Title
Disposition and covalent binding of acyl glucuronides

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Publication Date
1985

Peer reviewed|Thesis/dissertation

DISPOSITION AND COVALENT BINDING OF ACYL GLUCURONIDES: STUDIES WITH ZOMEPIRAC GLUCURONIDE

by

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B.S. Pharmacy, University of Illinois - Chicago, 1978

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

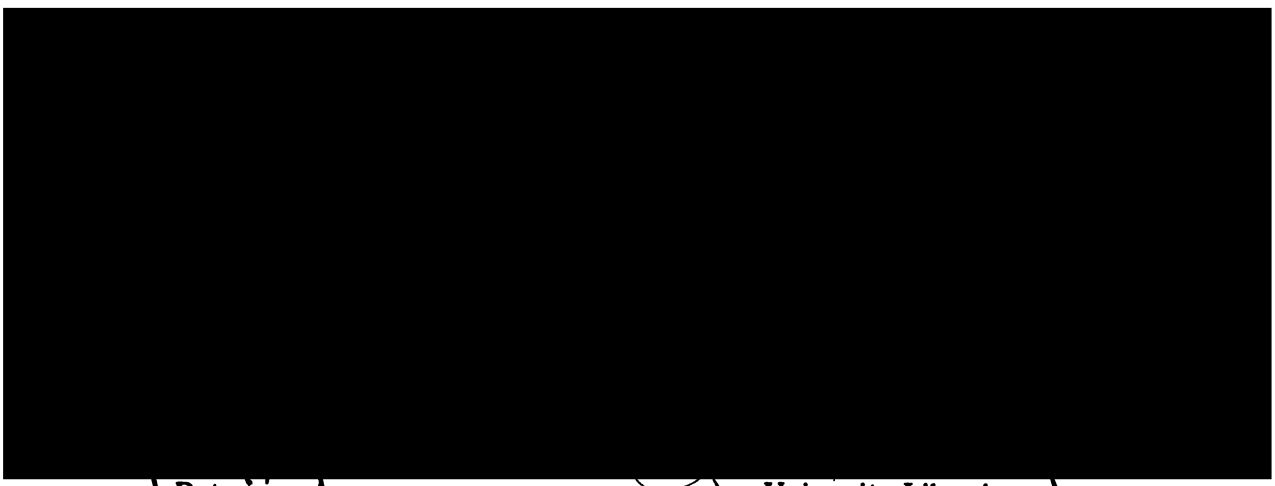
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



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DEDICATION

To

my mother, Irene,

with the acheivements of all her children,

she would be proud.

ACKNOWLEDGEMENTS

I would like to extend my thanks to the following persons for their support and encouragement during my graduate studies:

Dr. Leslie Z. Benet for his support and patience as my graduate research advisor, and of course special thanks for performing my wedding ceremony.

Drs. Svein Oie, Alma Burlingame, David Zakim, Kathleen Giacomini and Wolfgang Sadee for their time, discussion, and efforts in reviewing my thesis work.

Dr. Jiro Hasegawa for his astute observation in the laboratory which provided the nucleus for this project, his scientific discussion, and continuing friendship.

Pim Langendijk for his contribution to the clinical studies in this thesis, his enthusiasm and friendship.

Dr. Antony McDonagh for his contribution to the studies of covalent binding of acyl glucuronides to proteins, his stimulating discussions and enduring enthusiasm.

Dr. Roger Ketchum and his wife Doris for their warm friendship and constant hospitality.

The entire Benet research group and others in the Department of Pharmaceutical Chemistry for their support and friendship, especially Dr. Bruce Mico, Dr. Carol Gloff, Sue Tsang, Linda Gustavson and Dr. Bobbe Ferraiolo.

The American Foundation for Pharmaceutical Education and the Merck Corp. for their Fellowship support of my graduate education.

To my family for their unquestionable support during my long graduate training and for their occasional loans which were also not questioned.

Finally, the most significant contributor, which made my graduate studies more pleasant and enduring, my wife, Laurene, whose love, understanding and scientific discussions helped make this thesis possible.

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ABSTRACT

DISPOSITION AND COVALENT BINDING OF ACYL GLUCURONIDES: STUDIES WITH ZOMEPIRAC GLUCURONIDE

Philip Charles Smith

Xenobiotics which contain carboxylic acid functional groups are often metabolized in human and animals by conjugation with glucuronic acid. Previous literature suggested that the ester or acyl glucuronide so formed may be unstable, with degradation occurring *in vitro* and *in vivo* either by hydrolysis or intramolecular acyl migration about the uronic acid. Evidence from studies with bilirubin glucuronides have also shown that acyl glucuronides can react with albumin forming an irreversible adduct of the aglycone and albumin. Using the nonsteroidal antiinflammatory compound zomepirac (Z), 5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid, and its acyl glucuronide, ZG, as model compounds, we examined the hypothesis that acyl migration and enzymatic hydrolysis of ZG occurred *in vivo* and that these routes of degradation for ZG were significant in the disposition of Z. The relationship between exposure to the labile and reactive ZG and the extent of irreversible binding of Z to plasma proteins was investigated *in vitro* and *in vivo* in animals and humans.

ZG was isolated and purified from human urine after an oral dose of Z. The isomeric conjugates of ZG formed by acyl migration were unambiguously identified using fast atom bombardment mass spectrometry and high field ^{1}H-NMR. The rate of acyl migration of ZG was dependent upon pH, temperature and solvent, with maximum stability for ZG at pH 2. ZG degraded faster in blood and plasma than in aqueous solutions. Reductions of temperature and pH reduced the rate of ZG loss prior to and

during analysis by liquid chromatography. Previous analytical methods for assaying Z in plasma did not control for ZG stability and may have overestimated Z concentrations. The disposition of Z and ZG in humans after a 100 mg oral dose of Z was examined using an assay method which minimized degradation of ZG. Rapid hydrolysis of ZG in guinea pig liver homogenates was observed and found to be inhibited by 1mM phenylmethylsulfonyl fluoride (PMSF) or physostigmine, however, 20 mM 1,4-saccharolactone had no effect. After an intravenous dose of ZG to the guinea pig or rabbit, the conjugate was rapidly eliminated with a large fraction of the dose hydrolyzed to form Z *in vivo*. Concurrent intraperitoneal dosing of 50 mg/kg PMSF inhibited ZG hydrolyis by esterases in the guinea pig and caused a marked decrease in the plasma clearance of ZG. When PMSF was given to the guinea pig prior to Z, the plasma clearance of Z was increased three fold. Together these data support the hypothesis that hydrolysis of acyl glucuronides *in vivo* does occur and also proves that the cleavage is an enzymic process due to esterase, not β-glucuronidase.

Time and pH-dependent irreversible binding of Z to albumin occurred *in vitro* when ZG or its isomeric conjugates were incubated with albumin. The extent of irreversible binding of Z to albumin through ZG was determined by exhaustive washing of the protein to remove reversibly bound compounds, then hydrolysis of the Z-protein adduct with base with subsequent extraction and chromatographic analysis of Z. Irreversible binding of Z also occurred *in vivo* in humans and guinea pigs and the extent of binding correlated with exposure to ZG as measured by the area under the plasma concentration vs. time curve.

CHAPTER I

INTRODUCTION

DISPOSITION AND COVALENT BINDING OF ACYL GLUCURONIDES: STUDIES WITH ZOMEPIRAC GLUCURONIDE

A. INTRODUCTION

Carboxylic acid is a ubiquitous functional group, present in many endogenous compounds, drugs and other xenobiotics. Besides being present in compounds prior to administration, carboxylic acids may also be produced *in vivo* by Phase I (1, 2) metabolic pathways such as oxidation or hydrolysis (1). Carboxylic acids are often further metabolized *in vivo* by Phase II (1) conjugative processes producing conjugates with glucuronic acid, glycine, glutamine and taurine (1). Conjugation with glucuronic acid is a common route for the elimination of many other functional groups as well as carboxylic acids. Glucuronides have been identified with carboxylic acids, aliphatic alcohols, phenols, amines, thiols, hydroxylamines and even with activated carbon centers (3). Glucuronides of carboxylic acids are esters, i.e. acyl glucuronides, which are known to be unstable (3, 4, 5) and which can hydrolyze in urine at physiological pH of 6 to 8 (6). Besides chemical hydrolysis, reports have accumulated in the literature documenting or suggesting other means for loss of acyl glucuronides *in vitro* and *in vivo*. Intramolecular acyl migration, producing isomeric conjugates of the initial β-1-glucuronide, has been shown to occur for bilirubin glucuronide under mild alkaline conditions (7). Also, data from several laboratories have suggested that 1-β-acyl glucuronides are cleaved rapidly *in vivo* in

animals by enzymatic, rather than chemical hydrolysis (8, 9). If hydrolysis and/or acyl migration of acyl glucuronides does occur *in vivo*, it may have a significant effect on the disposition of not only the conjugate, but also the parent compound, because hydrolysis of the conjugate results in nonproductive, futile cycling of the drug. This possiblility should be considered when studying the pharmacokinetics of the parent compound. Moreover, problems in the interpretation and analysis of pharmacokinetic data will occur if hydrolysis or acyl migration of glucuronides takes place *in vitro* after the biological samples are obtained. These potential effects and problems associated with the dispositional characteristics of acyl glucuronides were investigated using the model compounds, zomepirac (Z), 5-(4-chloro(-)-benzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid, (Fig. I-1) and its acyl glucuronide (ZG), the latter found by our laboratory to be hydrolytically labile and to undergo acyl migration *in vitro* at physiological pH.

