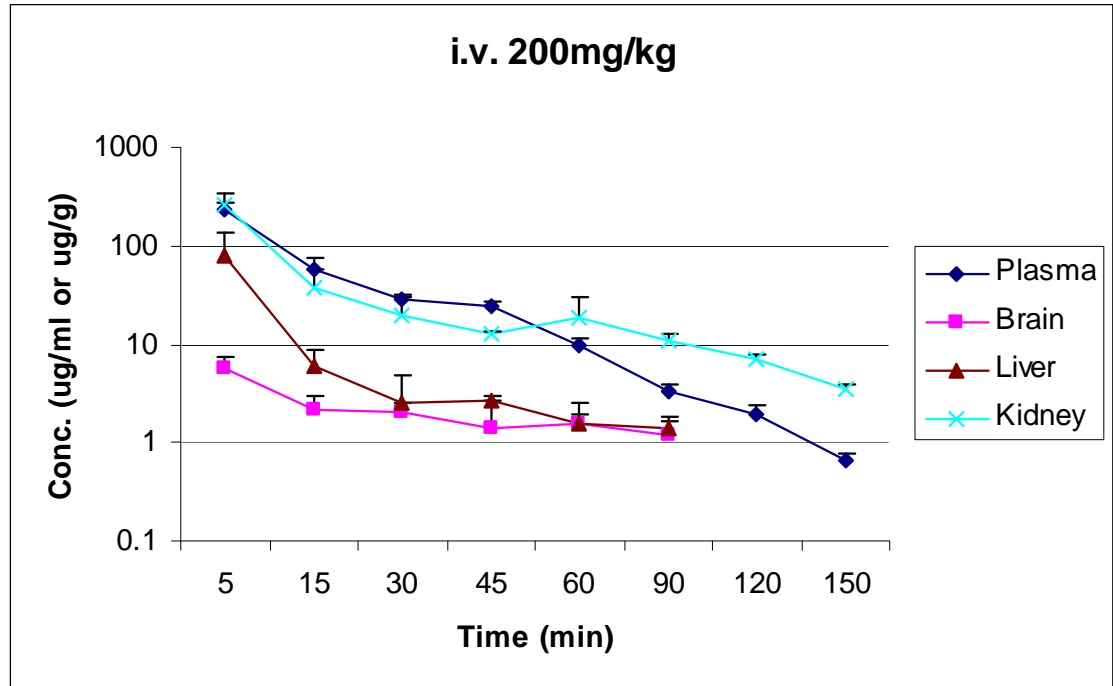


Fig. 5.3. Concentration vs. time curve of (-)-carbodine in plasma, brain, liver and kidney after 200 mg/kg IV bolus dose of (-)-carbodine.

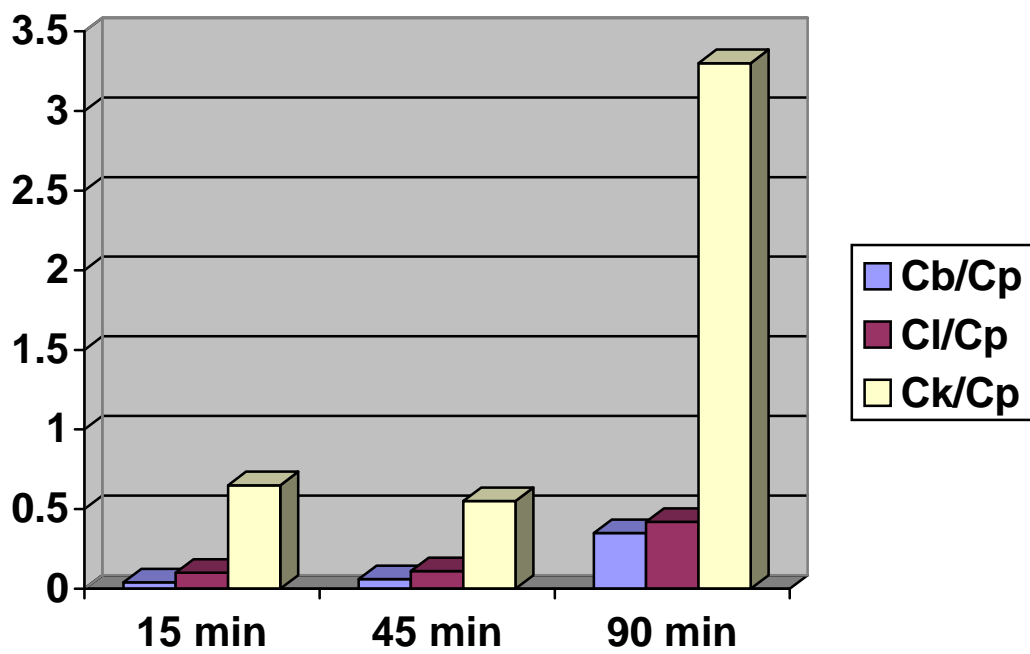


Fig 5.4. Tissue/Plasma concentration ratios at 15, 45, and 90 min after 200 mg/kg IV administration of (-)-carbodine.

