STUDIES OF CARBAMAZEPINE METABOLISM

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ABSTRACT

The objective of this study was to examine aspects of carbamazepine metabolism, in order to contribute to a long term goal of a thorough examination of how the metabolism of carbamazepine is influenced by other drugs.

The first set of experiments were designed with the intent of determining values for the pharmacokinetic parameters of carbamazepine metabolism in male New Zealand white rabbits. Values were obtained for t_{max} (60-90 min), $t_{1/2}$ (90-122 min), clearance (46.2-142.4 ml/min/kg), and the elimination constant (0.0057-0.0077 min^{-1}) in five test cases. In the remainder of cases, unexpected results were observed which did not allow calculation of these parameters. The plasma carbamazepine concentration was either delayed in reaching its peak concentration or it reached an apparent peak, but maintained that level for an extended period of time. It is thought that these differences between rabbits may have been due to differences in the rates of gastric emptying, a factor that may have been influenced by the food eaten by the animals in a period in excess of the 12 hours that some of the rabbits were fasted prior to the experiments. Alternatively, the time period of required sampling may have been underestimated. In addition, it is also possible that some degree of enterohepatic circulation is taking place. The relative positions of the curves for carbamazepine and for carbamazepine-10,11-epoxide suggest that there may be differences in the activities of hepatic monooxygenases and glucuronysyl transferases responsible for the metabolic fates of carbamazepine.

The second set of experiments examined the influence of isoniazid and some of its principal metabolites on the conversion of carbamazepine to carbamazepine-10,11-epoxide in the S9 fraction of rat liver homogenate. This study is a prelude to planned in vivo studies of the interaction in rabbits. Three concentrations of each of isoniazid, acetylhydrazine, acetylisoniazid, hydrazine, and isonicotinic acid were tested in a system containing constant concentrations of carbamazepine and of essential co-factors. The results indicated that there was a concentration dependent inhibition of carbamazepine metabolism by isoniazid, hydrazine, and isonicotinic acid. These types of experiments should expanded to include a range of carbamazepine concentrations so that an evaluation of the type of inhibition can be determined, as can be done Michaelis-Menton kinetics.

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LIST OF ABBREVIATIONS

AcHz	Acetylhydrazine
AcINH	Acetylisoniazid
CBZ	Carbamazepine
CE	Carbamazepine-10,11-epoxide
Cl	Clearance
Hz	Hydrazine
INA	Isonicotinic acid
INH	Isoniazid
$t_{1/2}$	Apparent half-life
V_d	Volume of distribution

1 PHARMACOKINETICS OF CARBAMAZEPINE IN RABBITS:

1.1 INTRODUCTION:

The first portion of this thesis examines the pharmacokinetics of carbamazepine in rabbits. In our laboratory, there is an ongoing study of the hepatotoxicity of isoniazid in rabbits. One of the future goals of that study is to examine the influence of carbamazepine. To do so, a knowledge of the pharmacokinetics of carbamazepine, in the same animal model, is required. Since the appropriate pharmacokinetic parameters were not available in the literature and since an appropriate liquid chromatographic assay procedure had been developed, the study was done as part of this project.

1.1.1 PHARMACOKINETICS:

The study of pharmacokinetics involves the investigation of the kinetics of drug absorption, distribution, and elimination (i.e., metabolism and excretion). There are sensitive, accurate and precise analytical methods for the direct measurement of drugs in biological samples, such as plasma and urine. These measurements of drug concentrations can be used to determine pharmacokinetic parameters such as bioavailability, the elimination rate constant, the apparent volume of distribution, and the elimination half-life.

Bioavailability is defined as the fraction of unchanged drug reaching the systemic circulation following administration by any route (Benet, 1987). Thus, for an intravenous dose, the bioavailability will be equal to one. For an oral dose, the bioavailability may be less than one for any of several reasons. The most obvious reason is the incomplete absorption of

the drug. As well, the drug may be metabolized during absorption. The intestinal mucosa contains sulphate-conjugating enzymes which may inactivate certain drugs during their absorption. The drugs may also be susceptible to the effects of gastrointestinal secretions and they may be subject to metabolism by bacteria residing in the intestines. Drugs may also undergo metabolism in the portal blood or in the liver without ever being able to reach the systemic circulation. As a result, the bioavailability can be significantly decreased from unity.

The apparent volume of distribution (V_d) of a drug is the volume of fluid it would occupy if the total amount in the body were in a solution at the same concentration as in the plasma. The volume of distribution may vary widely depending on the pK_a of the drug, the degree of plasma protein binding, the partition coefficient of the drug in the fatty tissues, and the degree of binding to other tissues within the body. For example, a drug that is tightly bound to plasma protein may have a volume of distribution of 0.06 L/kg, which corresponds to the plasma volume per kilogram of body weight. In contrast, some drugs that are selectively bound to constituents of tissues or are taken up selectively by cells can have an apparent volume of distribution that is several hundred times body volume.

The elimination half-life ($t_{1/2}$), an expression of the relationship between volume of distribution and clearance ($t_{1/2}=0.693 \times V_d/Cl$), is a useful kinetic parameter in that it indicates the time required to attain steady state or to decay from steady state conditions after a change (i.e., starting or stopping) in a particular rate of drug administration (dosing regimen). However, it has little value as an indicator of drug elimination or distribution.

Clearance is the measure of the body's ability to eliminate a drug. The

organs of elimination can only clear drug from the blood or plasma that is in direct contact with the organ. Thus, the time course of drug in the body will depend on both the volume of distribution and the clearance.

1.1.2 CARBAMAZEPINE:

Carbamazepine [5-carbamyldibenz-(b,f)-azepin], an iminostilbene derivative related to imipramine (Figure 1), was first synthesized by Schindler in the late 1950's and it was patented in 1961 (Schindler, 1961a). It was first introduced for the treatment of trigeminal neuralgia (Blom, 1962, 1963), and it was approved for use as an anticonvulsant agent in the United States in 1974. Carbamazepine has been studied extensively, particularly with respect to its metabolism and pharmacokinetics, its toxicities, and its potential mechanisms of action.

1.1.2.1 DEVELOPMENT AND CHEMISTRY:

Iminodibenzyl (10,11-dihydro-5H-dibenzo[b,f]azepine), shown in Figure 2, first described by Theil and Holzinger in 1899, may be considered historically as the precursor of carbamazepine. Schindler and Hafliger (1954) synthesized a number of iminodibenzyl derivatives that possessed local anaesthetic and antihistaminic properties and some modest anticonvulsant activity. When a carbamyl (carboxamide) group was added at the 5-position of iminodibenzyl, considerable anticonvulsant activity was observed. The strongest anticonvulsant properties were observed when a carbamyl side chain was combined with iminostilbene (Figure 3), a structure analagous to imino-

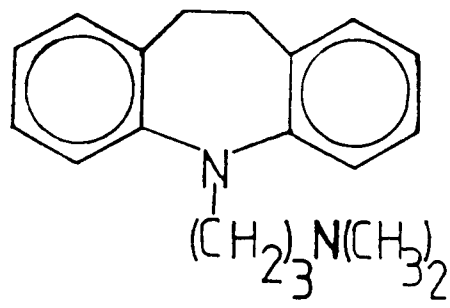


Figure 1: Imipramine

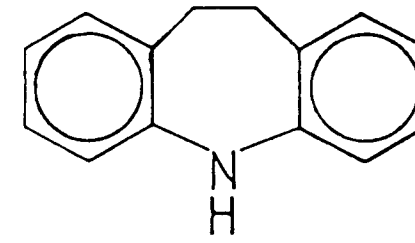


Figure 2: Iminodibenzyl

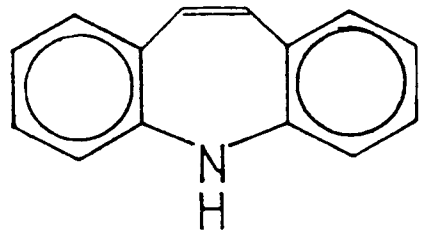


Figure 3: Iminostilbene

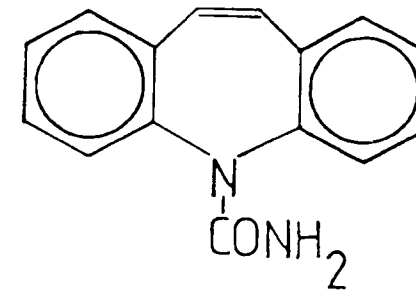


Figure 4: Carbamazepine

dibenzyl, but having a double bond between the 10 and 11 positions. This structure has become known as carbamazepine, and its synthesis was described in 1961 (Schindler, 1961b).

Carbamazepine (5-carbamyl-5H-dibenzo[b,f]azepine; 5H-dibenzo[b,f]azepine-5-carboxamide) (Figure 4), an iminostilbene derivative with an empirical formula $C_{15}H_{12}N_2O$ and a molecular weight of 236.26 g/mol, appears as a white crystalline compound with a melting point between 190°C and 193°C (Kutt and Paris-Kutt, 1982). It is a neutral lipophilic substance. It is virtually insoluble in water, but it dissolves in ethanol, chloroform, dichloromethane, and other solvents.

X-ray diffraction studies of carbamazepine have produced measurements that are characteristic of tricyclic psychoactive drugs. In the three dimensional structure the angle of flexure, α, is 53°, the angle of annelation, β, is 30°, and the angle of torsion, γ, is 3° (Figure 5). The distance between the centres of the benzene rings measured 4.85 Angstroms (Gagneux, 1976). The similarity to imipramine is obvious in the steric parameters, except for the torsion angle which is 20° for imipramine.

1.1.2.2 THERAPEUTIC USES:

Carbamazepine was first introduced in the early 1960's, when it was administered to patients suffering from trigeminal neuralgia (Blom, 1962, 1963), a disorder that is also referred to as tic douloureux. By far the most dramatic, if not the most common, of all the painful disorders that afflict the human face, trigeminal neuralgia was described in detail by Fothergill (1773), who compiled, with thoroughness, the clinical features of fourteen patients with trigeminal neuralgia. In a short time following Blom's reports, a number of clinical investigators published similar obser-

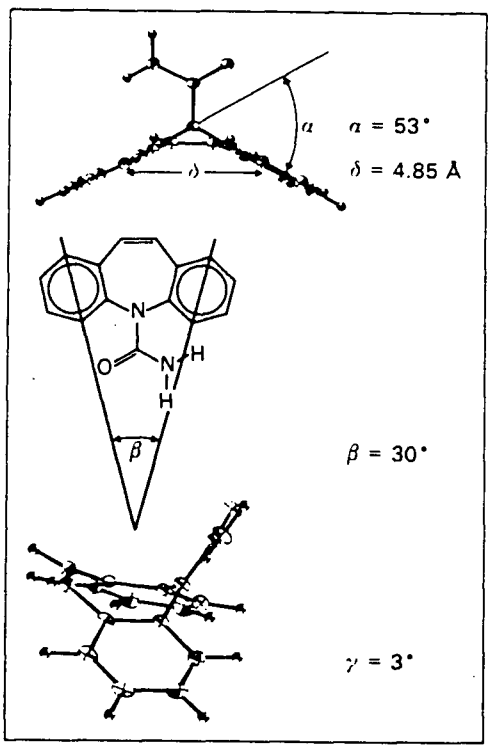


Figure 5: Three-dimensional structure of carbamazepine as revealed by X-ray diffraction.
Above: frontal view
Middle: top view
Below: side view
Note that the carbamazepine molecule is bent and slightly twisted. (from Gagneux, 1976)

vations (Taylor, 1963; Bonduelle, et al., 1963; Dalessid and Abbott, 1966; Amols, 1966). Carbamazepine is now the drug of choice in the treatment of trigeminal neuralgia. Later investigations indicated that carbamazepine is also effective in the treatment of glossopharyngeal neuralgia (Ekborn and Westberg, 1966).

Carbamazepine has also been used to treat lightning pains associated with tabes dorsalis (Ekbom, 1966, 1972). The basis of carbamazepine use in this disorder was the similarity between tabetic lightning pains and the pains in trigeminal neuralgia. The pains are paroxysmal, brief, and very intense. Tabes dorsalis presents symptoms and signs of demyelination of the posterior columns, dorsal root, and dorsal root columns of the spinal cord.

In 1974, carbamazepine was approved for use in the United States as an anticonvulsant. It is useful in patients with generalized tonic-clonic and both simple and complex partial seizures. Its efficacy is comparable to that of phenytoin and phenobarbital for the treatment of grand mal epilepsy and especially for psychomotor attacks (Meinardi, 1972; Cereghino, et al., 1974; Livingston, et al., 1974). Carbamazepine is now a first-line drug in the treatment of most forms of epilepsy.

In recent years, the therapeutic indications for carbamazepine have broadened. Carbamazepine has been suggested for use in cases of bipolar depression (Post, et al., 1984), excited psychosis (Klein, et al., 1984), and alcohol withdrawal syndrome (Ritola and Malinen, 1981).

1.1.2.3 MECHANISMS OF ACTION:

The elucidation of the mechanism of action of carbamazepine is not an easy task. Carbamazepine has several clinical effects, but the time course of onset of action and the dose required for these effects varies greatly.

This suggests that there may be different mechanisms of action for the different clinical effects. In fact, carbamazepine exerts a plethora of biochemical effects on a variety of neurotransmitter, neuromodulator, second messenger, and neuropeptide systems (Post, 1988). The differential time course of carbamazepine's anticonvulsant, antinociceptive, antimanic, and antidepressant effects may provide insight into the mechanisms of action relevant to each syndrome.

The anticonvulsant and antinociceptive effects of carbamazepine are readily apparent within hours to days, whereas improvement in sleep occurs in the first week, and maximal antimanic and antidepressant effects tend to occur within two and three weeks, respectively (Post, 1988). The availability of suitable animal models for seizure and pain disorders has allowed partial elucidation of the anticonvulsant effects of carbamazepine. The anticonvulsant effects appear to be closely tied to mechanisms involving "peripheral-type" benzodiazepine receptors (Weiss, et al., 1986), inhibition of sodium currents (Willow, et al., 1984; MacDonald, et al., 1985), noradrenergic potentiation (Post, et al., 1985), and, possibly, decreased glutamate responsivity (Olpe, et al., 1985; Volger and Zeiglgansberger, 1985). As well, chronic treatment with carbamazepine has been associated with a significant reduction in cerebrospinal fluid somatostatin in affectively ill patients (Rubinow, et al., 1985) and in epileptic patients (Steardo, et al., 1986).

It has been suggested that the antinociceptive effects of carbamezepine are exerted via a mechanism that involves $GABA_B$ receptors (Terrence, et al., 1983). Foong and Satoh (1985) also implicated noradrenergic and dopaminergic mechanisms.

The relative lack of suitable animal models for mania and depression makes the task of linking the psychotropic effects of carbamazepine to specific biochemical mechanisms difficult. Carbamazepine affects almost every neurotransmitter-modulator system hypothesized to be involved in mania and depression (Post, 1988), though a significant dose range is required, ranging from therapeutic to high to toxic. The time course analysis performed by Post (1988) suggests that adenosine and substance P should be added to the large range of candidates for its putative antimanic and antidepressant effects.

1.1.2.4 BIOTRANSFORMATION:

The term "biotransformation" is applied to the chemical changes which substances undergo in biological systems. These changes are almost invariably catalysed by enzymes, so the form they take and the rate at which they occur are dependent on the physico-chemical properties of the substance concerned and the enzymatic complement of the biological system (Faigle, et al., 1976).

As stated previously, carbamazepine is classified as a neutral lipophilic substance based on the characteristics of its solubility and partition in organic and aqueous media. Since the lipophilicity of carbamazepine is a property conducive to diffusion of the active substance through the body's various lipid membranes and barriers, it also facilitates the transport of the drug to its sites of action. The body possesses no mechanism by which exogenous lipophilic substances, particularly those of a neutral character, can be readily excreted in unchanged form (Weiner, 1967). In fact, at most 2 percent of a dose of carbamazepine can be recovered unchanged in human

urine or bile (Levy, et al., 1975; Pitlick, 1975). As a result, such substances must first be transformed within the organism to more highly hydrophilic metabolites which can be more readily excreted through the kidney. Thus, biotransformation is important for both the intensity and the duration of pharmacologic effects, since the elimination of carbamazepine from the organism is controlled by the primary metabolic reactions rather than by renal or biliary excretion of the unchanged drug (Faigle and Feldmann, 1982).

Carbamazepine was introduced in the early 1960's but the first identification of a metabolite was in 1972, when the 10,11-epoxide was identified (Frigerio, et al., 1972). Since then, more than thirty metabolites have been identified (Lertratanangkoon and Horning, 1982). While the overall rate of biotransformation in man is drastically different from that in animal species, as reflected by the elimination half-lives of carbamazepine determined in plasma, it would appear that they metabolize carbamazepine by the same basic mechanisms, making it apparently permissible to extrapolate certain biochemical findings from animal models to man (Faigle and Feldmann, 1982).