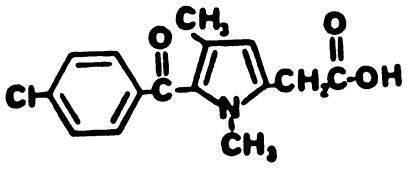


Figure I-1. The structure of zomepirac.

While the studies described in this thesis were ongoing, an additional mechanism for the loss of labile acyl glucuronides *in vitro* and *vivo* was documented for bilirubin acyl glucuronides. The formation of covalent adducts of bilirubin to albumin in plasma was proven to require bilirubin acyl glucuronides as a necessary precursor (10, 11).

Once bilirubin was bound irreversibly to protein *in vivo*, the bilirubin-protein adduct was cleared from plasma very slowly which resulted in its accumulation (11, 12). The significance of irreversible binding of Z to protein through ZG was therefore studied *in vitro* and *in vivo*. Covalent binding of reactive metabolites to macromolecules *in vivo* is often correlated with the toxicity of the parent compound (13, 14). The finding that binding of the parent drug to proteins occurs via formation of an acyl glucuronide may be of toxicological importance and merits thorough investigation.

B. BACKGROUND

Significance of acyl migration and hydrolysis of acyl glururonides

If the glucuronide conjugate is not eliminated rapidly relative to its rate of formation, it can accumulate to a significant extent *in vivo*. The commonly held generalization that all glucuronides are cleared so efficiently that their concentrations in plasma are negligible is not necessarily correct. There is a growing list of examples of glucuronides which do accumulate in healthy humans and especially in patients with compromised excretory function, such as renal failure (15, 16) or cholestatic hepatic disease (12). The plasma concentrations of alcoholic or phenolic glucuronides of acetaminophen (17), lorazepam (18), oxazepam (19) and diflunisal (20, 21) are measurable in individuals after administration of the parent compound and become elevated in patients with renal failure (15). There are also reports of acyl glucuronides that are detectable in human plasma after administration of clofibric acid (22), indomethacin (23), ketoprofen (24) and zomepirac (25, 26). Especially well documented is the rise in the plasma

concentrations of bilirubin acyl glucuronide during cholestatic liver disease (10, 11, 12, 27). However, this list is not comprehensive, and undoubtedly reflects only a small fraction of the compounds whose glucuronides accumulate in the plasma, since few studies have attempted to measure the conjugates in vivo. The lack of data from, or interest in, studies of the disposition and accumulation of glucuronides in vivo probably results from the belief that glucuronides are frequently devoid of pharmacological activity and, as mentioned above, are not thought to accumulate in vivo to a significant extent.

If accumulation of acyl glucuronides in vivo does occur, it is of little significance unless the conjugate has pharmacological or toxicological properties. From the preliminary data of the irreversible binding of bilirubin to plasma proteins through its acyl glucuronides (10, 11, 12), there is reason to believe that a correlation exists between exposure to the conjugate in vivo and the extent of irreversible binding. The possible effects of such binding will be discussed below.

In the past, the reactivity of the acyl glucuronides in biological fluids was not well appreciated by many investigators. Thus, precautions were seldom taken to prevent the loss of the acyl glucuronide after sampling of the biological fluids. The loss of glucuronide in vitro after samples are collected will result in artifactually low concentrations of the β-1-conjugate, but high concentrations of the parent compound or the products of acyl migration. Acyl migration of the natural β-1-glucuronide results in the formation of isomeric conjugates which are not susceptible to cleavage by β-glucuronidase and may have different chromatographic properties (7, 28). The commonly employed method for the analysis of glucuronide concentrations in

biological fluids, is to measure the increase in the parent drug concentration after treatment with β-glucuronidase. This method would not be able to detect the presence of the isomeric conjugates of acyl glucuronides. Even with direct chromatographic measurement of the β-1-glucuronide, the isomeric conjugates might not be detected since their different retention times upon chromatography may complicate the analysis or the resultant peaks may be erroneously considered as interferences from substances of no interest.

Hydrolysis of the β-1-glucuronide or its isomeric conjugates *in vivo* decreases the concentration of conjugates with resultant regeneration of the parent compound. This potential futile cycling between the parent and the conjugate results in nonproductive metabolism which increases the exposure of the body to the pharmacologically active parent compound. Measurements of the absolute metabolic conversion or clearance of the drug to form the metabolite will be underestimated if the measurement is based upon the total conjugate excreted, the rate of loss of the parent drug, or the rate of appearance of the metabolite. An increase in the residence time of the acyl glucuronide *in vivo*, caused by inhibition or reduction of elimination of the conjugate, will result in an increase of the extent of hydrolysis or acyl migration of the conjugate *in vivo*. Therefore, decreased excretion of the conjugate and the resultant increased hydrolysis would appear to reflect a decrease in the metabolic clearance of the parent to form glucuronide, even though conjugation ability of UDP-glucuronyltransferase had not been reduced. This phenomenon is observed when comparing drug disposition in renal failure between the drugs metabolized to labile acyl glucuronides and the drugs metabolized to more stable phenolic

glucuronides. Clofibric acid (29, 30) and ketoprofen (31) which are primarily eliminated by man as acyl glucuronides, exhibit decreased clearance of the parent drug in renal failure. In contrast, acetaminophen (17) and oxazepam (19) exhibit no change in plasma clearance in renal failure even though their glucuronides accumulate to high levels.

An understanding of the factors which affect acyl migration and the hydrolysis of acyl glucuronides *in vivo* and *in vitro* will allow proper analysis and interpretation of pharmacokinetic data for drugs and other xenobiotics metabolized to these labile conjugates. At present, many of the studies in the literature on compounds metabolized to acyl glucuronides have not considered the possible effects of either acyl migration or hydrolysis of the labile conjugates, not to mention the possible covalent binding to protein. This may have resulted in considerable errors in the published pharmacokinetic parameters for the disposition of these compounds.

Selection of zomepirac glucuronide as a model compound

Acyl migration and hydrolyis of acyl glucuronides are probably general phenomena. The relative and absolute rates of the degradation via the two routes may vary among different aglycones. Acyl migration has been documented for many acylated sugars and all but the most sterically hindered acyl groups will migrate about the sugar ring (32). Whether many acyl glucuronides accumulate *in vivo* is not known, the reasons for which have been mentioned above. Previous data in the literature have shown that after an oral dose of Z, ZG is present in the plasma of human at concentrations near those of Z (25, 26). Early studies in our laboratory had indicated that 1-β-ZG was unstable in

human urine, forming putative isomeric conjugates of ZG. We had also developed some ability to assay Z and 1-β-ZG in human urine. From these preliminary data, we selected Z and 1-β-ZG as the parent compound and acyl glucuronide metabolite to be used as the model compounds for studying the disposition of acyl glucuronides and the effect of acyl migration and hydrolysis of acyl glucuronides on the disposition of its parent compound. It was crucial for our studies to be able to obtain sufficient quantities of 1-β-ZG to allow dosing of the conjugate to animals. Later development of a method to isolate and purify 1-β-ZG from human urine satisfied this requirment.

Possible toxicological significance of acyl glucuronides

Zomepirac (Zomax®) was withdrawn from distribution in the U.S. in March, 1983, because of unexplained anaphylactic reactions which led to several reported deaths (32, 34). Z is representative of a large and growing group of nonsteroidal antiinflammatory drugs (NSAIDs) of the aryl alkanoic class which include tolmetin, indomethacin, diflunisal and ibuprofen (35). Because of the toxicity of Z, as well as that of benoxaprofen (Oraflex®) (36, 37), this group of drugs has come under increasing scrutiny by both the public and the government (38). The well documented toxicity of NSAIDs, i.e. gastric irritation, nephritis and acute renal failure (39, 40), were not responsible for the withdrawal of Z. Instead, a high incidence of immunological reactions and anaphylaxis which may be unique to Z and other NSAIDs (41) led to its removal from the market (34). Not unique to Z or unusual for NSAIDs is their elimination by metabolic conjugation of the ubiquitous carboxylic acid side chain with glucuronic acid to form acyl

glucuronides. This metabolic pathway is the major route of elimination for Z with up to 90% of a ^{14}C-labelled dose excreted in the urine as ZG (25). Another well known, but unrelated compound, that is metabolized to unstable acyl glucuronides is bilirubin. In cholestatic liver disease bilirubin becomes irreversibly bound to albumin (42, 43) and recent studies have shown that this reaction occurs via the acyl glucuronide metabolites of bilirubin (10, 11). Although bilirubin glucuronides have only been reported to accumulate in human plasma during liver disease (12), ZG has been reported to reach a plasma level similar to that of Z after administration of a single oral dose of Z to healthy subjects (25). Since both bilirubin and Z appear to form similar reactive and unstable acyl glucuronide metabolites (7, 28, 44), it seemed possible to us that covalent binding of Z to protein might occur for Z via its glucuronide metabolite.