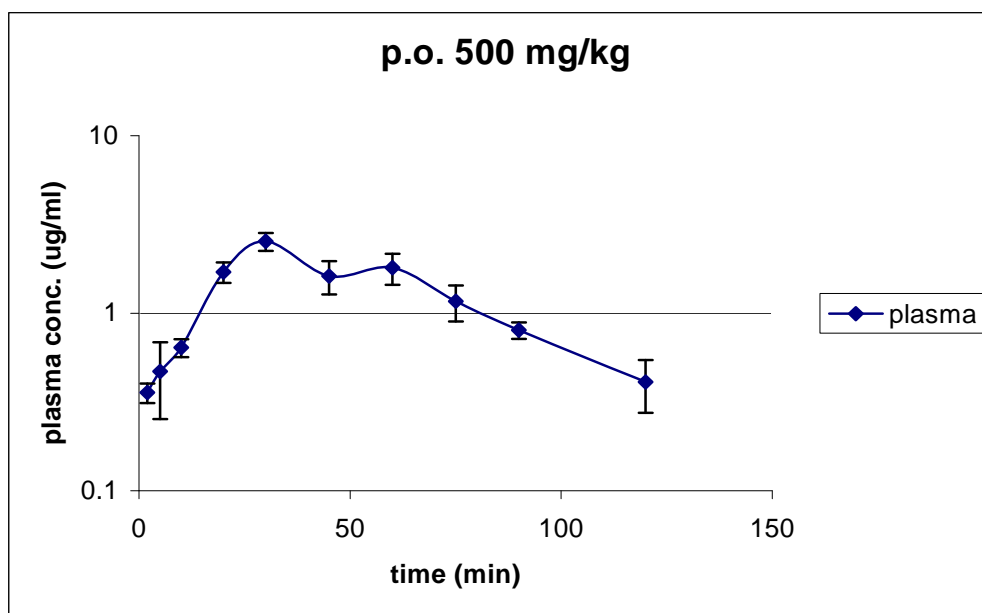


Fig. 5.5. Concentration vs. time curve of (-)-carbodine in plasma after 500 mg/kg oral dose of (-)-carbodine.