Radiotracer studies (Faigle and Feldmann, 1975; Faigle, et al., 1976; Richter, et al., 1978) of carbamazepine administration reveal metabolite structures that suggest that the biotransformation of carbamazepine in man proceeds by four major pathways (Figure 6). Taking the total urinary radioactivity as 100 percent, the following approximate percentages are attributable to the different pathways or the corresponding metabolites: epoxidation of the 10,11 double bond of the azepine ring, 40 percent; hydroxylation of the six-membered aromatic rings, 25 percent; direct N-glucuronidation at the carbamoyl side chain, 15 percent; and substitution

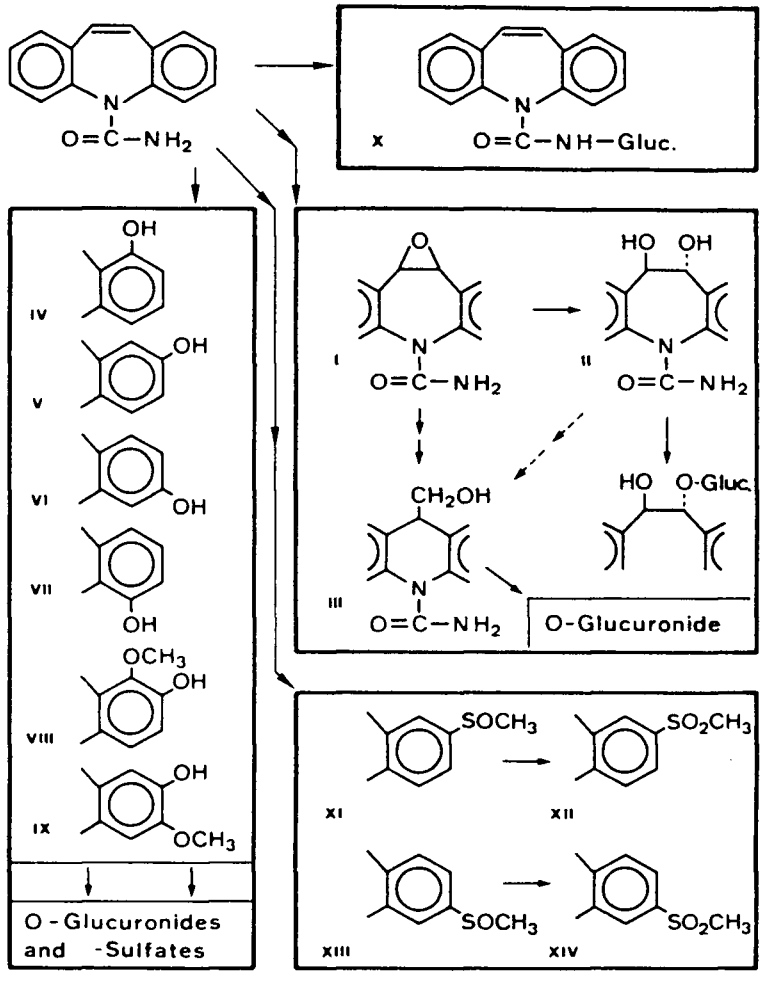


Figure 6: Structures of carbamazepine metabolites isolated from human urine and major pathways of biotransformation. (from Faigle and Feldmann, 1982)

of the six-membered rings with sulfur-containing groups, 5 percent. The remaining radioactivity excreted by the kidneys can be attributed to intact drug and to products of combined metabolic attack. For example, there may be metabolites that have been both hydroxylated and N-glucuronidated.

The reaction producing carbamazepine-10,11-epoxide (the first intermediate of pathway 1) is catalyzed by hepatic monooxygenase (Frigerio, et al., 1976). Most of the epoxide is enzymatically converted to trans-10,11-dihydro-10,11-dihydroxycarbamazepine in the liver by epoxide hydrase (Oesch, 1973). The epoxide accounts for only one percent of excreted radioactivity, while the diol accounts for approximately 35 percent. In urine, the diol is partly present as such and partly as its mono-O-glucuronide. A smaller portion of the epoxide intermediate is converted to a ring-contracted compound, 9-hydroxymethyl-10-carbamoylacridan by a mechanism that has yet to be elucidated. In addition, it is not yet known whether this reaction proceeds directly or via the diol. The acridan is almost completely conjugated with glucuronic acid at the hydroxymethyl group before excretion (Faigle, et al., 1976).

The second pathway, also thought to be catalyzed by monooxygenases, starts with the hydroxylation of carbamazepine at various positions of the six-membered rings. Single substitution results in all four possible phenols, i.e., 1-, 2-, 3-, and 4-hydroxycarbamazepine. Two other intermediates of this pathway carry a hydroxy group in postion 2 and, additionally, a methoxy group in position 1 or in position 3 (Richter, et al., 1978). The bulk of these metabolites are excreted by the kidney as O-glucuronate and O-sulfate conjugates in a ratio of about 2:1, while only trace amounts of the phenols are excreted unconjugated. Additional

metabolites have been found by other investigators, including three hydroxymethoxy compounds and three dihydroxy compounds (Lynn, et al., 1977, 1978), all of which were glucuronidated.

The third important route of biotransformation is direct conjugation of carbamazepine with glucuronic acid. In the conjugate, the ligand is bound to the amino group of the carbamoyl side chain. It has been assumed that the conjugation is metabolized by a hepatic glucuronyl transferase (Faigle and Feldmann, 1982). The enzyme β-glucuronidase is able to cleave the glucuronide from most conjugated species, but it is unable to do so in this case.

The fourth major pathway involves the introduction of a sulfur-containing substituent into one of the six-membered rings of the carbamazepine molecule. Four products resulting form this pathway were found in human urine: 2- and 3-methylsulfinylcarbamazepine and 2- and 3-methylsulfonylcarbamazepine (Faigle and Feldmann, 1982). The mechanism by which these conjugates are generated is unknown.

Additional products formed by pathways 1, 2, and 4 have been described by Lertratanangkoon and Horning (1982). Such products will reduce the unidentified fraction in urine, but they are relatively minor components.

A study has been done to examine the steric course of the enzymatic hydrolysis of carbamazepine-10,11-epoxide, a primary metabolite of carbamazepine (Bellucci, et al., 1987). During the study, the intention was to subject the epoxide to the action of microsomal epoxide hydrolase from animal liver *in vitro*. However, attempts with microsomes from rabbit, rat and guinea pig and with cytosolic fractions from rat and guinea pig showed that their hydrolytic activity on the epoxide was very low, with only traces of the diol being formed after protracted incubations. As a result, the

investigators were forced to isolate the diol from the urine of patients under carbamazepine treatment. Both the free diol, and that obtained after treatment with β-glucuronidase/arylsulfatase, were found to be formed in an enantiomeric excess of 80 percent, the prevalant enantiomer having the (-)-10S,11S absolute configuration. This finding is an indication of pronounced enantioselectivity of the microsomal epoxide hydrolase toward meso and racemic substrates, but is in contrast with the prevalent formation of (R,R)-diols in most other known cases of enzymatic hydrolysis of epoxides (Bellucci, et al., 1987).

1.1.2.5 ADVERSE EFFECTS:

It has been estimated that 33 to 50 percent of adults and children being treated with carbamazepine experience side effects and/or toxicities. Side effects seem to be more common with polytherapy than with monotherapy (Masland, 1982). In general, the adverse effects of carbamazepine can be divided into two classes - clinical side effects and laboratory abnormalities.

Most of the clinical side effects are mild, transient, and reversible if the dosage is reduced or if initiation of treatment is gradual. The most common side effects include nausea, drowsiness, vertigo, ataxia, blurred vision, diplopia, and slurred speech. All but the first are neurotoxic in origin. These side effects are serious enough to warrant discontinuation of therapy in only five percent of cases (Pellock, 1987). The carbamazepine-associated side effect that is most frequently reported to Geigy Pharmaceuticals and the FDA in the United States are skin and allergic reactions such as Stevens-Johnson syndrome, Lyell's syndrome, exfoliative dermatitis, and

erythema multiforme. Certain movement disorders and seizure increases have been attributed to carbamazepine therapy. The movement disorders, which include chorea, dystonia, asterixis, and myoclonus, are very rare and have usually been observed in conjunction with toxic plasma levels of carbamazepine, most commonly in patients receiving polytherapy for hard-to-control seizures and having significant neurologic dysfunction (Masland, 1982). There have been a number of reports of carbamazepine associated seizure increase (Shields and Saslow, 1983; Johnson, et al., 1984; Sachedo and Chokroverty, 1985; Snead and Hosey, 1985; Hurst, 1985; Horn, et al., 1986). Most of these involved patients with generalized nonconvulsive seizures with slow spike and wave generalized electroencephalographic abnormalities. The seizures that arise are generally atonic, myoclonic, and absence-type seizures, but these can progress to generalized tonic-clonic seizures.

A variety of laboratory side effects have been observed with carbamazepine therapy, the most important of which are hematologic and hepatic abnormalities. Although hematologic reactions to carbamazepine are rare, they are very important, since carbamazepine can produce serious and potentially fatal cases of protracted bone marrow depression. Agranulocytosis and aplastic anemia are perhaps the most serious hematologic side effcts, but they are not the most common. Those most frequently reported to Geigy Pharmaceuticals, in order of frequency, include thrombocytopenia, aplastic anemia, agranulocytosis, pancytopenia, and bone marrow depression. As well, leukopenia is a condition estimated to occur in approximately 10 percent of children and adults treated with carbamazepine (Hart and Easton, 1982). The most common hepatic and pancreatic abnormalities reported to Geigy Pharmaceuticals include hepatitis, abnormal liver function tests, jaundice/cholestatic icterus, liver dysfunction, hepatomegaly/hepatosplenomegaly, and

pancreatitis. The most common observations are transient elevation of liver enzymes, reported to occur in 5 to 10 percent of patients receiving carbamazepine (Pellock, 1987).

In view of the known cytotoxic, teratogenic, mutagenic, and carcinogenic properties of some aromatic polycyclic hydrocarbons (Oesch, 1976), carbamazepine and some of its metabolites have been examined with respect to these factors. Carbamazepine-10,11-epoxide was found to be neither cytotoxic to human cells *in vitro* and to mice bearing leukemia L1210 (i.e., there was no effect on the survival rate of the mice) (Frigerio and Morselli, 1975) nor mutagenic to bacterial tester strains (Glatt, et al., 1975). In teratogenicity studies in mice, some studies reported positive results (Eluma, et al., 1981) while other studies yielded negative results (Fritz, et al., 1976; Wray, et al., 1982). In humans, no increase in congenital malformations was observed in offspring of mothers treated with carbamazepine during pregnancy (Livingston, et al., 1974; Nakane, et al., 1980). Recently, the mutagenic effects of carbamazepine have been studied by examining its ability to induce sister-chromatid exchanges (SCE) and structural aberations in the chromosomes (Schaumann, et al., 1985). In such studies, there was no correlation observed between chromosome breaks and SCE in either *in vivo* or *in vitro* studies. The studies produced negative *in vivo* results, indicating an absence of detectable chromosome damaging effects of carbamazepine in monotherapy in epileptic human subjects who had been administered carbamazepine at therapeutic levels for a minimum of 18 months. The serum carbamazepine concentrations of the subjects in the study ranged from 4.3 to 9.0 μg/ml.

1.1.3 PHARMACOKINETICS OF CARBAMAZEPINE:

The pharmacokinetics of carbamazepine in humans has been studied extensively, and very thorough reviews of the information are available (Morselli and Frigerio, 1975; Bertilsson and Tomson, 1986). In addition, the pharmacokinetics of carbamazepine have been examined in the rhesus monkey (Wedlund and Levy, 1983; Levy, et al., 1984), in the rat (Chang and Levy, 1986) and in the rabbit (Siegers, et al., 1982; Sumi, et al., 1987).

In healthy human subjects, the apparent plasma half-life, following single doses, has been reported to be 35-37 hours (Palmer, et al., 1973), 40-41 hours (Faigle and Feldmann, 1975), 31-55 hours (Morselli, et al., 1975), a mean of 35 hours with a range of 20-65 hours (Strandjord and Johannessen, 1975), and 26.2 ± 6.1 hours (Eichelbaum, et al., 1985). The immense variation of this parameter between individuals is obvious. The volume of distribution has been reported as 1.3 L/kg (Palmer, et al., 1973) and as 0.82-1.04 L/kg (Morselli, et al., 1975). This value can be explained in part by the level of plasma protein binding of carbamazepine being 70 to 80 percent (Bertilsson, 1978) and the hydrophobicity of the drug which could lead to its partitioning into fatty tissues.

There have been two studies involving the pharmacokinetics of carbamazepine in rabbits. In one study carbamazepine was administered intravenously (Sumi, et al., 1987) and in the other it was administered orally (Siegers, et al., 1982). The intravenous administration produced a half-life of 0.648 ± 0.143 hours, a clearance rate of 1.219 ± 0.470 L/hr/kg, and a volume of distribution of 1.086 ± 0.287 L/kg. When carbamazepine was administered orally, the half-life was 2.35 ± 1 hour. Since these studies either used

the wrong route of administration or did not determine all of the desired parameters for the experiments planned for our laboratory, the current study was initiated.

1.1.4 INDUCTION OF CARBAMAZEPINE METABOLISM:

It is well established that carbamazepine is subject to autoinduction and to heteroinduction. During long term therapy, carbamazepine induces its own metabolism (Bertilsson, et al., 1980; Eichelbaum, et al., 1975). Concomitant treatment with phenobarbitone or phenytoin further induces the metabolism (Christiansen and Dam, 1973; Eichelbaum, et al., 1979, 1985). The epoxide-diol pathway is the metabolic route that is induced during both auto- and heteroinduction (Eichelbaum, et al., 1985). There are indications that it is not only the epoxidation but also the formation of the trans-diol metabolite that is induced (Bourgeois and Wad, 1984; Eichelbaum, et al., 1985; Tybring, et al., 1981; Wedlund, et al., 1982).

The time course of autoinduction of carbamazepine kinetics has been studied in three children with a recently developed psychomotor epilepsy (Bertilsson, et al., 1980). Tetradeuterium-labelled carbamazepine ($CBZ\text{-}D_4$) was given as a single dose before maintenance therapy, and on three occasions, part of the regular carbamazepine dose was replaced by $CBZ\text{-}D_4$. On day 6 (second dose of carbamazepine given during maintenance therapy), the clearance of $CBZ\text{-}D_4$ was greater than it was for the initial $CBZ\text{-}D_4$ dose. The clearance of $CBZ\text{-}D_4$ was doubled from 21 to 36 days and was not further increased after five months, when the last $CBZ\text{-}D_4$ dose was given. Thus, in children at least, the autoinduction of carbamazepine metabolism seems to be complete during the first 3-5 weeks of treatment.

Autoinduction of carbamazepine metabolism has also been studied in human adults (Eichelbaum, et al., 1985). The first group in the study consisted of healthy volunteers that received a single oral dose of 200 mg of carbamazepine. The second group consisted of epileptic patients who had received carbamazepine monotherapy for at least six months. Compared with healthy subjects, the plasma clearance was 3-fold higher in patients on carbamazepine monotherapy. The increased plasma clearance was mainly attributed to the induction of the epoxide-diol pathway. Thus, in human adults, a significant degree of induction occurs within six months. Unfortunately, the influence prior to the six month time point is not as clearly defined.

The induction of microsomal enzymes in rat liver by carbamazepine has also been examined (Wagner and Schmid, 1987). The animals were treated for four days with daily oral equimolar doses of 315 μmol/kg. The rats were killed by decapitation 24 hours after the last dose of carbamazepine. The livers were excised, and it was found that carbamazepine significantly increased the liver weight and the concentration of cytochrome P_{450}, but not the concentration of microsomal protein. This indicates that, in rats, an inductive effect is occurring within four days.

1.1.5 STATEMENT OF PROBLEM:

As stated at the beginning of this introduction, the goal of this study was to determine the pharmacokinetic parameters associated with carbamazepine metabolism in rabbits. The information presented in the introduction indicates that knowledge of the parameters for this species is sparse, despite the fact that the metabolism has been studied extensively in man and in other species. Thus, the basis of this study is justified.

1.2 EXPERIMENTAL:

1.2.1 MATERIALS:

Carbamazepine, heparin, and ethanol were obtained from Sigma Chemical Co. Acetonitrile and methanol were obtained from BDH Chemicals. Dextrose (5 percent) was obtained from Abbott Laboratories, and Hoffman-LaRoche was the supplier of nitrazepam. NaH_2PO_4 was obtained from Fisher Scientific Co.

Male New Zealand white rabbits (2-3 kg) were obtained from the Animal Care Unit of the University of British Columbia.

1.2.2 ADMINISTRATION AND SAMPLING:

Carbamazepine, suspended in 1mL of Tween 20 (0.5 mg/mL) and 5 percent dextrose as required, was administered orally to male New Zealand white rabbits (2-3 kg). This size of rabbit was chosen because animals from the local supplier weighing under 2 kg are susceptible to a frequently lethal subclinical respiratory infection when transported and handled. Administration of the suspension was by syringe to the back of the throat of the rabbit. The rabbits were administered doses of 12.5 mg/kg or 25 mg/kg, and they were either fasted or fed ad lib. prior to administration of the drug. The doses were chosen based on the fact that rats are often administered a dose in the range of 80 mg/kg and that rats have a much more rapid rate of metabolism. In the one available reference in which a comparable study was done (Siegers, et al., 1982), the carbamazepine dose was 40 mg/kg. Incidently, the existence of the study by this group was not discovered until the current study was essentially complete. In studies in which

carbamazepine was administered intravenously, the dose was generally about 10 mg/kg (Rimerman, et al., 1979; Sumi, et al., 1987). As further justification for the doses used, in one experiment of the current study a dose of 250 mg/kg was accidently administered to a rabbit, but the maximum plasma carbamazepine obtained was very similar to the concentration obtained as a result of a 25 mg/kg dose, as was the rest of the time profile of carbamazepine concentrations. Each rabbit underwent two administrations, with at least seven days separation between the administrations. This period allowed complete elimination of the drug so that there was no carryover effect, allowing each animal to be used as its own control.