Covalent binding of xenobiotics, per se, need not result in toxicity. However, there has been a great deal of evidence showing good correlations between the covalent binding of reactive chemicals to proteins or nuclear material and the carcinogenic, mutagenic (14) or immunogenic (13) potencies of the chemicals. Examples of reactive glucuronides, the N-hydroxy-glucuronides of phenacetin (45) and N-acetylaminofluorene (46), have been reported and implicated in the carcinogenicity of the parent compound. Acyl glucuronides appear to be electrophiles, with other electrophilic reactions occurring besides hydrolysis and acyl migration. Salmon and Fenselau (47) have found that the labile glucuronides of bilirubin undergo solvolysis in aqueous methanolic solutions forming methyl esters. Reports from this same laboratory have indicated that thiol containing compounds, such as

ethanethiol and glutathione, react with clofibric acid glucuronide *in vitro* producing the respective thiol esters (48). Thus, acyl glucuronides behave as electrophiles, which may provide a mechanism for the irreversible binding of bilirubin to albumin. It is interesting to speculate that such covalent binding may be related to the immunological toxicity observed for Z.

C. OBJECTIVES

The specific objectives of the studies presented here were to:

1. Prove that the degradation of ZG *in vitro* is primarily due to acyl migration by isolating and characterizing the products of acyl migration of ZG.
2. Characterize the factors affecting the degradation of 1-β-ZG *in vitro* and *in vivo* and to develop techniques to prevent such loss *in vitro*.
3. Prove that a futile cycle exists between Z and 1-β-ZG by studying the disposition of Z and 1-β-ZG in a small animal model.
4. Examine the dispostion of Z and 1-β-ZG in humans after a single oral dose of Z.
5. Determine whether Z binds irreversibly to plasma proteins, *in vitro* and *in vivo*, through its glucuronide, 1-β-ZG.

CHAPTER II

ACYL MIGRATION OF ZOMEPIRAC GLUCURONIDE; ISOLATION AND CHARACTERIZATION OF ISOMERIC CONJUGATES

A. INTRODUCTION

Zomepirac (Z), is a nonsteroidal antiinflammatory drug (NSAID) approved by the FDA in October, 1980, but withdrawn from the U.S. market in March, 1983, due to a high incidence of immunological reactions (33, 34). Metabolism (49) and disposition studies (26) conducted by the manufacturer, McNeil Pharmaceuticals (Springhouse, PA) have shown that the major metabolite of Z in human is the glucuronide conjugate (Fig. II-1). Subsequent studies in humans by other investigators have found similar metabolic profiles for Z (25, 50) which will be discussed in greater detail in Chapter III. While studying the pharmacokinetics of Z in our laboratory, unusual artifacts attributed to zomepirac glucuronide (ZG) were noted during analytical method development. Liquid chromatography (HPLC) of Z and putative ZG revealed time and pH dependent loss of ZG from human urine samples collected after an oral dose of the drug. The loss of putative ZG, however, resulted in the appearance of other unidentified peaks in the HPLC chromatogram besides Z. Characterization of these compounds formed in urine and the reasons for their formation was investigated.

Because ZG decreased as the unidentified HPLC peaks increased, it was apparent that ZG was being transformed into compounds other than the parent compound, Z. Glucuronidation is a major Phase 2 metabolic

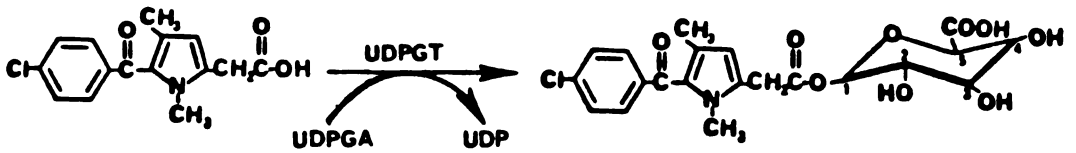


Figure II-1. Metabolic pathway for zomepirac forming zomepirac glucuronide by the enzyme Uridine diphosphate glucuronyltransferase (UDPGT). UDP-glucuronic acid (UDPGA) is consumed liberating UDP.

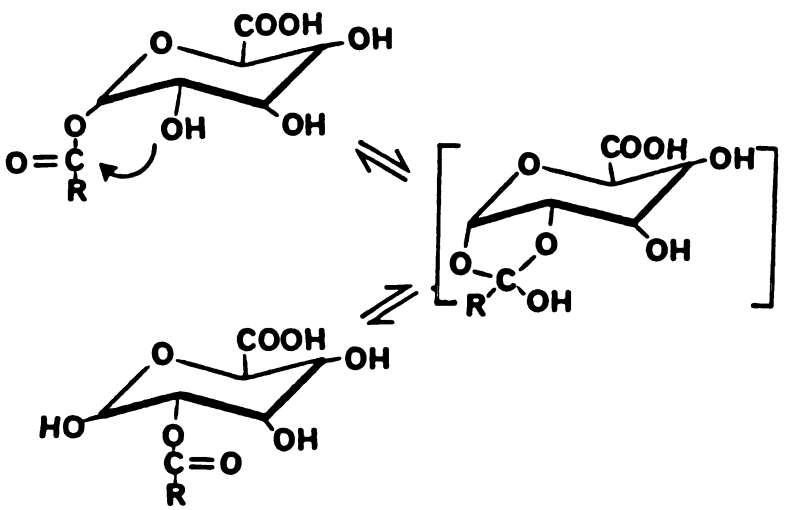


Figure II-2. Postulated mechanism for acyl migration of a 1-O-acyl glucuronide to form the 2-O-acyl glucuronide through an unstable orthoacid intermediate.

pathway for many xenobiotics and endogenous compounds (51, 52, 53, 54). The chemical stability of glucuronides has also been well documented (3, 4, 5). The coupling of the sugar, glucuronic acid, by the enzyme UDP-glucuronyltransferase (E.C. 2.4.1.17) can occur with many polar groups such as phenols, alcohols, carboxylic acids, thiols, amines and even activated carbon compounds (3). The resultant conjugates vary in chemical structure, stability and succeptability to β-glucuronidase (4). Phenol, alcohol, thiol and carbon linked conjugates are quite stable under mild alkaline conditions, whereas similar conjugates of glucuronic acid with carboxylic acids and amines yield esters and aminals, respectively, which are easily hydrolyzed under such conditions (4). Instability of acyl glucuronides to mild base is thus well understood and accepted, however, acyl glucuronide decomposition to products other than those that would occur due to chemical or enzymatic hydrolysis, i.e. glucuronic acid and the parent drug, was not well documented in the drug metabolism literature at the time this investigation was initiated. A possible degradation route for acyl glucuronides is via intramolecular transesterification, i.e. acyl migration of the β-1-acyl conjugate to form isomeric conjugates where the ester is on the 2, 3 or 4 positions of glucuronic acid (Fig. II-2). Carbohydrate chemists have known, and thoroughly described, acyl migration reactions in sugars (32). The mechanism of acyl migration would likely explain the anomolous peaks observed for ZG in mildly basic urine.

Acyl migration

The phenomenon of acyl migration has only recently been realized by investigators in drug metabolism to be a potential problem for acyl

glucuronides of drugs. In contrast, the carbohydrate literature is replete, with reviews of the topic about every ten years (32, 55, 56, 57, 58) since it was first correctly proposed by Emil Fischer in 1920 to occur through an orthoacid intermediate (59). It was subsequently proven to be an intramolecular rearrangement by Doerschuk in 1952 (60). Figure II-2 shows the acyl migration of a β-1-acyl glucuronide conjugate forming the anomerized α/β-2-acyl isomer through a postulated unstable orthoacid intermediate.

The first proof of acyl migration for drugs or endogenous compounds of biological interest came from studies conducted with the important product of heme catabolism, bilirubin. Compernolle et al. (7) proved the migration of acyl glucuronides of bile azopigments from dog in 1977 by using mass spectrometry to verify the position of the acyl group. Later, this group identified the same phenomenon in human bile (44, 61). Clofibric acid yielded two separable conjugates in urine, both of which hydrolyze to clofibric acid in the presence of dilute alkali, though only one conjugate was susceptible to β-glucuronidase (E.C. 3.2.1.31) hydrolysis (22, 62). However, another report on clofibric acid by Caldwell and Emudianughe (63) found only one conjugate, and that was cleaved by β-glucuronidase. A later analysis by Sinclair and Caldwell (64, 65) confirmed that the discrepency observed between the laboratories was due to acyl migration of clofibric acid glucuronide. In contrast, Hignite et al. (66) believed that the β-glucuronidase resistant conjugates of clofibric acid were due to the formation of α/β-furanose and α/β-glucuronides which were identified by GC-MS. They discounted possible acyl migration since the urine pH was below 6.8 when collected. Probenecid, which is eliminated to a large extent by

glucuronidation, has also been shown to yield other glucuronic acid products, which may be due to acyl migration (67). In this case ^{13}C-NMR was utilized to determine the location of the acyl linkage for the probenecid glucuronide isomers. A recent communication on isoxepac, an acidic NSAID, found β-glucuronidase resistant compounds which were formed from the putative β-1-glucuronide and that the rate of loss of the glucuronide was pH-dependent (68). Since the publication by our laboratory of the results presented here for ZG (28), several other reports have appeared which prove or suggest that acyl migration occurs with glucuronides of other drugs. An investigational NSAID, Wyeth #18,251 (69); another NSAID, diflunisal (70, 71); and most recently, the antiepileptic, valproic acid (72) have been shown to exhibit acyl migration of their acyl glucuronide metabolites.