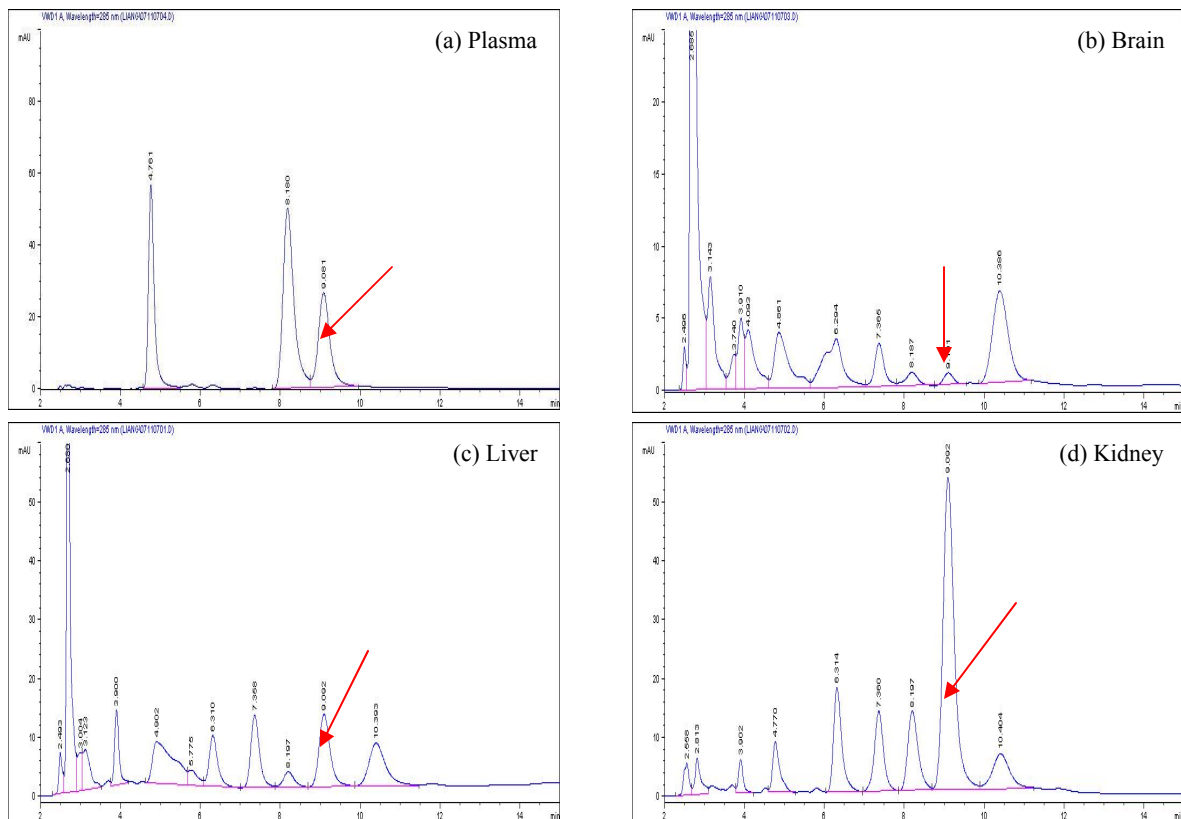


Fig. 5.6. Chromatographs of mice plasma, brain, liver and kidney sampled 30 min after 200 mg/kg i.v. bolus administration of (-)-carbodine. Arrows represent peaks of metabolite.

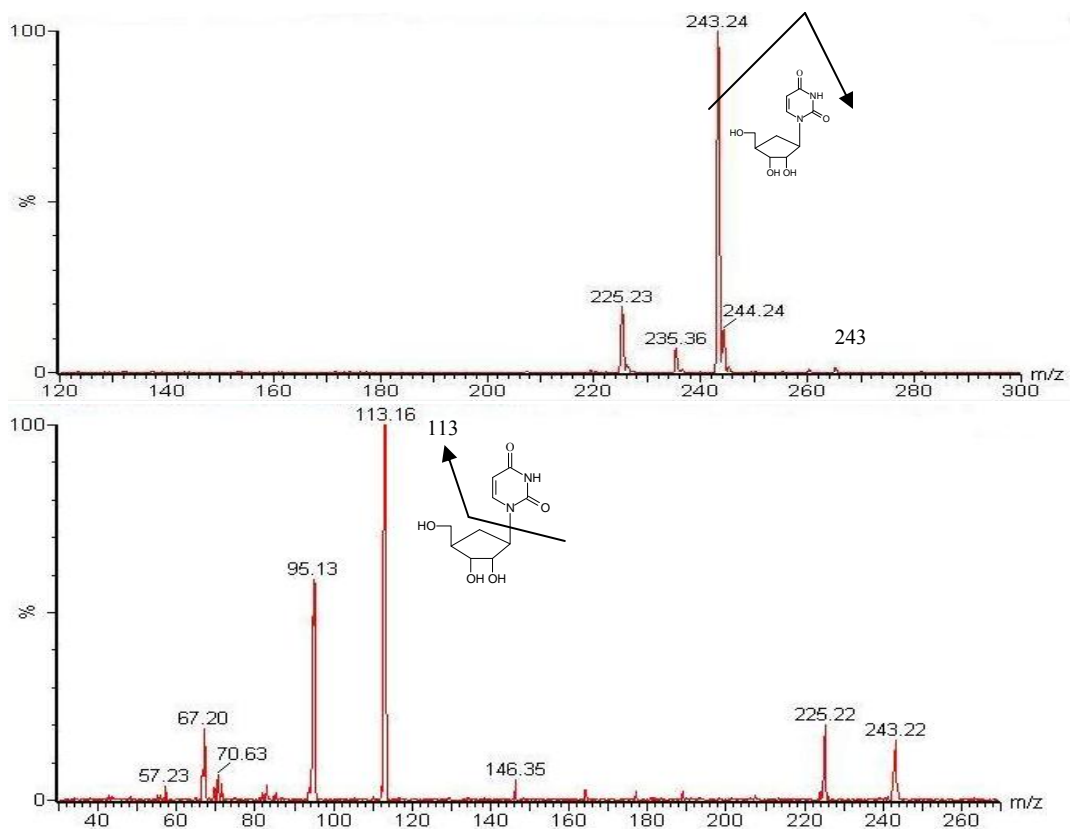


Fig. 5.7. Primary (top) and secondary (bottom) MS graphs of (-)-carbodine's major metabolite.