Ten blood samples were taken over a period of up to six hours, to monitor the levels of carbamazepine and of its primary metabolite, carbamazepine-10,11-epoxide, over time. Blood samples (approximately 0.7-0.8 mL) were taken from the ear vein through a catheter (Jelco 22g) that remained in place for the duration of the experiment. Blood volume was kept relatively constant by flushing the catheter with a volume of heparinized saline that was approximately equivalent to the volume of blood removed. The blood samples were centrifuged in microcentrifuge tubes for one minute using an Eppendorf Centrifuge 3200. The plasma was collected and was stored at -20°C until extracted.

1.2.3 EXTRACTION:

Carbamazepine and carbamazepine-10,11-epoxide were extracted from the plasma samples using a solid phase extraction procedure described by Hartley, et al. (1986). This type of extraction was chosen over a liquid-liquid extraction because the latter have a tendency to form emulsions that decrease

the efficiency of the extractions. On the other hand, the column method was a relatively simple procedure that was quick and reasonably efficient. Hartley and co-workers reported recoveries in the range of 90 to 93 percent, and it was these values and the ease of the technique that led to the decision to use this method. The recoveries obtained during the current study were similar to those reported by Hartley's group. Reversed-phase octadecylsilane bonded silica columns, with a 2.8 mL capacity, were used. Both Bond-Elut (manufactured by Analytichem International, Harbor City, CA) and Clean-Up (manufactured by Worldwide Monitoring, Horsham, PA) C_{18} columns were utilized, with no significant difference in efficiency between column types. The vacuum apparatus used was a Baker-10 SPE system (J.T. Baker Chemical Co., Phillipsburg, NJ). The columns were conditioned immediately prior to use by drawing through, under vacuum, two column volumes each of acetonitrile and water. With the vacuum released, 250 µL of the plasma sample and 25µL of internal standard (nitrazepam, 100 µg/mL, in ethanol) were applied and allowed to equilibrate for one minute. The vacuum was applied to transport all of the plasma into the column. Upon release, there was a two minute equilibration period before washing with one volume of water and one volume of water/acetonitrile (80:20). The compounds of interest (Carbamazepine and its epoxide metabolite) were eluted with 750 µL of ethanol, and this extract was evaporated to dryness under a nitrogen stream at 55˚C. The residue was reconstituted in 250 µL of mobile phase and stored at -20˚C until analysed. Nitrazepam was chosen as the standard in this procedure since it is similar to carbamazepine and the epoxide with respect to hydrophobicity and will, as a result, elute from the extraction column under the same conditions and provide a similar recovery. In

addition, the compounds of interest have similar extinction coefficients at the wave length used for detection following their separation by liquid chromatography.

1.2.4 ANALYSIS OF SAMPLES:

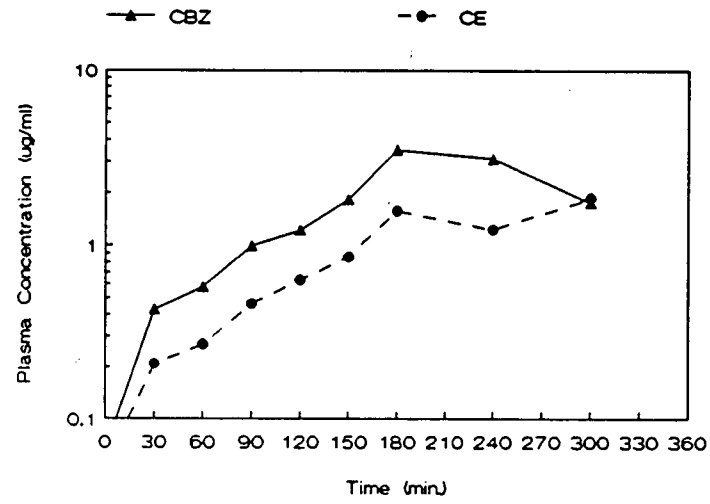
The samples were analysed by reversed-phase high performance liquid chromatography (HPLC) using a Spectra-Physics SP8000B liquid chromatograph. A 125 x 4.6 mm bore column was packed with Spherisorb 5 μm ODS2. The oven temperature was set at 48°C. The mobile phase was 42 percent methanol with a 0.01M NaH_2PO_4 buffer. The flow rate was 1mL/minute. Detection was by a Spectra-Physics SP8400 uv/vis detector set at 210 nm, allowing the detection of both carbamazepine and carbamazepine-10,11-epoxide, its primary metabolite (Rambeck, et al., 1981). The results were obtained as peak areas as determined by the data system of the chromatograph.

1.3 RESULTS:

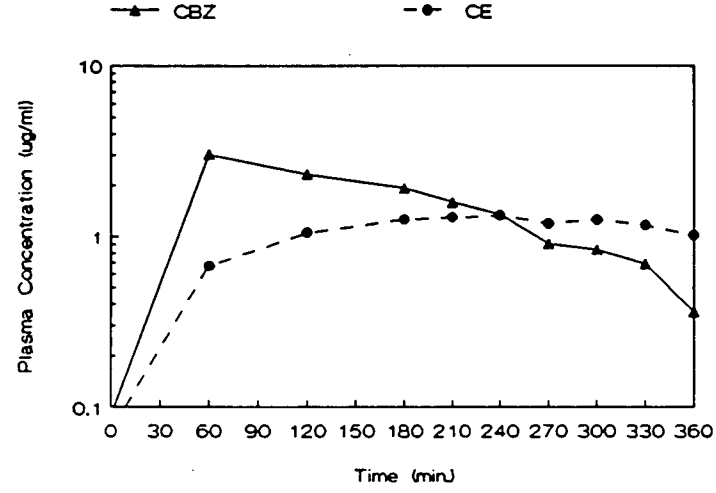
The results of the study of carbamazepine pharmacokinetics in rabbits are shown in Figures 7, 8, and 9, with a sample chromatogram being displayed in Figure 10. Figure 7 shows the results in animals administered a dose of 25 mg/kg and allowed free access to food. For those trials shown in Figure 8, the animals were also given a dose of 25 mg/kg, but they were fasted. The animals used to get the data in Figure 9 were also fasted, but they were administered a dose of only 12.5 mg/kg.

Initially, the period over which samples were taken was five hours. When this did not seem adequate for rabbit D1 (25 mg/kg, fed ad lib.), the time period was extended to six hours for rabbit D2 (25 mg/kg, fed ad lib.),

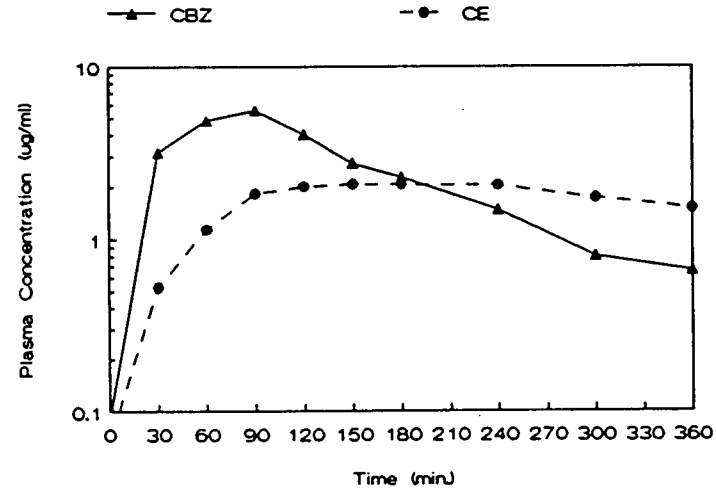
RABBIT D1
25 mg/kg; ad lib.



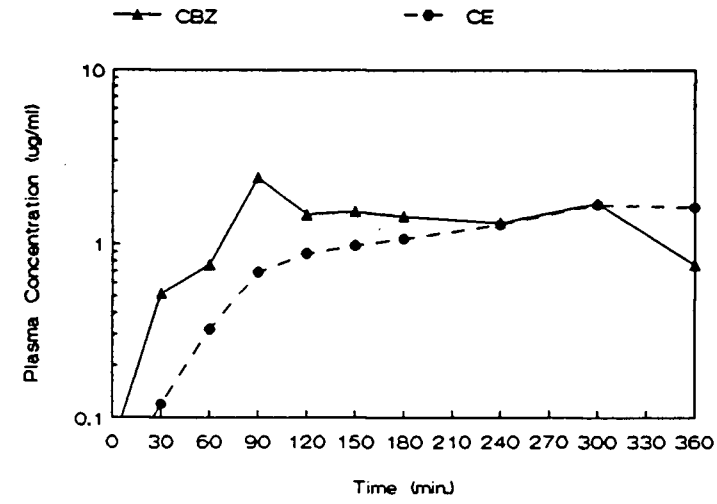
RABBIT D2
25 mg/kg; ad lib.



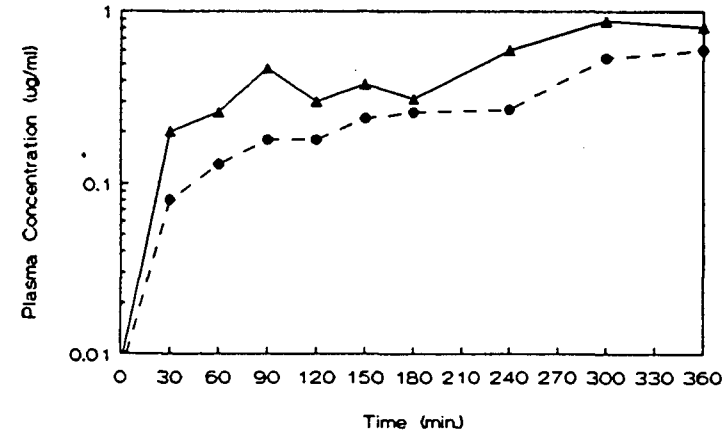
RABBIT D3
25 mg/kg; ad lib.



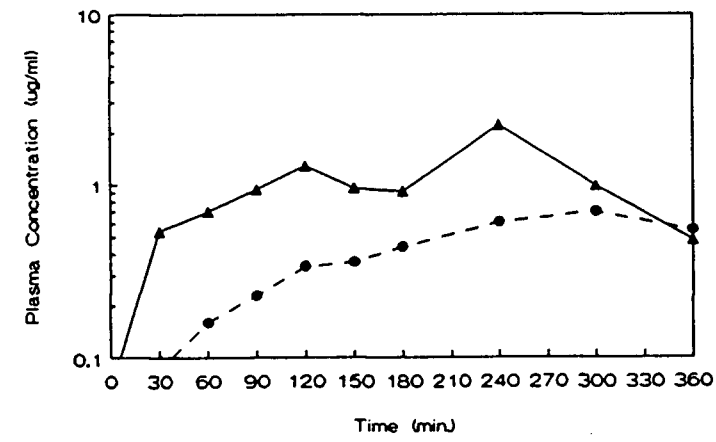
RABBIT D4
25 mg/kg; ad lib.



RABBIT D5
25 mg/kg; ad lib.



RABBIT D6
25 mg/kg; ad lib.



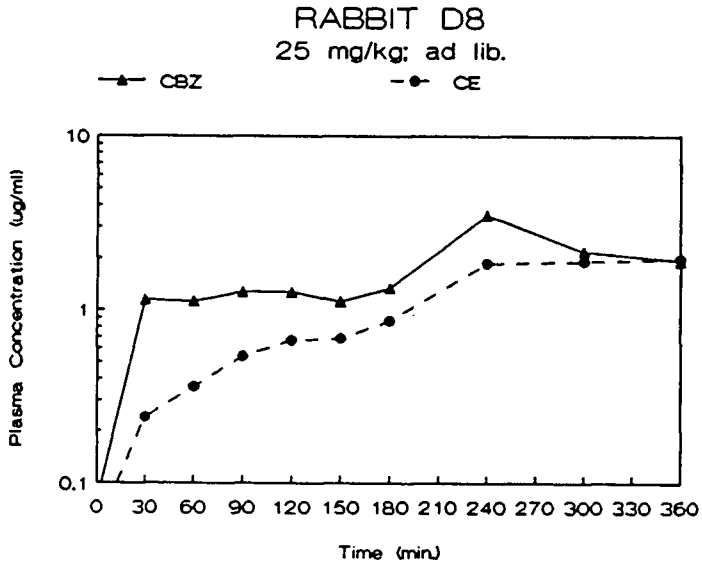
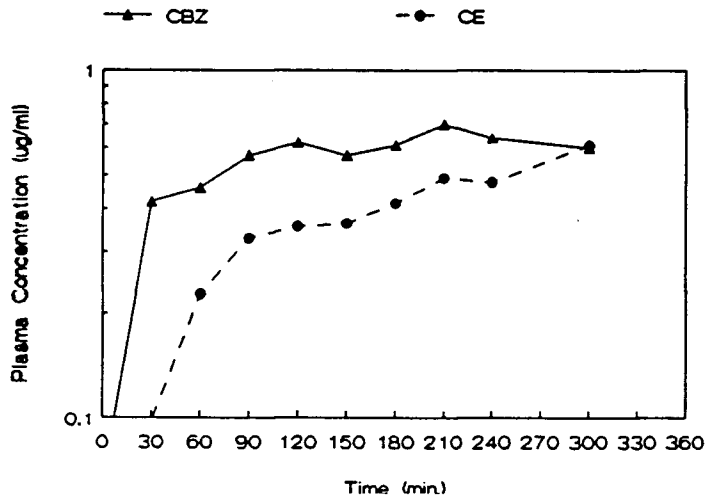
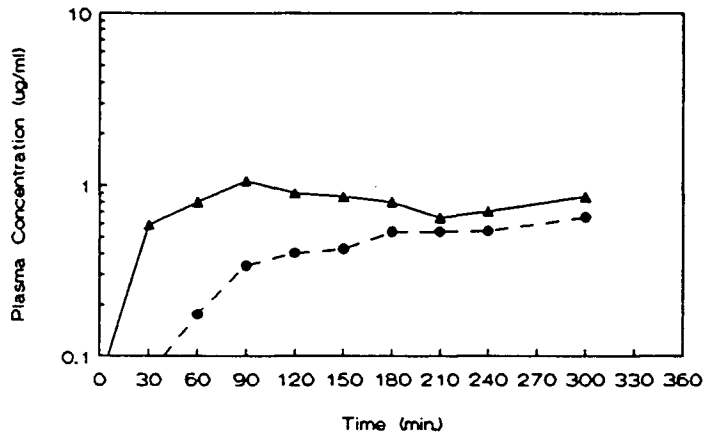


Figure 7: Seurm carbamazepine and carbamazepine-10,11-epoxide in rabbits administered carbamazepine (25 mg/kg) and fed ad lib. The data for each animal is presented in an individual frame.