Caldwell et al. has also observed the rearrangement of the acyl glucuronide of another NSAID, fenclofenac, (data unpublished) and has stated that acyl migration is suspect for many other acidic compounds studied in their laboratory which are metabolized to acyl glucuronides (64, 65). Their suspicion of acyl migration is based upon higher recoveries of parent drug from urine after base hydrolysis than after incubation with β-glucuronidase. Upton et al. (6) reported unidentified HPLC peaks from human urine following oral doses of ketoprofen and naproxen, which yield the parent compound when treated with aqueous base. Both of these compounds are similar to Z and isoxepac and are metabolized to acyl glucuronides which appear in the urine (73, 74).

Considering the bulk of chemical literature on acyl migration for sugars and the proof of its occurance for bile pigments in humans, the possibility of this phenomenon explaining the anomalous peaks derived

from ZG was investigated. The chemical structures of ZG and the other compounds formed from ZG were characterized and are presented in this chapter. The approach taken was to isolate and purify ZG from the urine of man, then to characterize the conjugate by accepted chemical techniques such as elemental analysis, mass spectrometry, NMR, chromatography and qualitative colorimetric reactions. The putative isomeric conjugates of ZG formed by acyl migration were produced from ZG, then purified by preparative HPLC and characterized as well.

B. MATERIALS AND METHODS

Chemicals

Zomepirac·Na·$2H_2O$ and 1,4-dimethyl-1H-5-(-methoxybenzoyl)-pyrrole-2-acetic acid (internal standard) were graciously supplied by Dr. J. Michael Grindel, McNeil Pharmaceutical (Spring House, PA). Bovine liver β-glucuronidase (Type B-10) was from Sigma Chemical Co. (St. Louis, MO). Sodium D-glucuronic acid, D-saccharic 1,4-lactone, and all deuterated solvents for NMR and tetramethyl silane (TMS) were purchased from Aldrich (Milwaukee, WI). Methanol was HPLC grade from Fisher Scientific (Pittsburg, PA). All other chemicals were reagent grade.

Purification of zomepirac glucuronide from urine

One of the investigators ingested about 200 mg of Z with 100 ml of water. Urine was collected hourly for four hours and frozen immediately. The combined urine was adjusted to pH 2 with dilute HCl, washed with 300 ml hexane, then extracted twice with 250 ml portions of ethyl acetate. The ethyl acetate fraction was washed with water, dried with magnesium sulfate, filtered and evaporated to dryness using a rotoevap-

orator. The residue was dissolved in about 5 ml of methanol and the mixture concentrated to about 1 ml under a stream of nitrogen; the glucuronide was then precipitated by slow addition of water. The filtered precipitate was again dissolved in 5 ml of methanol and the mixture was evaporated under a stream of nitrogen to yield an amorphous liquid which when dissolved in ethyl acetate and concentrated by evaporation resulted in 44.5 mg of coarse white crystals after drying over P_2O_5. Elemental analysis for ZG hemihydrate: Anal. calc. for $C_{21}H_{23}ClNO_{9.5}$, C 52.89, H 4.86, N 2.94. Found: C 52.81, H 4.83, N 2.95. The melting point of 152-153 °C, uncorrected, was determined with a Hoover capillary melting point apparatus (Philadelphia, PA).

Qualitative colorimetric reactions were performed on the purified ZG to support the presence of glucuronic acid. Benedict's and Fehling's tests for reducing sugars (75) and the naphthoresorcinol test for glucuronic acid (76, 77) were used with glucose and glucuronic acid as positive controls and Z as a negative control. Incubations of ZG and the other isomeric conjugates with β-glucuronidase, 3000 U/ml, were done at pH 5.0, 37 °C, in 0.10 M sodium phosphate. Saccharic acid 1,4-lactone, a specific inhibitor of β-glucuronidase (78), was used to evaluate the specificity of the enzymatic hydrolysis at a concentration of 50 mM.

Purification of the isomeric conjugates of ZG

Zomepirac glucuronide, 250 mg, was dissolved in 200 ml of 0.10 M sodium phosphate buffer, pH 7.5 and incubated at 37 °C for 60 minutes to allow ZG to degrade to its isomeric conjugates. The pH was then adjusted to 2.0 with HCl and the solution was extracted twice with 300 ml

portions of ethyl acetate. The ethyl acetate extract was dried with magnesium sulfate, filtered and evaporated to dryness. The residue was dissolved in HPLC mobile phase and the fractions were purified by preparative HPLC (Fig. II-3). Peak fractions from each injection were combined; the pH of the eluent was adjusted to 2.0 with HCl and the volume reduced with a rotoevaporator which caused precipitation of the isolated compounds. The precipitates were filtered, washed with dilute HCl, pH 2.0, dried over P_2O_5 and used without further recrystalization. Purity of the isomers was estimated by taking an aliquot (100 μg) of the isomer in methanolic solution, drying the sample under a nitrogen stream, then hydrolyzing the sample in 4 ml of 0.1 N NaOH. After one hour at 37 °C a 0.4 ml aliquot was withdrawn, spiked with internal standard, and then analyzed for Z by analytical HPLC. The amount of Z liberated was compared to the theoretical value using peak area measurements assuming that the molar extinction coefficients of the isomers are the same as ZG. Strong base, not β-glucuronidase, was used for hydrolyzing the isomers because the isomers were not succeptible to the enzyme (see Chapter IV).

Chromatography

Liquid chromatography was performed with RSiL, C18, 10 μ reversed phase columns from Alltech (Deerfield, IL). Dimensions were 4 x 250 mm and 10 x 500 mm for the analytical and preparative columns, respectively. A Perkin-Elmer (Norwalk, CT) Series II pump and an LC-15 254 nm fixed wavelength UV detector were used for the HPLC. Manual injection was done with a Model 7105 HPLC injector (Rheodyne, Cotati, CA). Quantification was done by peak height using a strip chart recorder or peak

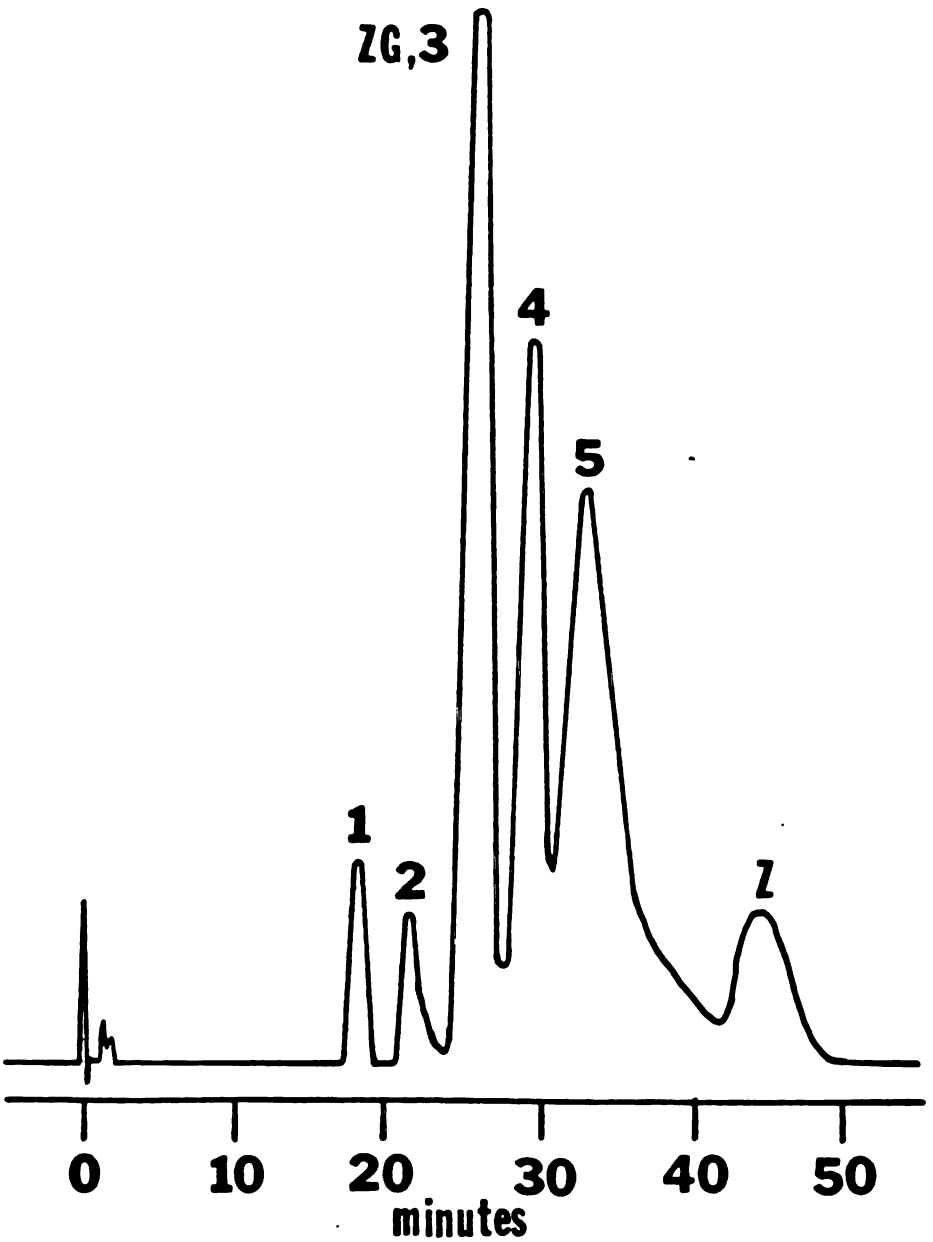


Figure II-3. Preparative HPLC of zomepirac glucuronide (ZG), the isomeric conjugates of ZG (fractions 1, 2, 4 and 5) and zomepirac (Z) after incubation of ZG in 0.1 M phosphate buffer, pH 7.4 at 37 °C for 60 minutes. The ordinate is UV absorption at 254 nm.

area when necessary using a 3380A integrator (Hewlett Packard, Palo Alto, CA). A methanol:0.01 M sodium acetate buffer, pH 5.1, was used as the mobile phase. A ratio of 40:60 (v/v) at a flow of 6 ml/min was employed for preparative separations, whereas a 35:65 (v/v) mobile phase mixture at 2 ml/min was used for analytical determinations.