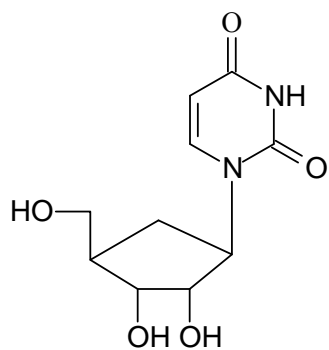


Fig. 5.8. The chemical structure of (-)-carbodine's major metabolite (a uridine analog).

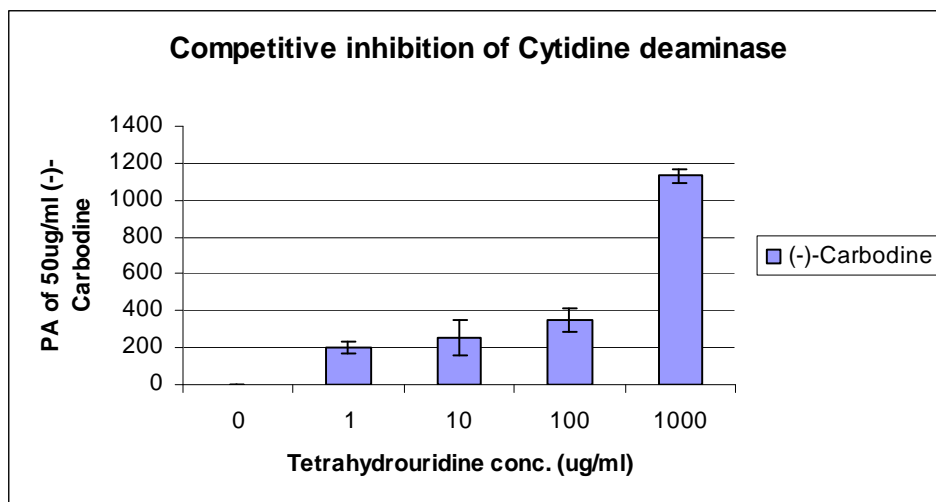


Fig. 5.9. Competitive inhibition of cytidine deaminase by tetrahydrouridine against (-)-carbodine.

CHAPTER 6

CONCLUSIONS

Bioavailability studies provide useful information on the development of new drug products before they are marketed. The evaluation of a drug product's bioavailability involves consideration of the drug product, subject, assay, experimental design and data analysis.

Bioavailability is affected by physicochemical factors of drugs (e.g. particle size, partition coefficient, polymorphism, and stability), physiological factors of subjects (e.g. gastric emptying, intestinal transit, blood flow, drug-food interaction, and disease states), and formulation factors. The choice of appropriate animal models and human subjects are important for preclinical and clinical studies.

Crossover design is extensively used in bioavailability studies. In this type of study, clearance, volume of distribution, and absorption, as determined by physiological variables, are assumed to have less variability compared to the variability arising from formulation performance. Therefore, differences between two products because of formulation factors can be determined. Pharmacokinetic parameters derived from measures of concentration, e.g. AUC, C_{max}, should be analyzed using analysis of variance (ANOVA). Other parameters such as T_{max}, t_{1/2}, CL, V_d, K_a, and F should be reported.

In chapter 3, the pharmacokinetics and bioavailability of a buccal formulation of S-carboxymethylcysteine in comparison to oral and IV routes of administration was described. The buccal formulation exhibited a 65% increase in bioavailability as compared to the oral tablet, indicating the buccal formulation greatly improved the absolute bioavailability and might be appropriate for many compounds, which undergo extensive first pass metabolism, or for patients who have difficulty in swallowing solid dosage formulation.

Chapter 4 studied the pharmacokinetics and bioavailability of an oral wafer formulation of N-acetylcysteine in comparison to a marketed capsule formulation. No significant differences in AUC, C_{\max} and T_{\max} were observed between wafer and capsule formulation. The new wafer formulation would be interchangeable with the standard formulation.

In chapter 5, an HPLC method was developed for the quantification of (-)-carbodine in mouse plasma, brain, liver and kidney, and the absolute bioavailability and tissue distribution of (-)-carbodine were determined in mice. (-)-Carbodine did not have extensive penetration across the blood-brain barrier as evidenced by the low AUC observed in the brain. However, brain concentrations were still above the EC_{50} throughout the experiment after the IV dose. Concentrations in tissues were not detectable after the oral dose. The oral bioavailability of (-)-carbodine was 1.2%, resulting from extensive first pass metabolism in the liver. Cytidine deaminase was identified as the metabolic enzyme responsible for the transformation of (-)-carbodine

into its uridine analog (-)-carboursidine. A prodrug which would increase oral bioavailability as well as increase penetration into the brain would decrease (-)-carbodine's total body exposure and increase the therapeutic index of the compound.