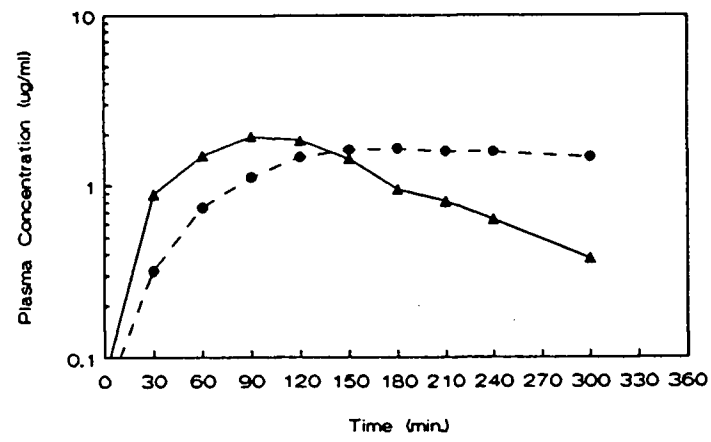
RABBIT D1
25 mg/kg; fasted



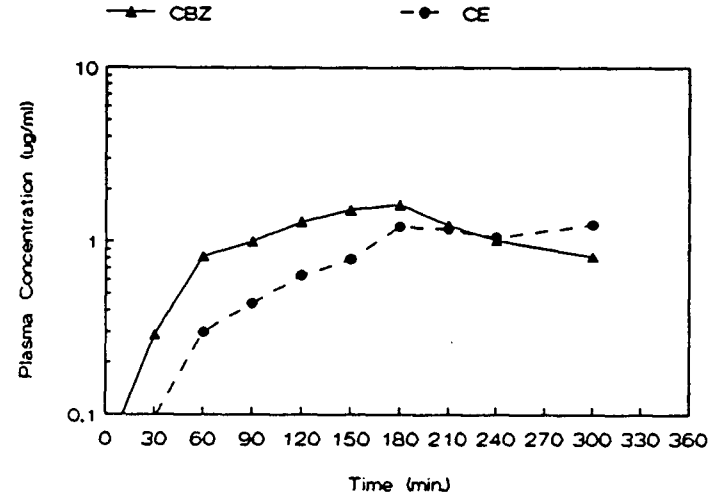
RABBIT D2
25 mg/kg; fasted



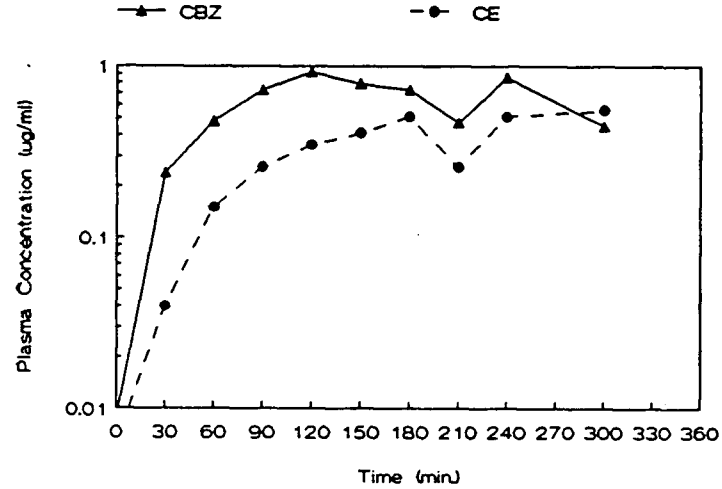
RABBIT D3
25 mg/kg; fasted



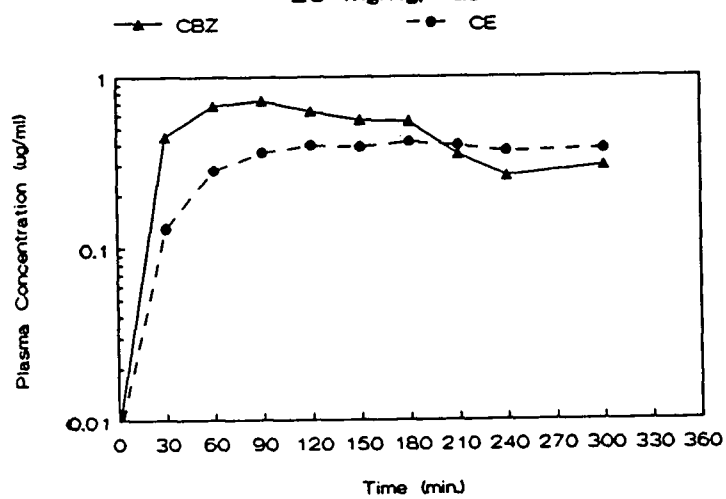
RABBIT D4
25 mg/kg; fasted



RABBIT D5
25 mg/kg; fasted



RABBIT D6
25 mg/kg; fasted



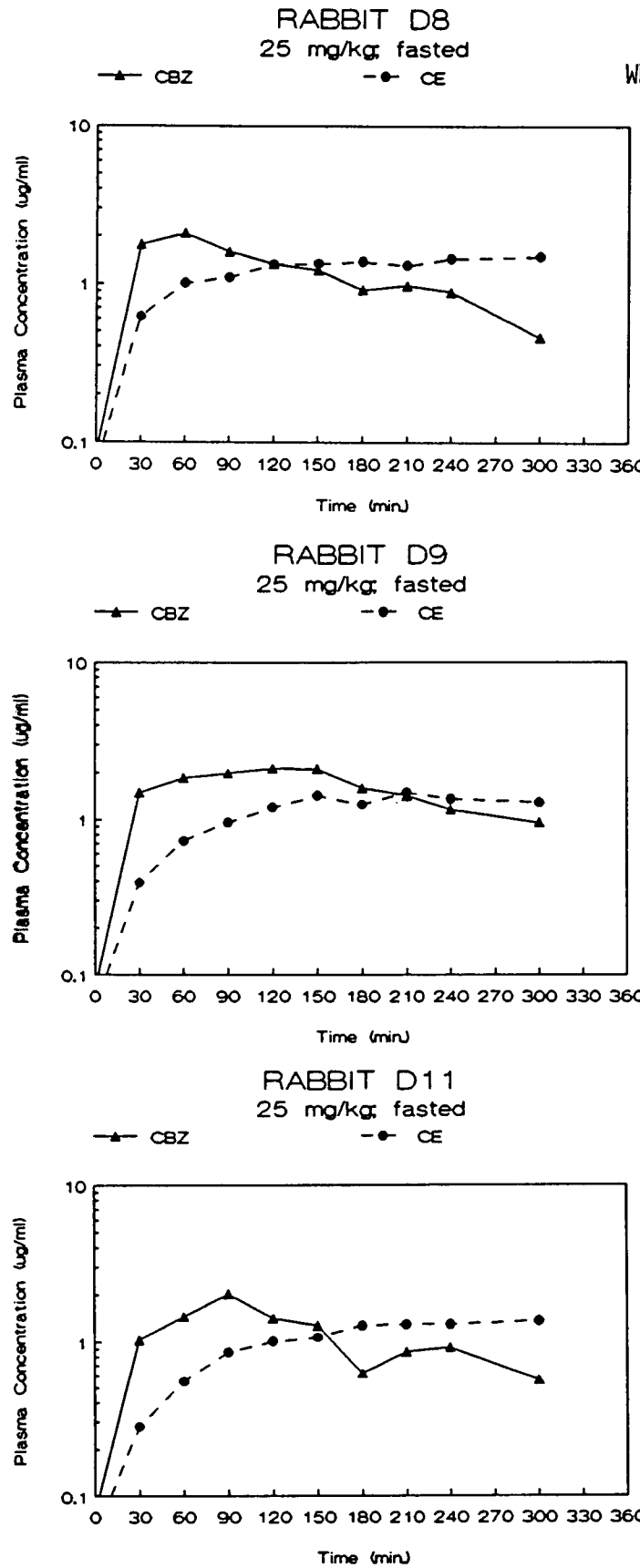
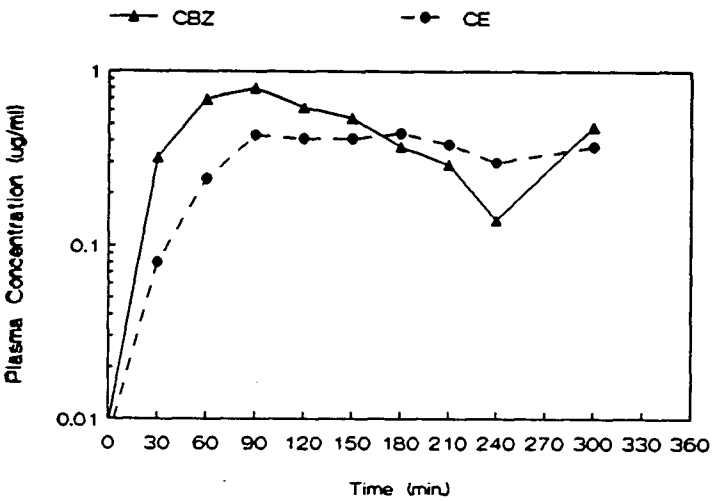
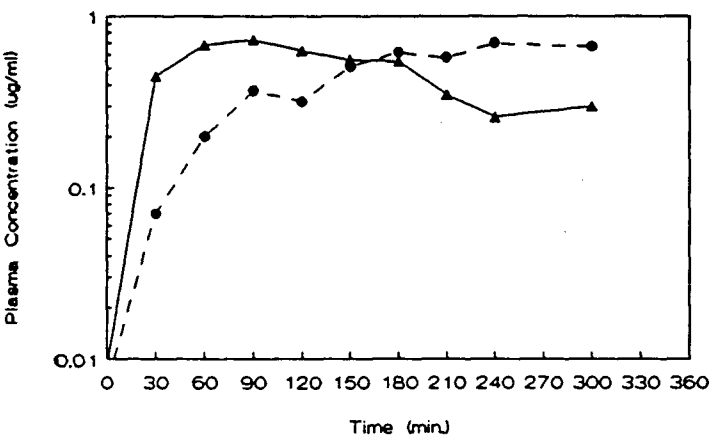


Figure 8: Serum carbamazepine and carbamazepine-10,11-epoxide in rabbits administered carbamazepine (25 mg/kg) and fasted. The data for each animal is presented in an individual frame.

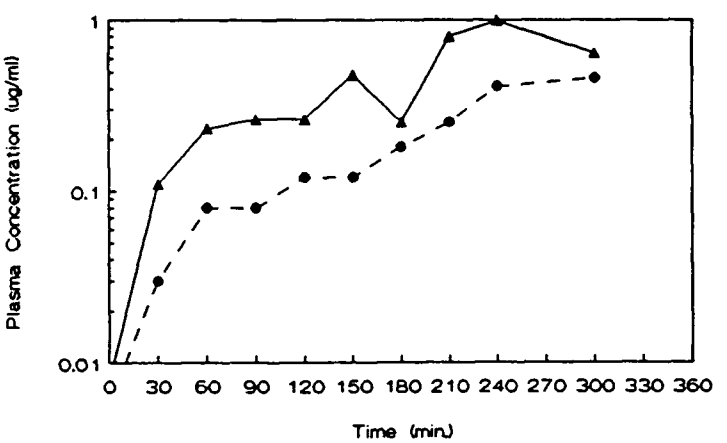
RABBIT D3
12.5 mg/kg; fasted

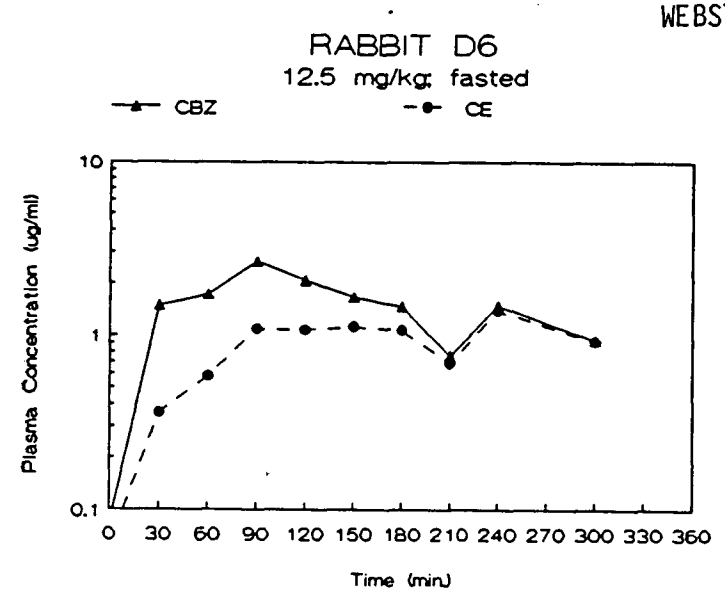


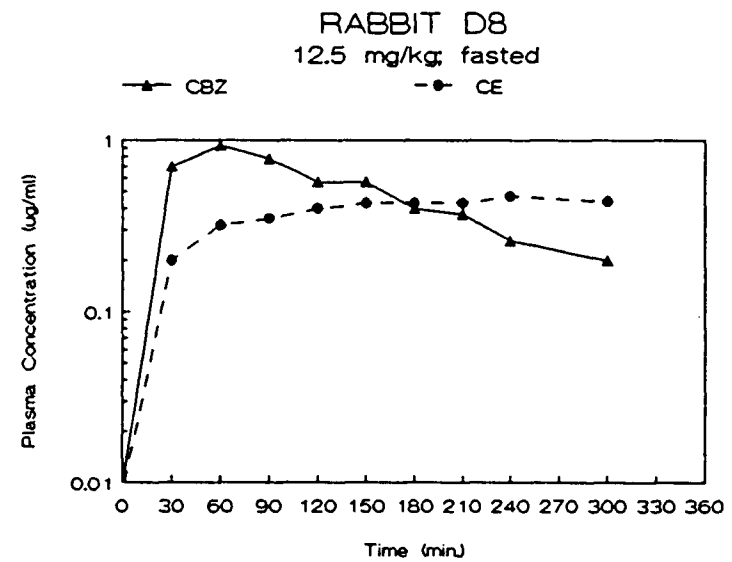
RABBIT D4
12.5 mg/kg; fasted

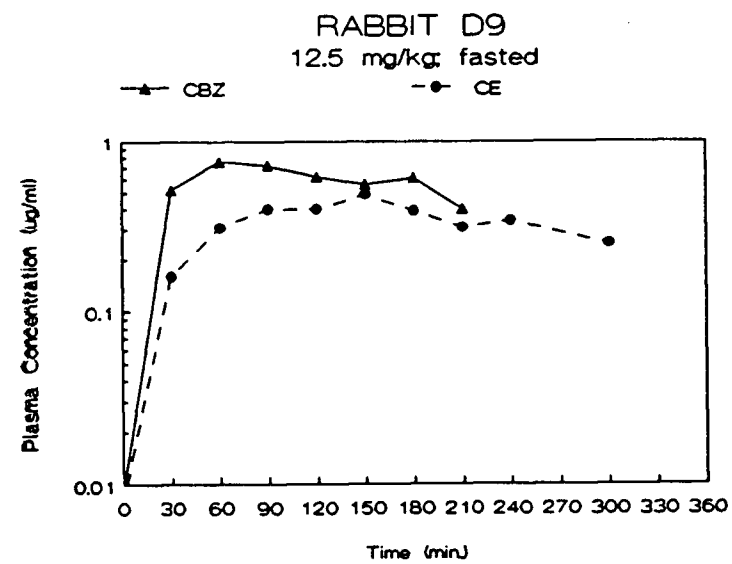


RABBIT D5
12.5 mg/kg; fasted




RABBIT D6
12.5 mg/kg; fasted
CBZ
CE
Plasma Concentration (ug/ml)
10
1
0.1
0 30 60 90 120 150 180 210 240 270 300 330 360
Time (min)


RABBIT D8
12.5 mg/kg; fasted
CBZ
CE
Plasma Concentration (ug/ml)
1
0.1
0.01
0 30 60 90 120 150 180 210 240 270 300 330 360
Time (min)


RABBIT D9
12.5 mg/kg; fasted
CBZ
CE
Plasma Concentration (ug/ml)
1
0.1
0.01
0 30 60 90 120 150 180 210 240 270 300 330 360
Time (min)

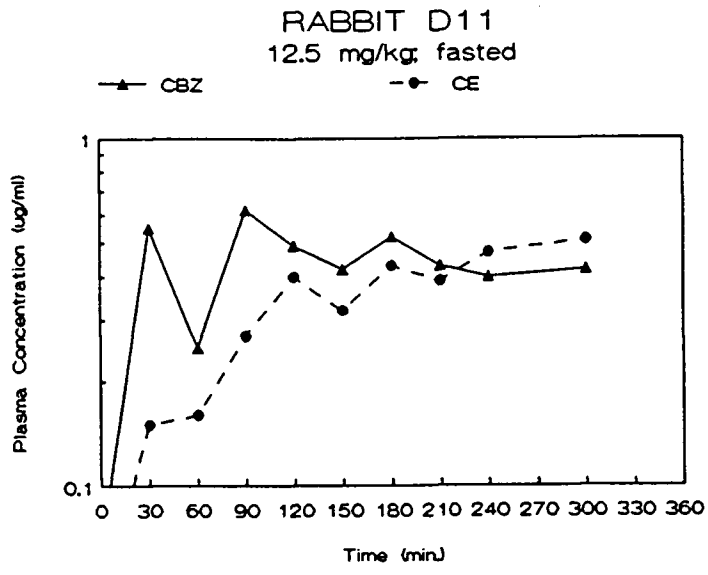


Figure 9: Serum carbamazepine and carbamazepine-10,11-epoxide in rabbits administered carbamazepine (12.5 mg/kg) and fasted. The data for each animal is presented in an individual frame.

Figure 10: Sample chromatogram from carbamazepine pharmacokinetics study in rabbits. The sample shown was taken at 120 min from a rabbit fed ad lib. and administered a dose of 25 mg/kg. The chromatogram shows carbamazepine-10,11-epoxide (293), nitrazepam (579), and carbamazepine (632).

at which time an appropriate profile was obtained. This was also observed for rabbit D3. But, as more animals were tested, it became obvious that a usable concentration-time profile could not be obtained in all cases. Subsequently, the tests were repeated with the rabbits fasted overnight prior being administered carbamazepine, and for at least one hour after drug administration (most animals did not eat during the period of blood sampling). The animals were allowed free access to water for the duration of the experiment. The idea was that the presence of food in the stomach may have delayed gastric emptying, and as a result delayed presentation of the drug to the site of absorption, namely the small intestine. But, an extended plateau was again seen in the pharmacokinetic profile for several animals (Figure 8). Tests at a lower dose (12.5 mg/kg) displayed a similar phenomenon (Figure 9).

As a result of the phenomenon that was observed, it was not possible to accurately determine the pharmacokinetic parameters, outlined in the objectives of this study, for all of the animals in the study groups. In the group that was fed ad lib. and administered a dose of 25 mg/kg (Figure 7), rabbits D2 and D3 produced a usable type of profile. For these tests, the values for t_{max} were 60 and 90 minutes, for the apparent half-life ($t_{1/2}$) were 90 and 98 minutes, for clearance were 91.6 and 46.2 mL/min/kg, and for the elimination constant (k_{el}) were 0.0077 and 0.0071 min^{-1}, respectively. Of the rabbits administered a dose of 25 mg/kg and fasted (Figure 8), rabbits D3 and D8 gave usable profiles. The values for t_{max} were 60 and 90 minutes, for the apparent half-life were 90 and 122 minutes, for clearance were 142.4 and 101.0 mL/min/kg, and for the elimination constant were 0.0077 and 0.0057 min^{-1} for rabbits D3 and D8, respectively. In the group administered a dose of 12.5 mg/kg (Figure 9), only rabbit D8

gave a standard profile. The t_{max} was 60 minutes, as in the previously mentioned test. The half-life was 110 minutes. The clearance rate was 121.8 mL/min/kg, and the elimination constant was 0.0063 min^{-1}. These results are summarized in Table I.

Table I: Pharmacokinetic parameters for those trials for which the parameters could be determined.