NMR and Mass Spectrometry

Low field ^{1}H- and ^{13}C-NMR spectroscopy was done using a Varian Associates (Sunnyvale, CA) FT-80 instrument. Chemical shifts are reported relative to internal tetramethylsilane (TMS) or to DMSO (2.500 ppm). High field ^{1}H-NMR was done with Nicolet equipment at the U.C. Davis NMR facility using field strengths of 360 or 500 mHz. Solvents and parameters employed for the NMR are indicated in Results, below. Solid samples, ca. 1 mg, were dissolved in about 0.3 ml deuterated solvent, then transferred to a 5 mm NMR tube. Samples were not deoxygenated. All purified isomeric conjugate fractions from preparative HPLC as well as ZG were analyzed by mass spectrometry using a Fast Atom Bombardment (FAB) ion source (79, 80) by the NIH Biomedical Mass Spectrometry Facility at U.C. San Francisco. The samples were dissolved in propylene glycol prior to application to the mass spectometer probe and the molecular beam used was xenon. Both the FAB ion source and the MS-50 spectrometer employed were Kratos instruments (San Diego, CA), operated in the positive ion mode.

C. RESULTS

The purified ZG provided satisfactory elemental analysis results which indicated the presence of a hemihydrate. Using analytical HPLC,

ZG exhibited a single peak at 23 minutes. The mass spectra of the underivatized compound obtained by FAB-MS and the low field ^{1}H-NMR supported the structure of ZG as being the β-glucuronide and will be discussed below in more detail. Upon hydrolysis by β-glucuronidase, the ZG HPLC peak was competely eliminated with the formation of Z which represented 94.4 ± 0.4 % (mean ± sd, n=3) of the possible theoretical value. This enzymatic hydrolysis was completely inhibited by saccharic acid 1,4-lactone, a specific inhibitor of β-glucuronidase (78). Colorimetric reactions were supportive for glucuronic acid, with positive Fehling's and Benedict's tests for reducing sugars and the formation of a blue color characteristic for glucuronic acid with the naphthoresorcinol test (76, 77).

Figure II-3 represents the preparative HPLC separation obtained for the isomeric conjugates of ZG. Retention times were 18.5, 21.8, 26.2, 30.0 and 33.5 min for fractions 1 through 5, respectively. Fraction 3 is ZG, and Z is eluted at 44.1 min with the conditions employed. Analytical HPLC gave a similar profile with Z eluted at 23 min. The purities of the fractions collected from preparative HPLC were: fraction 1, 100% (82); fraction 4, 91% (83); and fraction 5, 92% (84). The first number is based upon peak area by analytical HPLC assuming the molar extinction coefficients of the isomers are the same as ZG while the value in the parenthesis was calculated based on the amount of Z liberated by hydrolysis with NaOH. The amounts of purified isomers obtained were: fraction 1, 12.6 mg; fraction 4, 8.2 mg; and fraction 5, 16.6 mg. Fraction 2 was not stable enough to be isolated in solid form.

FAB mass spectrometry of the underivatized ZG is shown in Figure II-4. Ions at m/z of 468, 292 and 246 correspond to M+H, Z+H and Z-

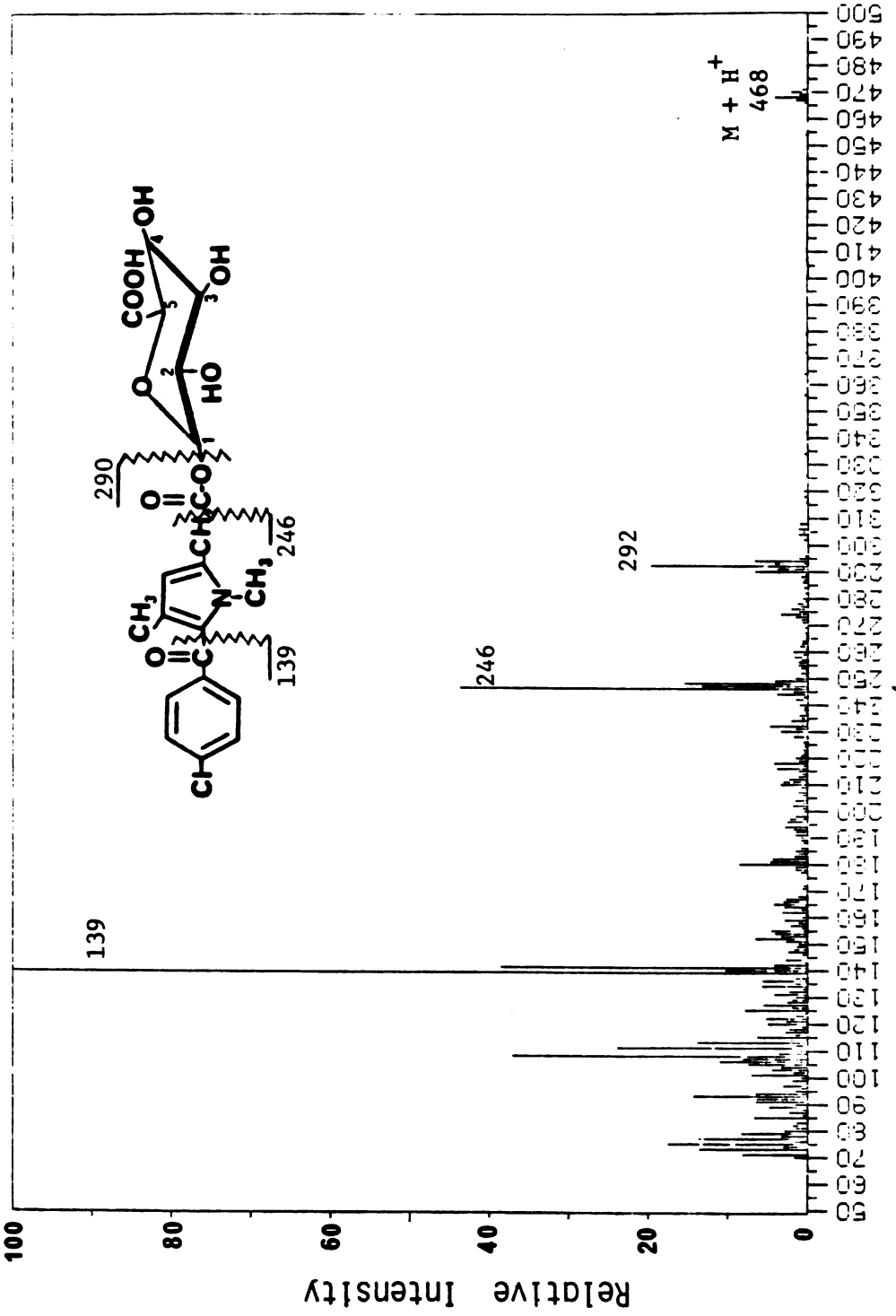


Figure II-4. Fast atom bombardment mass spectrum of underivatized zomepirac glucuronide.

COOH, respectively, all of which exhibited the characteristic isotopic cluster for one chlorine atom. Peak fractions 1, 4 and 5 from preparative HPLC produced similar mass spectra with the same parent ion at M+H of 468, and major peaks at 292 and 246. This supports the fact that these fractions are isomers of ZG. Fraction 2, which was unusual in that it was extremely labile, did not yield a satisfactory mass spectra.

Purified ZG in methanol-d_4 (TMS, δ=0.0) gave low field ^{1}H-NMR of: δ 7.55, m; δ 6.00, s; δ 5.57, d, J=7.3 Hz; δ 3.25-3.85, m; δ 1.72, s. Singlets for the methylene, δ 3.87; and the N-methyl, δ 3.69; were unresolved from the sugar protons. The assignments of Z and ZG made previously by Wu et al. (49) in DMSO, which are shifted slightly in methanol are: benzyl, δ 7.55; pyrrole, δ 6.00; β-1, δ 5.57; methylene, δ 3.87; N-methyl, δ 3.69; sugar ring, C2-C5, δ 3.25-3.85; and 4-methyl on pyrrole, δ 1.72. When ZG was compared to Z and glucuronic acid, the doublet at 5.57 ppm was determined to be the anomeric proton of glucuronic acid, having the same coupling constant as the 1-β proton of glucuronic acid, but about one ppm further downfield due to the acylation shift (81). The low field NMR, however, was insufficient to resolve the complex multiplet at 3.5 ppm which is due to the overlap of the other ring protons of glucuronic acid. Identification of the putative isomeric conjugates of ZG as being positional isomers of ZG is possible by NMR, but requires information on the nature of the linkage between the acyl group and the sugar. Although ^{13}C-NMR has much improved resolution of sugar structure than proton NMR and carbon does exhibit a similar shift upon acylation of the sugar hydroxyl (82, 83, 84), the limited quantity of material available precluded the use of the less sensitive ^{13}C-NMR technique. Instead, high field ^{1}H-NMR using field

strengths of 360 and 500 Mz was employed to resolve the proton envelope of glucuronic acid and allow definitive assignments.