Treatment	Rabbit	t_{max} min	$t_{1/2}$ min	clearance mL/min/kg	elimination constant min^{-1}
25 mg/kg Ad lib.	D2	60	90	91.6	0.0077
	D3	90	98	46.2	0.0071
25 mg/kg Fasted	D3	60	90	142.4	0.0077
	D8	90	122	101.0	0.0057
12.5 mg/kg Fasted	D8	60	110	121.8	0.0063

1.4 DISCUSSION:

The pharmacokinetics of carbamazepine in rabbits had been previously been examined by two teams of investigators. The first (Siegers, et al., 1982) administered the drug orally (40 mg/kg), as was done in the current study. But, the only parameters that were determined were the time at which the maximum plasma concentration was achieved (t_{max}), which was 2 hours, and the half-life ($t_{1/2}$), which was 2.35 hours. The values for t_{max} were similar to those obtained from animals exhibiting conventional one compartment pharmacokinetic profiles in the current study. This held true

for each of the dosing/feeding regimens. The half-lives measured in the current tests were all less than the average reported by Siegers and co-workers, but they still fell within the reported range. The profiles used to determine the values of the pharmacokinetic parameters in this study closely resembled those obtained by the Seigers group, which they described as conforming to the single compartment model. In single dose studies in man, those studies in which solutions or suspensions have been administered have displayed results that could be described by the one compartment model (Bertilsson, 1978; Pynnonen, 1979), whereas when commercially available tablets have been administered the disposition of the drug follows the two compartment model (Ronfeld and Benet, 1977). The second team of investigators (Sumi, et al., 1987) administered the carbamazepine intravenously (10 mg/kg) and they determined values for clearance (1.219 L/hr/kg), volume of distribution (1.086 L/kg), and half-life (0.684 hr). These values are all significantly less than those in the current studies. The differences in the values obtained in these various studies may be attributed, in part, to differences in strains of animals used.

When an intravenous dose is administered the entire dose enters the blood stream at once, giving an immediate peak concentration. From this point, the only activity occurring is elimination. On the other hand, when the drug is administered orally, there is a delay before the peak plasma concentration is attained. As well, elimination is occurring at the same time as uptake for a period of time. Normally, when there is rapid uptake of an orally administered drug, the elimination will be the same for both the oral and intravenous routes of administration. It is possible that, in the current situation, the uptake of the drug was slowed, resulting in concurrent uptake and elimination. If this is occurring, the values

obtained for clearance, volume of distribution, and half-life for the intravenous dose would all appear to be less than for an orally administered dose. This is the result that was actually observed, with respect to the values of the parameters.

A recurrent pattern emerged in several animals which indicated that the single compartment model was not satisfactory in all cases. In several cases, the plasma concentration of carbamazepine reached a peak at 60-90 minutes, began to decrease, and either plateaued or increased again. This pattern was observed for animals in each of the dosing/feeding groups that were tested.

The most likely explanation for this observation may be obvious if the experiments were to be repeated employing a longer time course of sampling. The reasoning behind this statement may be obvious if one considers the sequence of steps involved in the uptake of an orally administered drug. Following ingestion of the drug, it must be dissolved in the stomach, in the case of tablets. This is not a problem in the current case since the carbamazepine was administered as a suspension of fine particles. The stomach is lined by a thick, mucus-covered membrane with small surface and high electrical resistance. Its primary function is digestion. On the other hand, the epithelium of the small intestine has an extremely large surface area. It is thin, has low electrical resistance, and its primary function is facilitating the absorption of nutrients. Carbamazepine, being a hydrophobic drug, should be absorbed quite readily in the small intestine since it is highly lipid soluble. Thus, it will be quickly taken by the epithelial cells of the small intestine. The drug must then diffuse through the cell, again pass through the cell membrane, and then make its way across the membrane of the portal vasculature and into the portal circulation.

From this point the drug is transported to the liver, where it undergoes first pass metabolism, and then on to the systemic circulation. Of the processes described, the only one that displays any significant degree of variability is the rate of gastric emptying.

Since the stomach is the organ responsible for the majority of digestion, while the rest of the gastrointestinal tract is responsible primarily for absorption, the presence of food in the stomach will necessarily slow the rate of gastric emptying. Should this process be delayed, the rate at which the drug enters the small intestine and is able to be absorbed will also be slowed. It was thought that fasting the animals overnight prior to the experiment would be adequate to alleviate any worries about differential gastric emptying between animals, but it is possible that rabbits require a long period for the emptying of food contents from the stomach. As a result, the observed results may have been influenced by the feeding of the animals prior to the period of fasting.

It is also possible that carbamazepine itself may have been responsible for slowing gastric emptying in some of the animals. It is well known that fatty foods generally require a greater period to be passed through the stomach than do carbohydrates and proteins. This has been attributed to the caloric content of the food, but the exact mechanism by which the signal to slow gastric emptying is initiated is unknown. It is possible that there are "detectors" that are specific for some chemical feature of the different food types. If this is the case, perhaps a parallel can be drawn to carbamazepine. Since it is very hydrophobic, carbamazepine shares a key chemical property with fat. As a result, carbamazepine itself may be responsible for delayed gastric emptying. Alternatively, the presence of the surfactant Tween in the suspension may have been resposible, due its

structural similarity to fatty acids. If caloric content is really a major determinant of the rate of gastric emptying, it is possible that the dextrose used to make the suspension was sufficient to slow emptying.

One of the metabolic fates of carbamazepine is glucuronidation. Glucuronidated compounds can be excreted in the bile. Based on this fact, the possibility that enterohepatic circulation is occurring must be considered. Due to the lipophilicity of carbamazepine, most of its uptake occurs in the small intestine. If the glucuronide of carbamazepine is excreted in the bile, it is possible that intestinal microflora are able to hydrolyze the glucuronides using glucuronidase enzymes. This being the case, the parent drug can be regenerated in the small intestine and it can again be free for uptake. The result will be an apparently much slower elimination (the observed plateau) or even an increase in the circulating concentration of the drug. This postulation can and should be tested in the future. The amount of carbamazepine in the bile, presumably as the glucuronide conjugate, can be determined by cannulating the bile duct and collecting samples of bile. A technique for this type of procedure has been described for rats (Johnson and Rising, 1978), wherein biliary excretion and enterohepatic circulation can be assessed. This technique could easily be adapted for use in rabbits. The excretion of carbamazepine in the bile of man has been evaluated and only about 1 percent of the administered dose of carbamazepine was excreted in the bile (Terhaag, et al., 1978). Based on this information, enterohepatic circulation might ruled negligible in man. In fact, it has not been described as occurring to any measurable extent in man. Enterohepatic circulation cannot be ruled out in other species, such as the rabbits being used here, particularly since the bile measurements note the level of carbamazepine only and neglect to refer to conjugated

metabolites. In addition, the intestinal flora of the rabbit is very likely different from that in man, and it may be better able to hydrolyze the conjugating connection. This factor would increase the importance of there being a high glucuronysyl transferase activity, since if there were more conjugate excreted in the bile the degree to which enterohepatic circulation is occurring would be more pronounced. If one were to compare the profiles displaying a plateau with those that show the traditional type of profile, it would be reasonable to estimate that 15 to 20 percent of the dose is experiencing enterohepatic circulation in order to produce the observed plateau.

The differences between rabbits in this study are quite clear, in that some display usable single compartment concentration-time profiles, while others obviously do not. While the reasons for this observation are not entirely clear, it is quite possible that a genetic factor may be responsible. For example, those animals which are suspected of experiencing enterohepatic circulation may have a genetic complement that produces a greater rate of glucuronide conjugate formation (i.e., the activity of glucuronysyl transferase is greater). Alternatively, those rabbits that have a more conventional pharmacokinetic profile may have a slower rate of conjugation (or, possibly a faster rate of oxidation). Since the reaction forming the glucuronide conjugates is under enzymatic control, it is quite possible that a genetic influence on activity can exist. Thus, a rabbit producing a greater proportion of glucuronidated metabolites will excrete more metabolite in the bile and will experience a greater degree of enterohepatic circulation. On the other hand, a rabbit with a greater proportion of the metabolites excreted in the urine will not experience recirculation of carbamazepine and will display the conventional single compartment

profile. The presence of a genetic polymorphism, of the nature described above, is not often found within a strain of a given species. By definition, a strain should have a fairly high degree of genetic uniformity which would, in effect, negate the proposal presented in this paragraph. Despite the supposed uniformity of a strain, investigators in our laboratory have been able to obtain rabbits, from the same strain that was used in this study, that have had differential phenotypes with respect to rates of acetylation activity. This indicates that genetic polymorphisms do exist amongst the population of rabbits that were used in this study and that the explanation put forth is possible.

The apparent genetic polymorphism may also arise from the activity of the hepatic monooxygenases that convert carbamazepine to carbamazepine-10, 11-epoxide. Using Figure 7 as an example, differences in the relations of the epoxide curves to the carbamazepine curves can be seen. For rabbits D2, D3, and D4 the concentration of epoxide increases to a level that is greater than the concentration of carbamazepine at the same time and does not decrease in a manner that parallels the decrease of plasma carbamazepine concentration. It is possible that the animals noted above have a faster rate of oxidative metabolism which may be due to genetic differences in the cytochrome(s) P_{450} that is (are) responsible for the metabolism of carbamazepine in individual animals. It would be quite likely, if these polymorphisms do exist for glucuronysyl transferase and for hepatic monooxygenases, that the resultant observations are due to the combined influences of two genetic factors. Alternatively, it is possible that auto-induction is occurring over the time course of the experiment, though the time course seems to be too short for such an occurrence. That is, it is possible that carbamazepine is able to induce its own metabolism in the

rabbits noted. This effect may also have a genetic predisposition, since the apparent induction occurs in some animals and not in others, despite the fact that they were all administered the same dose. However an examination of the initial rates of reaction indicates that the former explanation is more likely to be true, since those animals displaying an apparently greater rate of oxidative metabolism over time also had the fastest initial rates of reaction. In addition, if, as stated in the introduction, the autoinduction of carbamazepine metabolism can take over 3 weeks in human children, the likelihood of it occurring over a single dose in rabbits is slim at best. In fact, Siegers and co-workers (1982) attempted to examine the carbamazepine autoinduction phenomena in rabbits and found no differences in the maximum concentrations at the expected t_{max}. The problem with their study was that only a single daily oral dose (10 mg/kg) was administered and blood samples were only taken prior to and 2 hours after drug administration. As a result, essentially complete clearance of carbamazepine is allowed between doses, and any inductive effects may have time to reverse. These phenomena must be examined further, since any insight can only be gained by increasing the data base significantly.

As this discussion indicates, the work in this field is by no means complete. A great deal of research must be done to answer the questions that remain. One might be tempted to explain the observations for which Figure 7 was used as an example by claiming that, since the animals were fed ad lib., there may have been differential uptake of the drug, based on the food content of the gastrointestinal tract. But, if Figures 8 and 9 are examined, it is obvious that similar patterns are seen for these trials where the animals were fasted overnight prior to and for at least one hour following drug administration (most animals did not eat during the period of

blood sampling after the carbamazepine was administered). Thus, as stated previously, the data base must be expanded using the method employed in the current study, a longer time course, and a longer period of fasting prior to the administration of carbamazepine. If a series of experiments were done under these conditions, the issue of the effect of gastric contents on the results can be conclusively settled. The situation regarding enterohepatic circulation in the rabbit should be examined in detail. In addition, the time course of carbamazepine auto-induction must be elucidated.

2 THE INFLUENCE OF ISONIAZID ON THE IN VITRO METABOLISM OF CARBAMAZEPINE:

2.1 INTRODUCTION:

The second portion of this thesis examines the influence of isoniazid on carbamazepine metabolism in rat liver microsomes, rather than the originally planned extension of the pharmacokinetic study to the examination of *in vivo* metabolic interactions between isoniazid and carbamazepine in the rabbit. The change in plans was a result of an illness that prevented me from working the intensive 12-15 hour days that would have been required.

Instead, an *in vitro* study using rat liver microsomes was done. The series of experiments are closely related to the original route of progression, but they did not require such extremely long working days. The *in vitro* model had been developed in our laboratory by undergraduate summer students Christian Band and Lawrence Selby. This study was able to provide a more complete analysis of the scenario than did their preliminary observations.

It has been suggested that isoniazid is an inhibitor of carbamazepine metabolism, as indicated by clinical reports wherein coadministration of normally therapeutic doses of carbamazepine and isoniazid produced signs of carbamazepine intoxication and/or isoniazid hepatotoxicity (Block, 1982; Wright, et al., 1982; Valsalan and Cooper, 1982; Barbare, et al., 1986). The observed signs of carbamazepine intoxication included headaches, nausea, vomiting, blurred vision, drowsiness, and confusion. The onset of these symptoms of toxicity occurred in conjunction with an increase in serum carbamazepine concentration to levels well above that which is generally considered sufficient for therapeutic effectiveness. Thus, it would appear valid to postulate that isoniazid either inhibits carbamazepine metabolism

or alters the distribution of carbamazepine. Inhibition of metabolism is a much more likely postulation since distribution tends to be a diffusionary process that is difficult to alter.

Carbamazepine is metabolized by a hepatic microsomal system that can be readily inhibited (Pippenger, 1987). Drug clearance is decreased, drug half-lives are prolonged, and steady state serum drug concentrations are elevated as a result of inhibition. Symptoms of drug intoxication appear as soon as the serum concentration rises above the minimum toxic concentration. The changes in metabolic pattern begin as soon as the inhibiting drug is added to the patient's therapeutic regimen. This situation occurred in the reports indicated above where the patients were undergoing chronic carbamazepine therapy for the control of seizure-causing disorders, and the administration of isoniazid, in addition to the carbamazepine, led to the observed symptoms of intoxication. Wright and co-workers (1982) reported a 45 percent decrease in carbamazepine clearance 3-5 days after isoniazid administration.

<u>In vitro</u> studies of the influence of isoniazid in rat liver microsomes (S9 fraction) indicated that the rate of metabolism of carbamazepine, in this system, is dependent on the concentration of isoniazid, with the rate being slowed by greater concentrations of isoniazid (Webster, et al., 1989). This thesis will examine the results of the studies with isoniazid and those of experiments with the primary metabolites of isoniazid, including acetyl isoniazid, acetyl hydrazine, hydrazine, and isonicotinic acid. The goal of testing the metabolites was to determine which were responsible for the inhibition of carbamazepine metabolism and, possibly, to relate this information to the production of hepatotoxicity by isoniazid.

2.1.1 CARBAMAZEPINE:

The development and chemistry, therapeutic uses, mechanisms of action, biotransformation, and toxicities of carbamazepine were discussed in the introduction to the previous section. As such, this information will not be repeated here.

2.1.2 ISONIAZID:

Isoniazid is the primary drug for the chemotherapy of tuberculosis, and all patients with tuberculosis caused by isoniazid-sensitive strains of tubercle bacillus should receive the drug if they can tolerate it (Mandell and Sande, 1985). Isoniazid, the hydrazide of isonicotinic acid was introduced in 1952 (Bernstein, et al., 1952). Despite a great deal of work since then, its mechanism of action has not been conclusively elucidated. Isoniazid has been tested for its therapeutic value in a number of disorders. The extensive use and testing of isoniazid has provided the opportunity for the observation and description of many toxicities and adverse effects, one of the most important of which is isoniazid-induced hepatotoxicity.

2.1.2.1 DEVELOPMENT AND CHEMISTRY:

The synthetic tuberculostats, i.e., those which were devised in the chemical laboratory, lend themselves readily to creative manipulation and limitless potential. For this reason, the first half of the twentieth century saw a great deal of synthetic chemistry performed in an attempt to produce a therapeutically effective agent against tuberculosis (Fox, 1953).

The first compound to exhibit marked *in vivo* activity against the tubercle bacillus was 4,4-diaminophenylsulfone, but it proved to be less effective in man than in laboratory animals. In addition, it exhibited considerable toxicity. Subsequently, many attempts were made to synthesize sulfones with increased activity and solubility and decreased toxicity. Most of the compounds that were synthesized were more soluble and less toxic, but they also lacked the necessary activity.

In the early 1940's, certain benzoates and salicylates were found to decrease the oxygen uptake and inhibit the growth of tubercle bacillus, the organism responsible for tuberculosis. The most common of these was p-aminosalicylic acid, commonly known as PAS (Lehmann, 1944). Many variations in the PAS structure were investigated, but none were found to be superior to the parent compound.

In the late 1940's, the tuberculostatic activity of the thiosemicarbazones was discovered as a result of a systematic investigation of the sulfa drugs in tuberculosis. Behnisch and his co-workers (1950) tested a series of thiosemicarbazones and found them to be strongly tuberculostatic. The best known compound of the series is p-acetamido benzaldehyde thiosemicarbazone (TbI), which was the most active synthetic tuberculostat in use until the advent of the hydrazides (Fox, 1953).

A series of pyridine carboxylic acid derivatives were synthesized and tested in the late 1940's. The tuberculostatic activity of nicotinamide, the parent compound of this group, was discovered by Kushner and his co-workers in 1948. This was followed by the discovery of pyrazinamide, a compound with approximately three times the tuberculostatic activity of nicotinamide or PAS. But, resistant strains rapidly emerged. The desire to study pyridine carboxylic acid derivatives closely related to nicotinamide

led to attempts to prepare isonicotinaldehyde thiosemicarbazone (Fox, 1952). Since isonicotinoyl hydrazide (isoniazid) (Figure 11) is a pyridine carboxylic acid derivative, and thus related to the structures being studied, it was investigated for tuberculostatic activity and was found to be more active than any known substance - whether synthetic or antibiotic. Extensive chemical and chemotherapeutic studies showed that, unlike 3-amino-isonicotinic acid or pyrazinamide, isonicotinoylhydrazide could be modified in many ways without abolishing its activity.

2.1.2.2 THERAPEUTIC USES:

Isoniazid is the primary drug for the chemotherapy of tuberculosis, and it has been since its introduction by Bernstein's group in 1952. In addition, isoniazid has been tested for its therapeutic value in several other disorders. The justification for testing isoniazid in different situations arises from the physiological influences of the drug in the subject to which it is administered. To an extent, this takes advantage of the structural relationship between isoniazid and endogenous compounds.

For example, isoniazid has been tested for its value in the treatment of Huntington disease (Perry, et al., 1979, 1982). In this disease, there is a marked loss of small neurons in the caudate nucleus and putamen, most of which probably belong to a population of cells that utilize γ-aminobutyric acid (GABA) as an inhibitory neurotransmitter. A marked decrease in GABA content in the affected areas has been observed in patients with the disease (Perry, et al., 1973). When large doses of isoniazid are given to animals, brain GABA content increases (Perry and Hansen, 1973; Perry, et al., 1974), a result that was thought to be due to the inhibition of GABA-aminotransferase, the first of two sequential enzymes that degrade GABA in the brain.

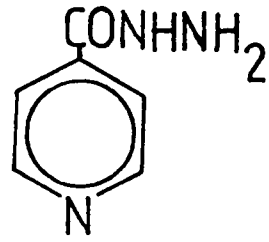


Figure 11: Isoniazid

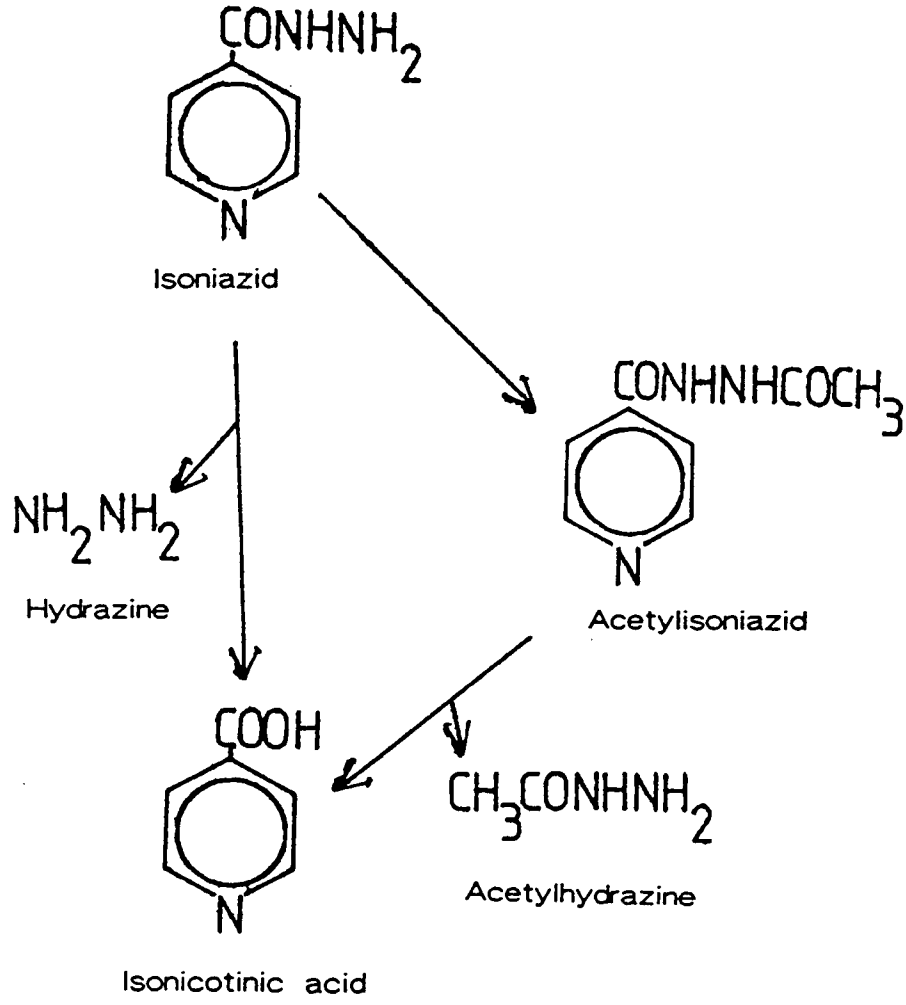


Figure 12: Relevant aspects of isoniazid metabolism.

However, in a double-blind clinical trial (Perry, et al., 1982), while the cerebrospinal fluid GABA concentrations were markedly increased during isoniazid therapy, there was a lack of clinical improvement in most Huntington disease patients.

In recent years, isoniazid has been examined for its usefulness against tremors associated with multiple sclerosis (Sabra, et al., 1982; Duquette, et al., 1985; Hallett, et al., 1985; Francis, et al., 1986; Bozek, et al., 1987). The early studies of this type gave contradictory results that were due, in part, to the mechanism of evaluation, which was subject to patient and observer bias. The later studies used techniques such as polarised light goniometry (Francis, et al., 1986) and tremograms (Bozek, et al., 1987) to evaluate the effectiveness of isoniazid. Polarised light goniometry demonstrated a two to three-fold reduction of tremor when standard methods of clinical assessment showed only marginal improvement. Bozek's group found that, although isoniazid appears to have a limited therapeutic role, a clinical trial is warranted in multiple sclerosis patients with postural tremor.

In addition to those disorders already discussed, isoniazid has been tested for its value in the therapy of Parkinson's disease (Gershanik, et al., 1988) and rheumatoid arthritis (when administered with rifampicin) (McConkey and Situnayake, 1988)

2.1.2.3 MECHANISMS OF ACTION:

The mechanism by which isoniazid is effective in the treatment of tuberculosis is unknown, but there are several hypotheses. For example, the cellular mycolate synthetase activity of Mycobacterium tuberculosis H37Ra

has been shown to be very sensitive to isoniazid (Wang and Takayama, 1972). α-Mycolic acid, a major mycolate component of Mycobacterium tuberculosis, is one of a homologous series of C_{74}-C_{84} fatty acids containing a long aliphatic chain at the α-position, a hydroxyl group at the β-position, and two cyclopropane rings (Minnikin and Polgar, 1967). These fatty acids are present in the cell wall. (Lederer, 1971). It has been shown that isoniazid inhibits the synthesis of saturated fatty acids greater than C_{26} and of unsaturated fatty acids greater than C_{24} (Takayama, et al., 1975). These fatty acids are thought to be precursors of the mycolic acids, and thus inhibition of their formation will disrupt the cell walls of growing cells.

Because of the structural similarity between isoniazid and nicotinamide, many of the proposed mechanisms of action involve pathways where nicotinamide adenine dinucleotide (NAD^+) or reduced NAD (NADH) are important intermediates. For example, relatively high concentrations of isoniazid inhibit NAD synthesis by cell-free extracts of both isoniazid sensitive and resistant Mycobacterium tuberculosis var. homonis H37Rv, the human virulent strain (Sriprakash and Ramakrishnan, 1969). In addition, isoniazid is able to inactivate the inhibitor of NAD glycohydrolase of H37Rv, the enzyme responsible for the degradation of NAD. The result is the release of NADase (Gopinthan, et al., 1966). The inhibitor present in an isoniazid resistant strain of H37Rv, however, is not inactivated by isoniazid (Bekierkunst and Bricker, 1967). Thus, a direct correlation exists between the lethality of isoniazid and the lowering of intracellular concentrations of NAD. DNA ligase of bacteria depends on NAD as a cofactor (Olivera, 1971) and since DNA ligase is essential for elongation of polydeoxynucleotide chains formed in DNA synthesis, the unavailability of NAD for this vital reaction may lead to inhibition of DNA synthesis.

It has been postulated that the bacteriostatic effect of isoniazid is related to its ability to complex certain essential heavy metals such as Cu and Fe. The inhibitory effects of isoniazid on hepatic catalase (Middlebrook, 1954; Arora and Krishna Murti, 1960), on the succinoxidase system of pigeon breast muscle (Arora and Krishna Murti, 1954), or on the organic nitroreductase of gram-negative bacteria (Arora, et al., 1959) are presumably related to sequestering of essential metal ion moieties from the enzymes.

2.1.2.4 BIOTRANSFORMATION:

Since the introduction of isoniazid for the treatment of tuberculosis in the early 1950's, there has been a great deal of work done to elucidate its metabolic pathway, particularly as it relates to isoniazid-induced liver injury. The relevant aspects of the currently accepted scheme of the metabolic fate of isoniazid are shown in Figure 12. The first published identification of isoniazid metabolites occurred in the same year as the introduction of the drug, when studies in dogs identified isonicotinic acid as a major metabolite (Kelly, et al., 1952). This was followed, in 1953, by the first studies in man (Cuthbertson, et al., 1953), in which nicotinic acid, isonicotinic acid, nicotinamide, isonicotinamide, nicotinic acid hydrazide, isoniazid, nicotinoylglycine, and isonicotinoylglycine were identified in urine.

By the mid 1960's it had been established that acetylation is the major route of isoniazid inactivation in man, with work having been done to purify and characterize the N-acetyltransferase enzymes responsible for catalyzing the conversion (Jenne, 1965). Production of acetylisoniazid was found to be

the step that is solely responsible for the differences in isoniazid metabolism between rapid and slow inactivators (Peters, et al., 1965). It was established that these differences in inactivation capacities are under genetic control.

In the mid 1970's, when much work was being done with respect to isoniazid-induced hepatotoxicity, α-ketoglutarate isoniazid hydrazone and pyruvate isoniazid hydrazone had been identified as metabolites (Mitchell, et al., 1975). At the same time, hydrazide metabolites, particularly acetylhydrazine, were identified as the metabolites responsible for production of the "reactive intermediate" which led to hepatic injury. Hydrazines and their derivatives were known to be hepatotoxins, mutagens, and carcinogens (Black and Thomas, 1970; Druckrey, 1973). Subsequently, it was observed that acetylhydrazine is eliminated from the body by at least three routes (Timbrell, et al., 1977). First, it is excreted in the urine as free acetylhydrazine and as the α-oxoglutaric acid and pyruvic acid hydrazones. Second, it is acetylated to diacetylhydrazine, which is excreted in the urine. Third, the acetylhydrazine is eliminated by metabolism by the hepatic microsomal enzyme system, a pathway that is thought to produce the reactive intermediate which is responsible for the hepatotoxicity (Mitchell, et al., 1976; Nelson, et al., 1975).

2.1.2.5 ADVERSE EFFECTS:

The adverse effects of isoniazid are dose related, with a rate of occurrence of 1 to 2 percent at conventional low dose therapy (3 to 5 mg/kg/day) and 15 percent at 10 mg/kg/day (Goldman and Braman, 1972). The most common reactions are rash, fever, jaundice, and peripheral neuritis (Mandell and

Sande, 1985). The range of reactions to isoniazid is quite broad, including: hypersensitivity reactions, such as fever, skin eruptions, hepatitis, and mobilliform, maculopapular, purpuric, and urticarial rashes; hematological reactions such as agranulocytosis, eosonophilia, thrombocytopenia, anemia, and vasculitis; and arthritic symptoms such as back pain, bilateral proximal interphalangeal joint involvement, and arthralgia of the knees, elbows, and wrists. There have also been reports of optic neuritis, muscle twitching, dizziness, ataxia, parasthesias, stupor, toxic encephalopathy (may terminate fatally), and mental abnormalities such as euphoria, transient impairment of memory, separation of ideas and reality, loss of self-control, and florid psychoses (Mandell and Sande, 1985).

One of the most common, and certainly the most extensively studied, of the side effects of isoniazid is toxic hepatitis, which occurs in less than 5 percent of those treated (Girling, 1982). It is manifested as hepatic necrosis, with increased levels of liver enzymes in the serum being evident. It was first suggested, in 1974, that the species responsible for the liver necrosis is acetylisoniazid (Snodgrass, et al., 1974). It was later suggested that the hepatocellular injury is due to the metabolic activation of acetylhydrazine to a reactive intermediate (Nelson, et al., 1976; Mitchell, et al., 1976; Timbrell, et al., 1980). Detoxification is by a further acetylation to the non-toxic diacetylhydrazine (Wright and Timbrell, 1978). Thus, the key determinant of the toxicity of isoniazid may be the balance between metabolic activation and acetylation, i.e., between acetylhydrazine production and metabolism to diacetylhydrazine (Lauterburg, et al., 1985). Based on this, one would expect acetylator phenotype to play a significant role in the incidence of toxicity, with rapid acetylators being at a greater risk (Mitchell, et al., 1975). But, there is abundant clinical evidence to

show that the risk of hepatic reactions during treatment with isoniazid is no greater in rapid than in slow acetylators (Dickinson, et al., 1981; Riska, 1976). In fact, some studies have shown that slower acetylators may be at higher risk (Lal, et al., 1972; Smith, et al., 1972), including one published by Lauterburg and co-workers (1985) who were involved in the studies proposing the opposite hypothesis. In support of the latter observations, there has been a recent report in which hepatic necrosis was histologically demonstrated in rabbits as a result of induction by hydrazine itself, rather than acetylhydrazine (Noda, et al., 1983). This is of interest since hydrazine is produced by a different metabolic pathway whose relative proportion of isoniazid metabolism is increased in slow acetylators.

2.1.3 STATEMENT OF PROBLEM:

As stated at the beginning of this introduction, the goal of this study was to examine the influence of isoniazid and its major metabolites on the metabolism of carbamazepine in rat liver microsomes. This will, hopefully, provide a better understanding of the nature of the clinical interaction and resultant toxicities that were noted.

2.2 EXPERIMENTAL:

2.2.1 MATERIALS:

Carbamazepine, $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. Isoniazid, acetonitrile, methanol, and KCl were obtained from BDH Chemicals. Fisher Scientific was the supplier of $MgCl_2$, hydrazine, tris (hydroxymethyl) aminomethane (TRIS) buffer, and NaH_2PO_4. Acetylhydrazine was obtained from Aldrich Chemical

Co. and isonicotinic acid was obtained from Eastman Kodak Co. Acetylisoniazid was synthesized from materials supplied by BDH, according to the method of von Sassen, et al. (1985), wherein isoniazid was reacted with a four-fold excess of acetic acid anhydride at room temperature for 1.5 hours with continous stirring, and the synthesized product was recrystallized in methanol - diethyl ether (1:4).

Male Sprague-Dawley rats (265-340g) were obtained from the Animal Care Unit of the University of British Columbia.

2.2.2 MICROSOME PREPARATION:

The microsome preparation and the subsequent incubation were based on the methods of Grasela and Rocci (1984).

Rats were sacrificed by a blow to the head, an incision through the neck to the spinal cord, and thorough exsanguination. The mid-section was exposed and the liver was slowly perfused (0.1M NaH_2PO_4 buffer, pH 7.4) via the portal vein. When the liver ceased to expand, the superior vena cava was occluded. Further perfusion allowed the liver to expand completely, attaining a tan colour. The liver was excised and was homogenized using a Potter-Elvejhem apparatus, with a 4:1 ratio of homogenizing buffer (20mM TRIS; 1.15 percent KCl) to liver (v:w). The homogenate was centrifuged at 10,000 g for 15 minutes at 4°C using a Beckman Model JA21 rotor. The S9 microsome fraction was obtained as the supernatant and kept on ice until used. The cytochrome P_{450} content of the preparations was determined using the method described by Mazel (1971). In this method, 2 mL of the microsome suspension was placed in each of two matched cuvettes with teflon stoppers. An LKB Ultrospec 4050 spectrophotometer was

used to determine the baseline absorptions at 450 and at 480 nm. Carbon monoxide was gently bubbled into the sample cuvette for 20 seconds, a few milligrams of solid sodium dithionite ($Na_2S_2O_4$) were added, the cap was placed on securely, the cuvette was inverted, and then the sample was gassed again with carbon monoxide for an additional 20 seconds. The reference was treated only with a few milligrams of sodium dithionite and mixed well. The absorptions of each cuvette were again measured at 450 and at 480 nm. The quantity of cytochrome P_{450} was calculated from the optical density difference (450-480 nm) and the molar extinction coefficient of 91 $mM^{-1}cm^{-1}$. Total protein concentration was determined using the method of Bradford (1976).

2.2.3 INCUBATION:

Each incubation mixture consisted of 150 µL of 10mM $NADP^+$ (0.721 mM final concentration), 150 µL of 60 mM glucose-6-phosphate (4.327 mM final concentration), 36 µL of 250 mM $MgCl_2$ (4.327 mM final concentration), 150 µL of 10 I.U./mL glucose-6-phosphate dehydrogenase (0.721 I.U./mL final concentration), 34 µL of 60 mM carbamazepine (0.981 mM final concentration), and one of 60 µL of homogenizing buffer, or 60 µL of 4 mM (0.115 mM final concentration) or 17 mM (0.490 mM final concentration) of isoniazid, or of one of its metabolites (acetylisoniazid, hydrazine, acetylhydrazine, or isonicotinic acid). For isoniazid and each metabolite there were three assays (control, 0.115 mM, and 0.490 mM) on microsome samples from each of four male Sprague-Dawley rats (that is, the sample size was four). The incubation mixtures were incubated in a shaking water bath (37°C) for 40 minutes, with 100 µL samples being removed at 0, 5, 10, 15, 20, 30, and 40

minutes and being placed into microcentrifuge tubes containing 200 µL of acetonitrile. The samples were then centrifuged in an Eppendorf Centrifuge 3200 for one minute. The supernatants were removed, placed in storage vials, and stored at -20°C until analysed.

2.2.4 ANALYSIS OF SAMPLES:

The samples were analysed using the same apparatus, solvents, and temperature as described previously for the rabbit pharmacokinetic study. The sole difference was that standards were run frequently to allow the production of standard curves and the determination of concentrations of the samples. An example of the chromatograms obtained is shown in Figure 13.