Initial NMR studies were conducted with ZG which is appreciably soluble in pyridine, methanol and DMSO (dimethylsulfoxide). Pyridine was chosen because of its ability to exchange with the hydroxyl protons of the sugar, which simplifies the spectra. There have also been studies documenting acylation shifts for glucose which differs from glucuronic acid at carbon 6. These studies were conducted using pyridine as the NMR solvent (82, 83, 84). However, when the putative isomeric conjugates of ZG were prepared for NMR, not all of the isomers were soluble in pyridine or methanol. Solubility of the isomers in both solvents decreased with their increasing retention on reversed phase HPLC, with fractions 4 and 5 being only slightly soluble. DMSO-d_6 was then utilized with a drop of trifluoroacetic acid-d_1 added to each NMR tube just prior to spectral accumulation (85). ^{1}H-NMR were obtained for ZG in pyridine-d_5 (Fig. II-5) and DMSO-d_6 (Fig. II-7), fraction 1 in pyridine-d_5 (Fig. II-6), and fractions 4 and 5 in DMSO-d_6 (Figs. II-8 and 9). Here the fraction number refers to the relative retention of the isomers on reversed phase preparative HPLC (Fig. II-3).

Assignments were made from successive homonuclear decoupling of the sugar region. ZG, or fraction 3 on HPLC, which is the β-1-glucuronide, gave only five sugar resonances with coupling constants of about 8 Hz which is characteristic for the β-anomer of sugars (82, 86) (see Figs. II-5 and 7). Assignments of chemical shifts observed for the sugar region of ZG and the isomeric conjugates in the Figures II-5 through 9 are given in Table II-1. The isomers of ZG in all cases were mixtures of the α/β-anomers, both of which were present in DMSO prior to addition

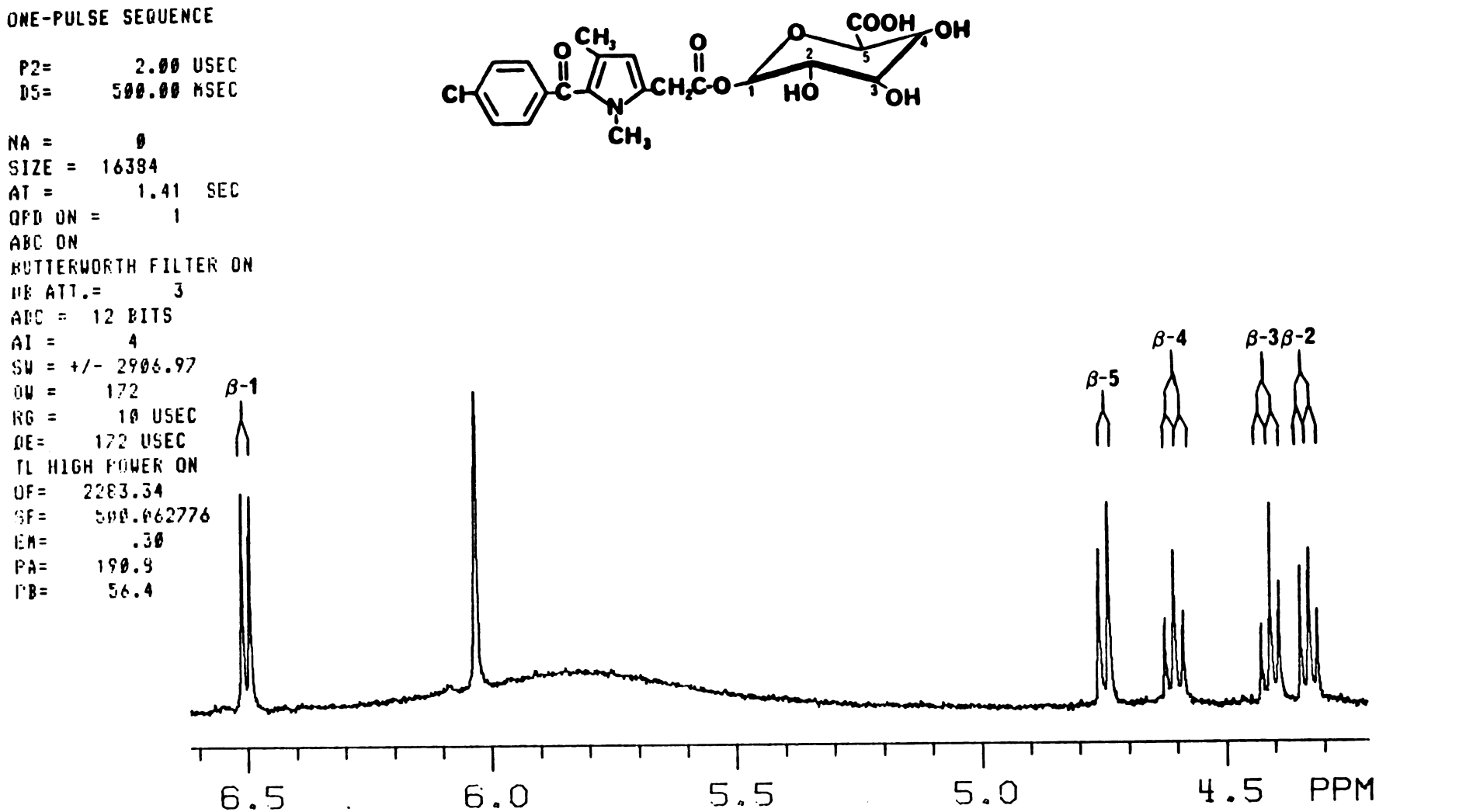


Figure II-5. ^{1}H-NMR of Zomepirac β-1-acyl glucuronide in pyridine-d_5 at 360 MHz. Only the sugar region of the spectra is presented.

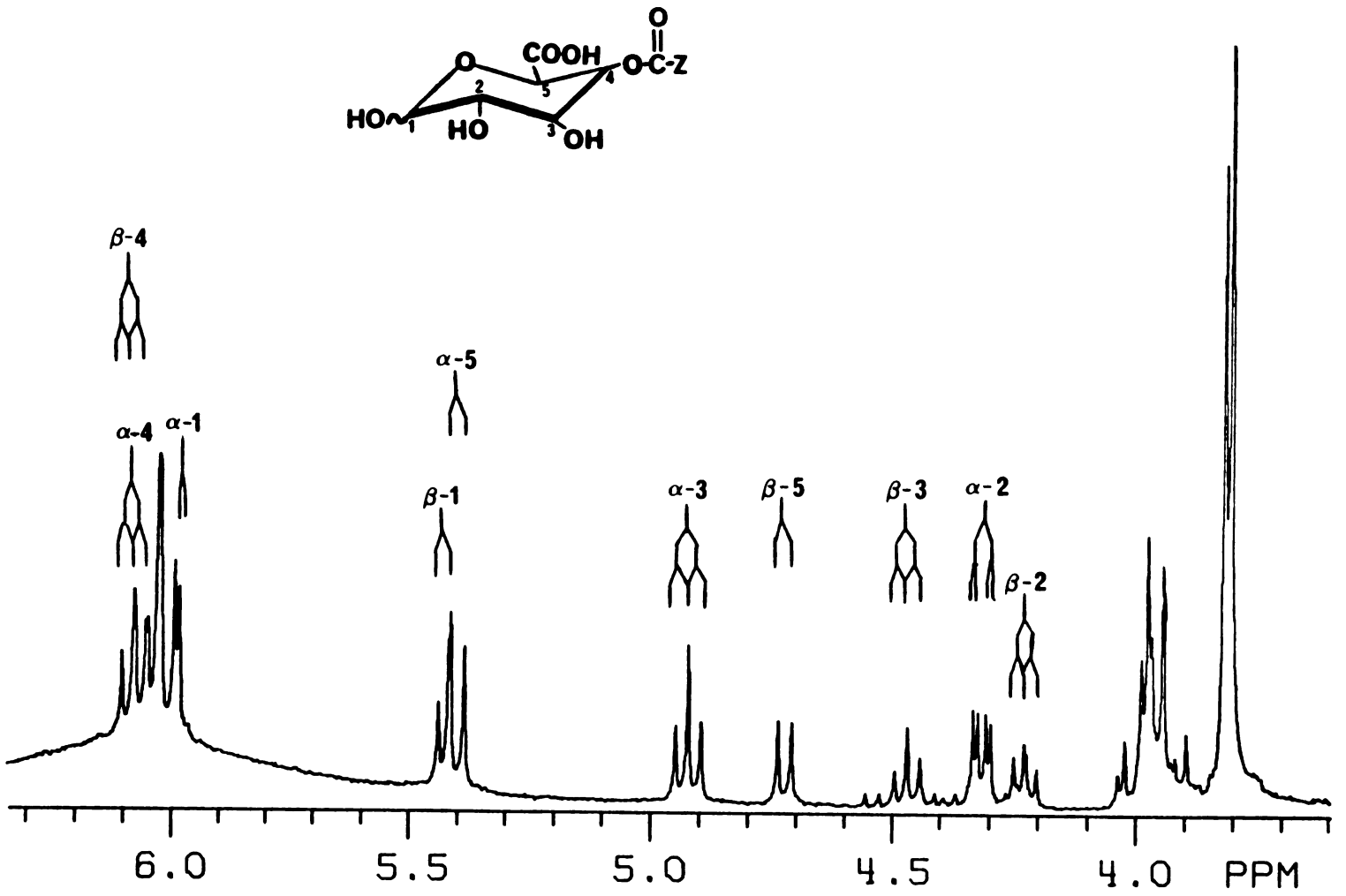


Figure II-6. ^{1}H-NMR of Zomepirac α/β - 4-acyl glucuronide in pyridine-d_5 at 360 MHz. Only the sugar region is presented. Parameters for accumulation are presented in Figure II-5.