2.3 RESULTS:

The epoxide metabolic ratio-time profiles in the S9 liver microsome preparations at three different concentrations of isoniazid, acetylisoniazid, acetylhydrazine, hydrazine, and isonicotinic acid are presented in Figures 14 to 18. In the trial with isoniazid (Figure 14), the metabolism of carbamazepine was apparently inhibited by isoniazid in a dose dependent manner. The lines representing the three concentrations on the figure diverge with the progression of time, a very good indicator of the trend. Significant differences were observed between the blank and 0.490 mM isoniazid samples at the 30 and the 40 minute marks (using student's t-test).

There were no points displaying significant differences in the trials with acetylisoniazid (Figure 15) or acetylhydrazine (Figure 16). In fact, the use of three concentrations of acetylisoniazid did not even produce a concentration related pattern. Instead, there is some degree of variability

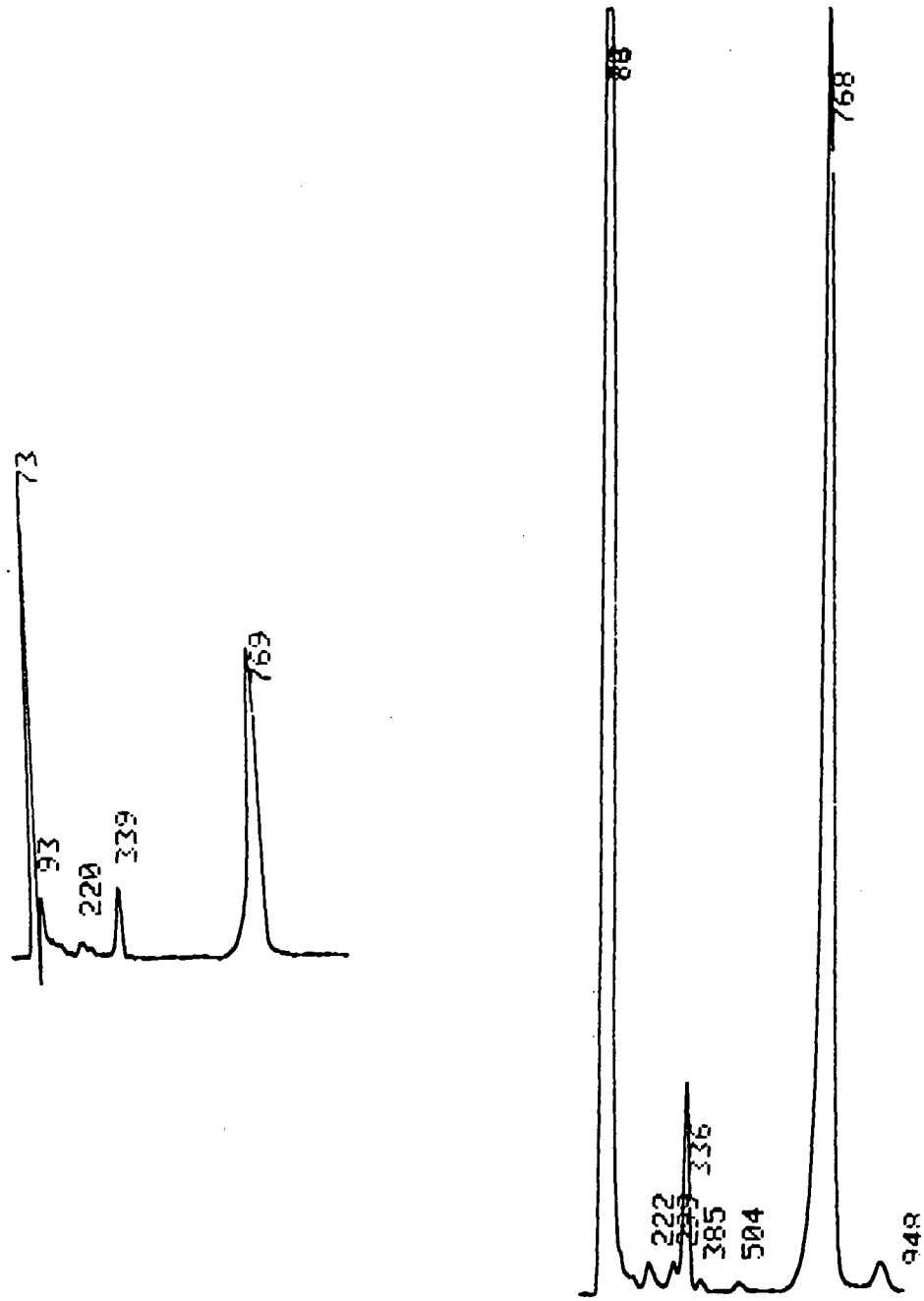


Figure 13: Sample chromatogram from the in vitro interaction study. On the left is a standard sample of carbamazepine (769) and carbamazepine-10,11-epoxide (339) and on the right is a sample taken at 40 minutes.

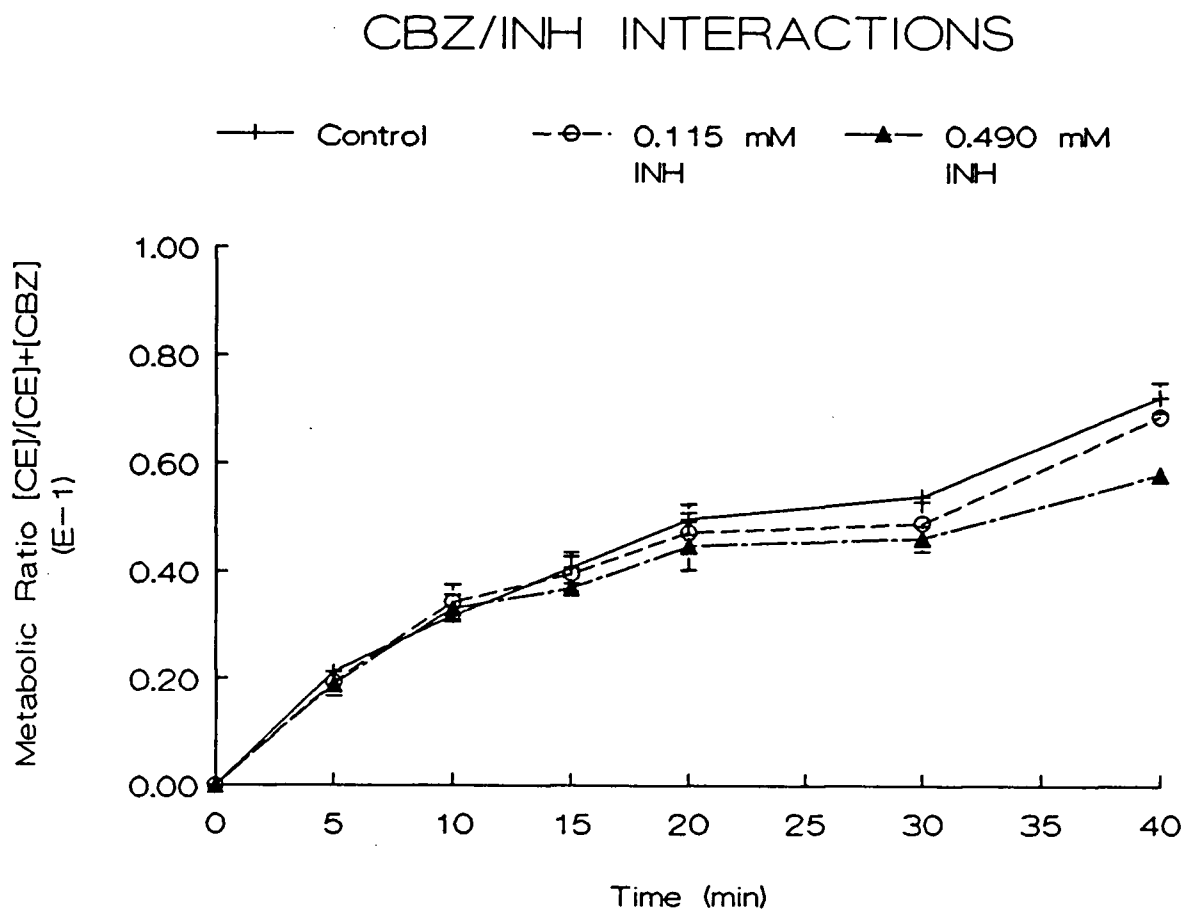


Figure 14: Influence of isoniazid (INH) on the conversion of carbamazepine (CBZ) to carbamazepine-10,11-epoxide (CE) in the S9 fraction of rat liver homogenate. Data represent mean +/- S.E.M. (n=4)

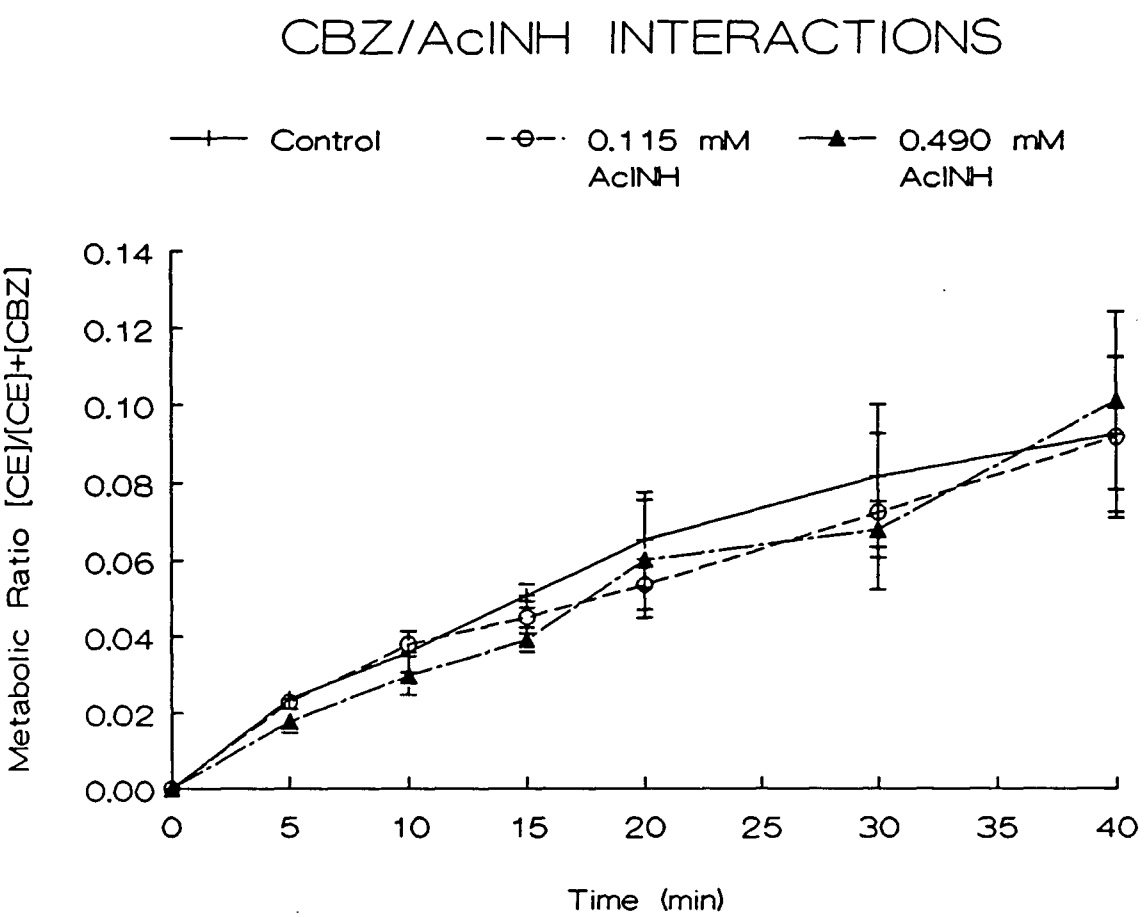


Figure 15: Influence of acetylisoniazid (AcINH) on the conversion of carbamazepine (CBZ) to carbamazepine-10,11-epoxide (CE) in the S9 fraction of rat liver homogenate.
Data represent mean +/- S.E.M. (n=4)

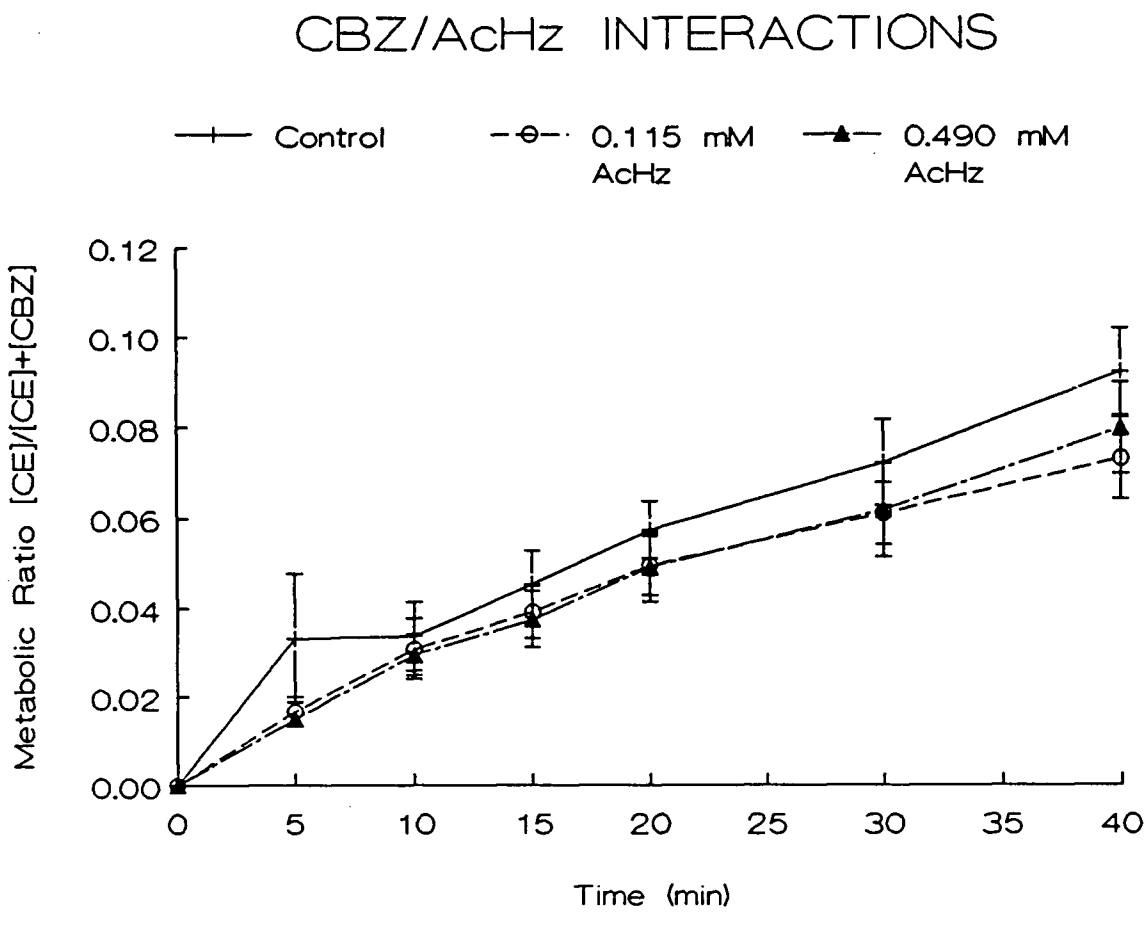


Figure 16: Influence of acetylhydrazine (AcHz) on the conversion of carbamazepine (CBZ) to carbamazepine-10,11-epoxide (CE) in the S9 fraction of rat liver homogenate.
Data represent mean +/- S.E.M. (n=4)

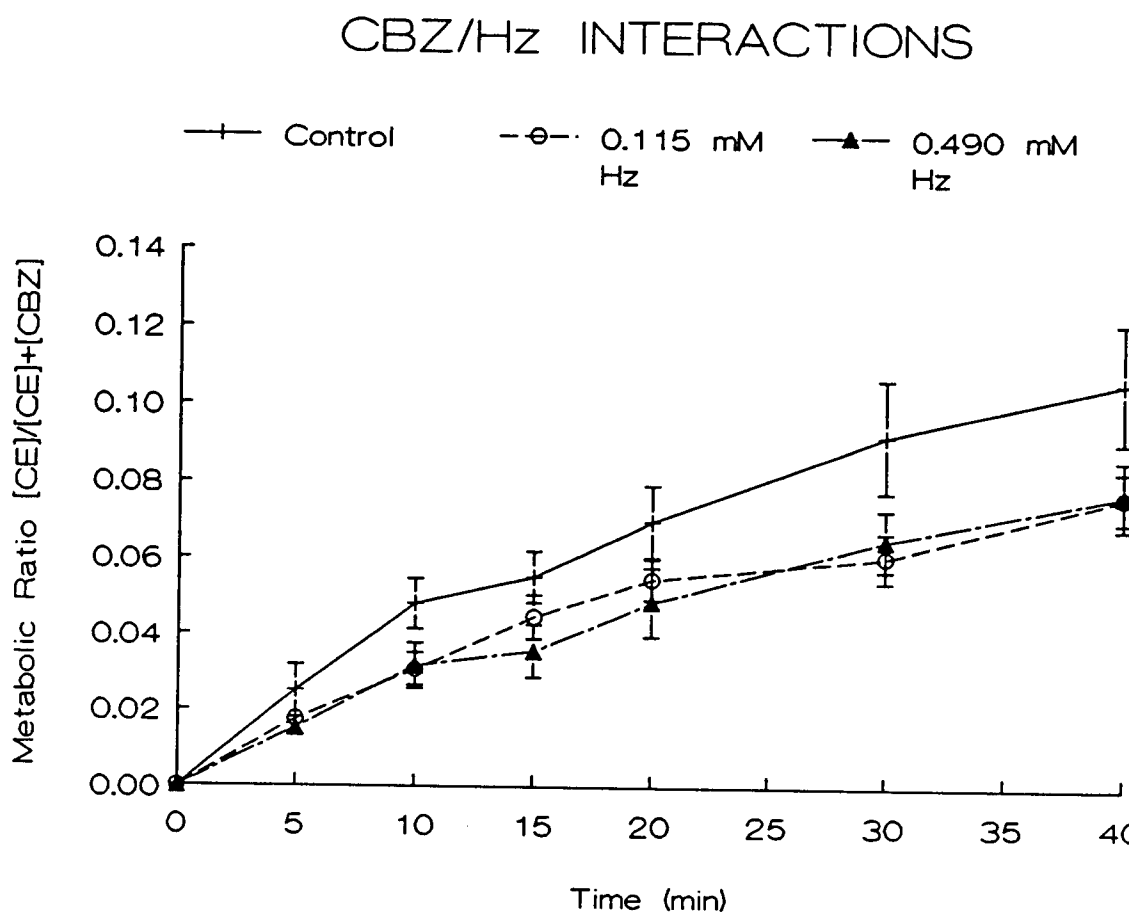


Figure 17: Influence of hydrazine (Hz) on the conversion of carbamazepine (CBZ) to carbamazepine-10,11-epoxide (CE) in the S9 fraction of rat liver homogenate. Data represent mean +/- S.E.M. (n=4)

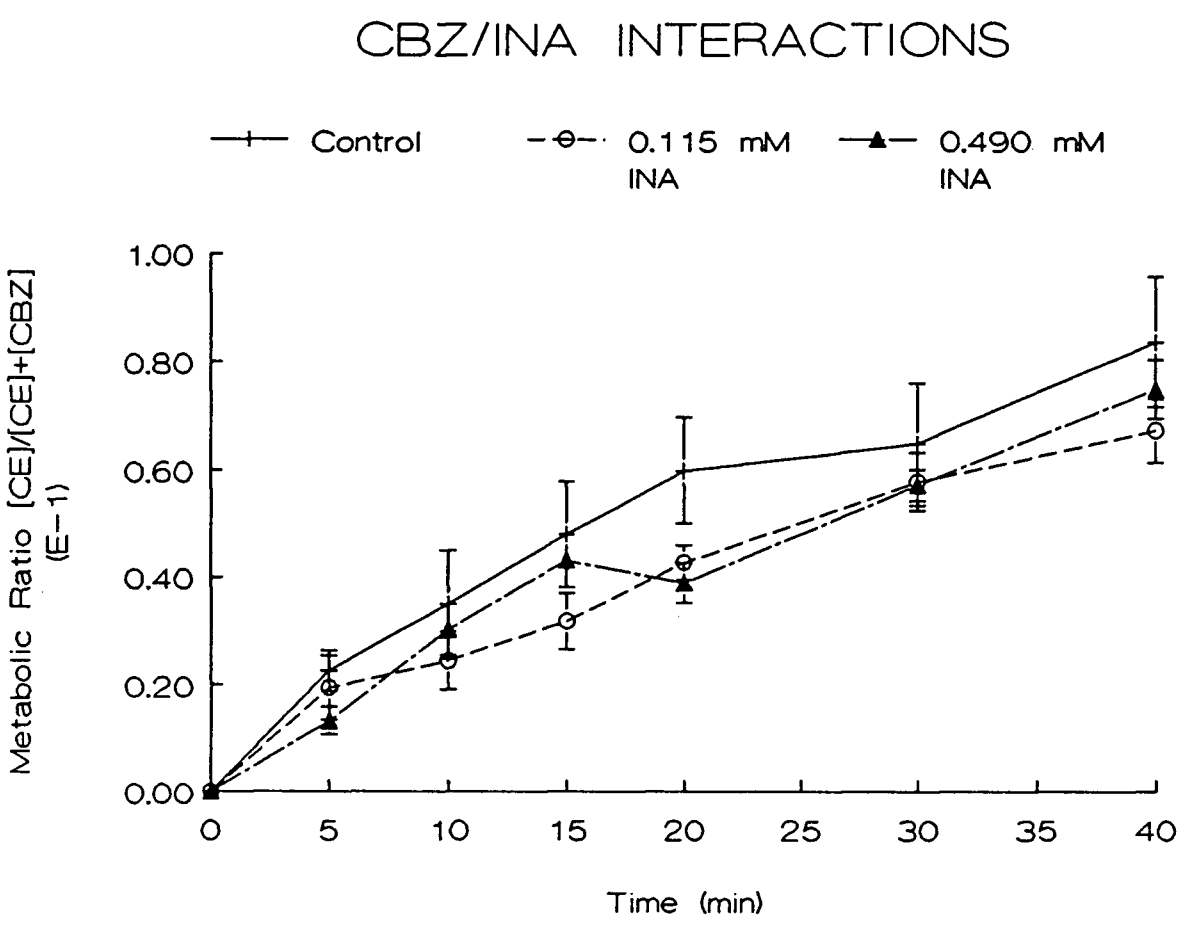


Figure 18: Influence of isonicotinic acid (INA) on the conversion of carbamazepine (CBZ) to carbamazepine-10,11-epoxide (CE) in the S9 fraction of rat liver homogenate.
Data represent mean +/- S.E.M. (n=4)

in the relative positions of the points at each time of measuremment. This is not true in the case of acetylhydrazine, where the control sample clearly has the greatest values for the metabolic ratio over the complete time course, although there are no significant differences between specific points.

In the cases of hydrazine (Figure 17) and isonicotinic acid (Figure 18), the line representing the control is clearly distinct from those representing incubation mixtures containing metabolite. In fact, in the hydrazine trials, there are significant differences at 30 and at 40 minutes, while in the isonicotinic acid trials, there is a significant difference observed at the 20 minute point. Thus, it would appear that both hydrazine and isonicotinic acid are capable of inhibiting the metabolism of carbamazepine.

In an effort to achieve a comparison of the whole spectrum of points between doses, another type of analysis was also attempted. A nonlinear estimation analysis was performed using the Systat software package with the modelling equation being $MR=a(1-e^{bt})$, where MR represents the metabolic ratio and t is the time. The variables a and b are the values for which estimates were obtained, with a representing the theoretical maximum metabolic ratio attained (i.e., the plateau value) and b representing a combined rate constant of several first order rate processes that infers the rate of exhaustion of essential components in the reaction mixture. The first order process was chosen because the results displayed what appears to be a satur- ating functioning and first order functions are the usual observed for this type of experiment. One would not expect to see a great deal of variation in the value obtained for a, since the level of isoniazid or of one of its metabolites would not be the limiting factor in the reaction. In contrast, one would expect to see differences in the value of

b, since if any inhibition is occurring the rate of reaction will necessarily be altered. Values were obtained for a and b for those tests in which the t-tests showed significance at one or more points. The estimates obtained using the software package are shown in Tables II - IV. If the numbers are examined closely, it is obvious that the software package was unable to make appropriate approximations. The values for a appeared to be less than the values of the metabolic ratio at the 40 minute time point. That is, the program seems to be ignoring the increase in the value of the metabolic ratio that occurs between 30 and 40 minutes.

The values of a and b for each individual run were tested by analysis of variance on each interaction group. By this method of analysis, significant differences were observed only for the group in which carbamazepine - isoniazid interactions were tested. For this group, the analysis of a yielded p.0.25, but for the analysis of b, p,0.0005. For the tests with hydrazine, the analysis yielded 0.10.p.0.05 for both parameters. For the tests with

Table II: Estimates of a and b for tests with isoniazid.

Trial	Parameter	Concentration of isoniazid		
		Control	0.115 mM	0.490 mM
1	a	0.041	0.041	0.037
	b	-6.897	-6.903	-6.892
2	a	0.052	0.053	0.046
	b	-6.927	-6.919	-6.896
3	a	0.043	0.034	0.032
	b	-6.912	-6.888	-6.875
4	a	0.042	0.044	0.044
	b	-6.911	-6.911	-6.907

Table III: Estimates of a and b for tests with hydrazine.

Trial	Parameter	Concentration of hydrazine Control	0.115 mM	0.490 mM
1	a	0.084	0.056	0.059
	b	-7.027	-6.940	-6.955
2	a	0.059	0.049	0.042
	b	-6.948	-6.929	-6.903
3	a	0.073	0.047	0.036
	b	-6.987	-6.916	-6.899
4	a	0.048	0.038	0.045
	b	-6.924	-6.895	-6.927

Table IV: Estimates of a and b for tests with isonicotinic acid.

Trial	Parameter	Concentration of isonicotinic acid Control	0.115 mM	0.490 mM
1	a	0.069	0.049	0.043
	b	-6.970	-6.924	-6.923
2	a	0.040	0.036	0.046
	b	-6.915	-6.908	-6.926
3	a	0.060	0.043	0.045
	b	-6.953	-6.903	-6.914
4	a	0.039	0.035	0.038
	b	-6.980	-6.885	-6.896

isonicotinic acid, the results of the analysis gave p=0.25 and p.0.25 for a and b, respectively. This is somewhat unexpected. If the figures for the tests done with each of these compounds are examined visually, it is quite apparent that over the course of the experiment there are dose related differences developing. The problem is that these differences are not reflected in the statistical procedures presented thus far.

If the probability procedure of run analysis is employed, the results are somewhat different. In this type of analysis, the probability of two points of adjacent lines being different is 0.5. These probabilities can be multiplied over the course of the line. For example, if this procedure were done on the curves from the carbamazepine/isoniazid interaction study, the p-value for the comparison of the control to the 0.490 mM curve would be $(0.5)^5$ or 0.03, since the points of the control line are above the points of the 0.490 mM line at 5, 15, 20, 30, and 40 minute points.

Table V: P-values from run analysis of in vitro interaction studies.

Treatment	P-value for: Control vs 0.490 mM	Control vs 0.115 mM	0.115 mM vs 0.490 mM
Isoniazid	0.03	0.03	0.02
Acetylizoniazid	0.03	0.03	0.06
Acetylhydrazone	0.02	0.02	0.06
Hydrazine	0.02	0.02	0.13
Isonicotinic acid	0.02	0.02	0.13

2.4 DISCUSSION:

The results presented in this study would appear to provide an in vitro confirmation of the clinical observations that isoniazid is able to inhibit carbamazepine metabolism, as reported by Wright and co-workers (1982), by Block (1982), and by Valsalan and Cooper (1982). In addition, the results suggest that hydrazine, isonicotinic acid, and possibly even acetylhydrazine are metabolites of isoniazid that inhibit carbamazepine metabolism by S9 liver microsome fractions.

It is not possible to determine the exact nature of the inhibition of carbamazepine metabolism at this point in time, but the concentration dependent nature of the inhibition would suggest a competitive interaction, particularly since isoniazid metabolism has been reported to be impaired when carbamazepine and isoniazid are coadministered in a clinical situation (Barbare, et al., 1986). It is also possible that an irreversible interaction is taking place, but a definite conclusion cannot be made without doing binding studies. An irreversible interaction is less likely, though, since plasma concentrations of carbamazepine seem to return to normal within a few days after isoniazid administration is ceased (Block, 1982; Wright, et al., 1982). Despite this observation, subsequent observations of the effects of isoniazid on acetaminophen metabolism suggest that isoniazid is a suicide inhibitor of certain cytochrome P_{450}'s capable of causing a 70 percent decrease in acetaminophen metabolism (Epstein, et al., in press).

The potent inhibiting capability of isoniazid described above was not observed in the current study. The carbamazepine concentration used in this series of experiments was chosen because it provided the best response in preliminary studies done in the absence of any inhibiting species. The lack of a more potent difference in the presence of isoniazid suggests that the chosen carbamazepine concentration may have been too high and not a reflection of what is occurring in the clinical situation. In fact, when compared with other studies where a similar procedure was used, the concentration used in this study were towards the high end, but not out of range. The concentrations of carbamazepine used in some other studies ranged from 0.05 mM (Tybring, et al., 1981) to 1.0 mM (van Boxtel, et al., 1977). The higher carbamazepine may have resulted in the systems adopting a zero-order, or close to zero-order, kinetic profile, with the inhibitor having very little effect.

When discussing these experiments, one must remain aware that the conversion of carbamazepine to carbamazepine-10,11-epoxide is not the only process that is able to occur in the system. In the S9 preparation epoxide hydrolase, the enzyme responsible for the conversion of the epoxide to the diol, is still present. As well, the enzymes responsible for glucuronidation and for the conversion of the epoxide to the acridan are also present, but these are minor. Each of these processes is first order with respect to the epoxide. Thus, a plateau of the metabolic ratio curve has to arise from a balance of processes. In fact, it is the activity of the epoxide hydrolase that is responsible for the curve not quite being exponential. If the reaction had been allowed to proceed for long enough, it is quite possible that the metabolic ratio may have decreased, since the ratio is the consequence of a balance of several enzymic processes.

Based on an examination of the structures of the two compounds, one might expect the aromatic ring structure to be an important recognition factor at the active site of the enzyme responsible for metabolism of the drugs, provided that the mechanism of inhibition is a competitive process. It is possible that the side chains of the two drugs, namely the carbamoyl side chain of carbamazepine and the hydrazide group of isoniazid, are, in part, responsible for positioning the compounds on the enzyme. If this combination of influences was occurring, one would expect to see inhibitory activity from the metabolites that result from the simple breakdown of the isoniazid compound, namely hydrazine and isonicotinic acid. Such an action was observed. If the mechanism of inhibition is not a competitive process, the same postulations about the relative importance of the structural features of isoniazid and its metabolites would likely hold true for a regulatory site, rather than for the active site.

The slight effect displayed by acetylhydrazine may be due to one of two reasons. First, the structural similarity between acetylhydrazine and hydrazine may play a role, with the presence of the acetyl group decreasing the effectiveness of acetylhydrazine at inhibiting carbamazepine metabolism. The second possible reason is that a small proportion of the acetylhydrazine is being converted to hydrazine in the preparation. Such a conversion has not yet been described, but it is possible that such a reaction may occur. Acetylisoniazid, on the other hand, displayed no apparent inhibition of carbamazepine metabolism. This is likely due to the prevention of access to the required recognition sites by the acetyl group, a factor not influencing the actions of isoniazid or isonicotinic acid.

An evaluation of the relative effectiveness of each of the compounds tested for their ability to inhibit carbamazepine metabolism reveals that the hydrazine moiety is likely to be most responsible for the activity. If the trials with hydrazine and isonicotinic acid (Figures 15 and 16) are compared, it is obvious that hydrazine exerted a greater effect. This might lead one to surmise that the hydrazide moiety of isoniazid is responsible for a greater proportion of the observed inhibitory activity.

The results obtained, with regard to the identity of the species primarily responsible for the inhibition of carbamazepine metabolism, do not correlate well with the results of studies examining the toxic effects of isoniazid and its metabolites. In studies of isoniazid-induced hepatoxicity, acetylhydrazine has been shown to be the metabolite that is primarily responsible (Mitchell, et al., 1975; Nelson, et al., 1976). There is evidence that acetylhydrazine is oxidized by a microsomal cytochrome P_{450} enzyme to produce the reactive acylating agents in <u>in vitro</u> studies with rat and human liver microsomes (Nelson, et al., 1976). Subsequent results

showed that the entire acetyl group of acetylhydrazine is trapped by cysteine, thereby eliminating ketene as the reactive acetylating agent formed during the oxidation of acetylhydrazine by liver microsomes (Nelson, Hinson, and Mitchell, 1976). Based on this information, acetylhydrazine would be expected to be the most effective of the metabolites tested at inhibiting carbamazepine metabolism, due to its ability to covalently bind proteins, such as cytochrome P_{450}'s that metabolize carbamazepine. But, in the experiments being presented currently, acetylhydrazine did not have a significant effect. Thus, it would appear that there is some degree of specificity with respect to the protein species that can be covalently bound as a result of acetylhydrazine reactivity. The enzyme responsible for carbamazepine metabolism would seem to be one such protein. The most likely reason for the exclusion of a protein from being covalently bound by the mechanism described would be the lack of exposed cysteine moeities, or at least of any that are positioned so as to alter carbamazepine metabolism if acylated. It cannot be confirmed if this in fact is the case, since the structure of the enzyme responsible for carbamazepine epoxidation has yet to be elucidated. It is reasonable to postulate, though that there are no cysteines present in the active or regulatory sites of the enzyme, since acylation at such a site would likely lead to the inhibition of carbamazepine metabolism.

The results obtained in this study are consistent with a number of studies wherein a metabolic interaction or competitiveness has been observed between carbamazepine and other drugs with cyclic moeities that undergo hepatic oxidative metabolism, such as erythromycin (Barzaghi, et al., 1987), imipramine (Daniel and Netter, 1988), and cimetidine (Grasela and Rocci, 1984). The obvious route to pursue in the future would be to continue

studying the interactions between compounds that fit the criteria stated above. It is hopeful that information derived from these types of studies will assist clinicians in making wise decisions about the co-administration of drugs. This may result in fewer patients that experience the adverse effects that sometimes result from drug interactions.

With respect to the system examined in the current study, it is necessary to examine the interactions over a wide range of carbamazepine concentrations. If this is done, it will be possible to examine the kinetics of the reaction more thoroughly by plotting Michaelis-Menten and Lineweaver-Burke plots. This should provide more information about the nature of the inhibition that is observed, i.e., the question of whether the inhibition by isoniazid is competitive or of a suicide-type should be answered. In addition, if studies are done with chemically synthesized compounds that are specifically designed for specific types of interactions at the active site of the enzyme, it should be possible to get a better idea about the structure of the active site.

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