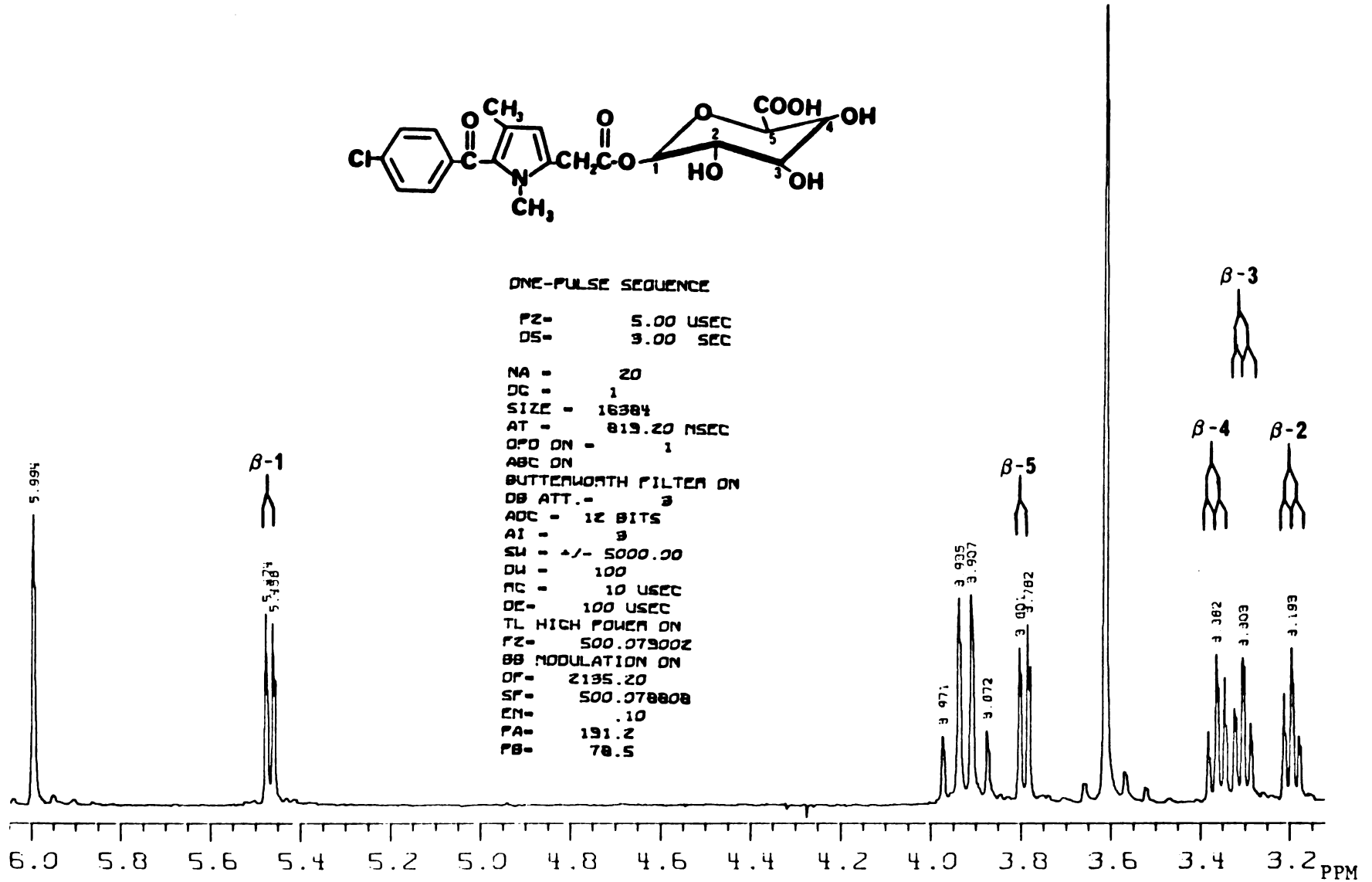


Figure II-7. ^{1}H-NMR of Zomepirac β-1-acyl glucuronide in DMSO-d_6/trifluoroacetic acid-d_1 at 500 MHz. Only the sugar region is presented.

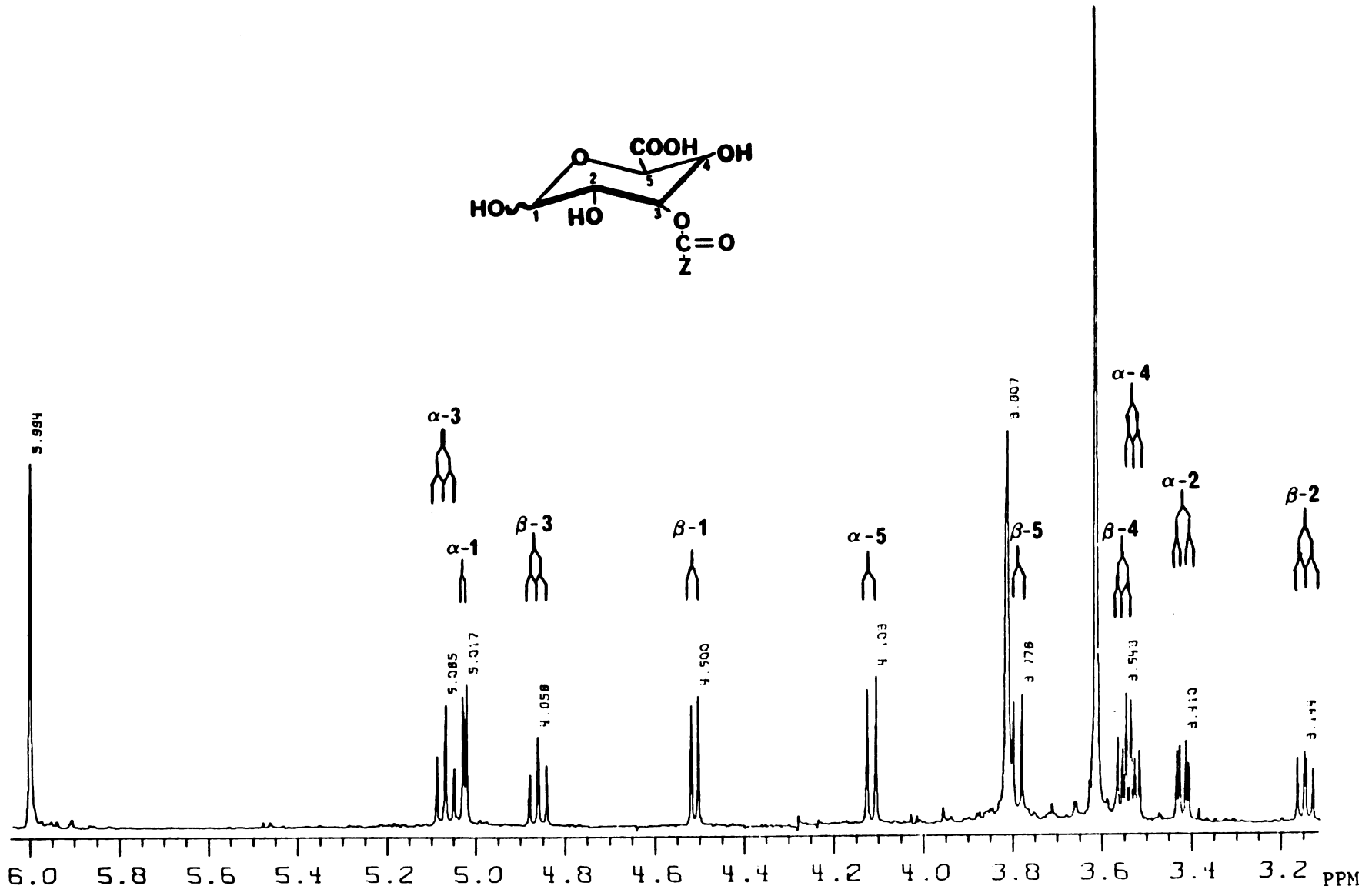


Figure II-8. ^{1}H-NMR of Zomepirac α/β -3-acyl glucuronide in DMSO-d_6/trifluoroacetic acid-d_1 at 500 MHz. Only the sugar region of the spectra is presented. Parameters for the accumulation are presented in Figure II-7.

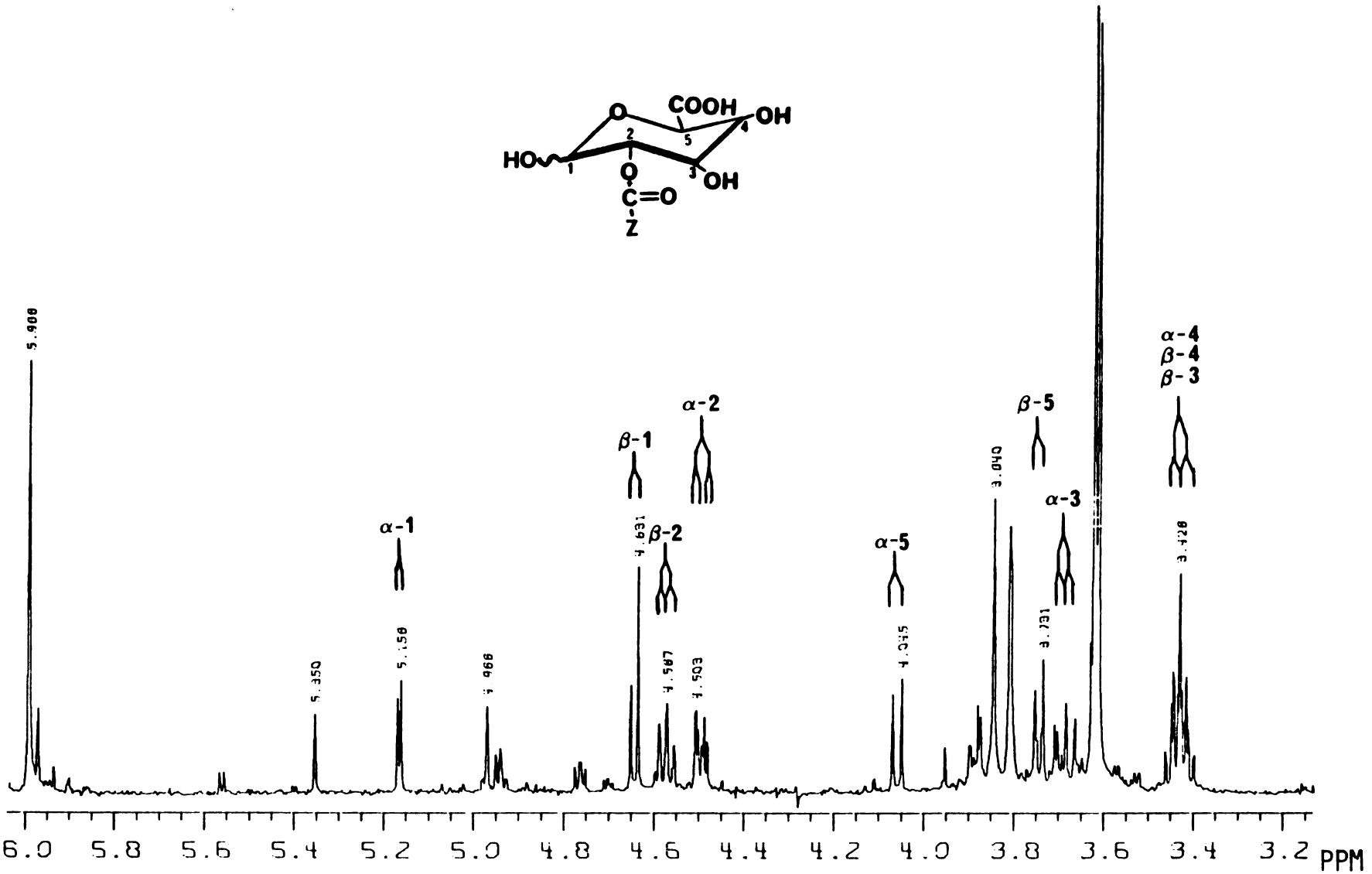


Figure II-9. ^{1}H-NMR of Zomepirac α/β -2-acyl glucuronide in DMSO-d_6/trifluoroacetic acid-d_1 at 500 MHz. Only the sugar region of the spectra is presented. Parameters for the accumulation are presented in Figure II-7.

Table II-1. **Proton chemical shifts[a] of glucuronic acid in zomepirac glucuronide (ZG), the 2-, 3-, and 4-O-acyl positional isomers of ZG.**

Isomer	anomer	H-1	H-2	H-3	H-4	H-5
β-1-ZG/ pyridine[b]	β	6.502 (8.1,d)[d]	4.329 (8.5,t)	4.408 (8.9,t)	4.605 (9.3,t)	4.748 (9.7,d)
4-acyl ZG/ pyridine[b]	β	5.442 (7.8,d)	4.240 (8.4,t)	4.483 (9.3,t)	6.08[e] (m)	4.733 (10.0,d)
	α	5.997 (3.5,d)	4.329 (3.6,dd) (9.5)	4.931 (9.2,t)	6.08[e] (m)	5.413 (10.3,d)
β-1-ZG/ DMSO + TFA[c]	β	5.466 (8.3,d)	3.193 (8.4,t)	3.299 (8.9,t)	3.362 (9.6,t)	3.792 (9.5,d)
3-acyl ZG/ DMSO + TFA[c]	β	4.508 (7.7,d)	3.142 (8.8,t)	4.856 (9.3,t)	3.54[e] (m)	3.786 (9.8,d)
	α	5.021 (3.4,d)	3.417 (3.4,dd) (9.9)	5.065 (9.9,t)	3.54[e] (m)	4.111 (9.9,d)
2-acyl ZG/ DMSO + TFA[c]	β	4.639 (8.0,d)	4.567 (8.3,t)	3.43[e] (m)	3.43[e] (m)	3.738 (7.2,d)
	α	5.162 (3.5,d)	4.494[e] (dd)	3.680 (9.6,t)	3.43[e] (m)	4.055 (9.9,d)

[a] Ppm from tetramethylsilane for solutions in pyridine-d_5, and referenced indirectly via DMSO (δ=2.500) for solutions in DMSO-d_6 with trifluoroacetic acid (TFA) added.

[b] Solution in pyridine-d_5 at 360 MHz. [c] Solution in DMSO-d_6 with TFA-d_1 added at 500 MHz.

[d] In parentheses (coupling constant in Hz, signal multiplicity). Signal multiplicities: d, doublet; t, triplet; dd, doublet of doublets; m, multiplet.

[e] Incompletely resolved multiplet.

of trifluoroacetic acid. This resulted in a more complex mixture of two sets of five proton spin system. The addition of the acid produced no noticeable degradation of ZG during the time of the NMR accumulation and subsequent HPLC analysis confirmed the stability. Identification was facilitated by the unique coupling of the α-isomer with coupling constants of about 3 HZ for protons 1 and 2 and by the characteristic downfield shift of about 1 ppm upon acylation (81). Fraction 5, whose purity was doubted because of its unsymmetrical peak shape on HPLC (Fig. II-3), did have NMR signals in the sugar regions which could not be attributed to either glucuronic acid or Z (Fig. II-9). The high field NMR analysis supported the following structural assignments: fraction 3 on HPLC, the compound originally isolated from human urine, is the natural, biosynthetic β-1-acyl glucuronide; fractions 1, 4 and 5 are the anomeric mixtures of the 4-, 3- and 2-O-acyl positional isomers of ZG, respectively, which are formed by sequential acyl migration of ZG.

D. DISCUSSION

Purification of ZG from human urine was first attempted using ethyl acetate extraction, then Sephadex chromatography as reported by Wu *et al.* (49). Low yields following the Sephadex procedure prompted trials of directly precipitating ZG from the residue of ethyl acetate extraction which were successful. ZG is the primary metabolite of Z in human urine accounting for most of the 200 mg dose excreted in the urine, which probably contributed to the favorable results obtained using our method. Attempts to derivatize ZG with TMS for GC and GC-MS analysis failed, although this method of derivatization has been employed by others for analyzing acyl glucuronides (66, 69, 72).

Mass spectrometry (Fig. II-4) and elemental analysis provide

evidence that the isolated fraction 3 is a glucuronide conjugate of Z. The melting point, 152 - 153 °C, is similar to that obtained by Wu et al., 146 - 148 °C (49). The colorimetric reactions also support and identify the presence of glucuronic acid. Hydrolysis of fraction 3 by β-glucuronidase and inhibition of the enzymatic hydrolysis by saccharic acid 1,4-lactone define the linkage of Z with glucuronic acid as being β-1 (78). Blanckaert et al. (44) found that for glucuronide isomers of bile azopigments only the β-1-glucuronic acid linked isomer was susceptable to β-glucuronidase, which agrees with our findings. Fractions 1, 4 and 5 were not cleaved by the enzyme and fraction 2 was too labile to perform an incubation with the enzyme at pH 5. Low field ^{1}H-NMR supports the structural proof, as the coupling constant, J=7.3 Hz, is characteristic for the β-anomer of sugars (82, 86). The high field NMR, though not absolutely necessary to prove that fraction 3 was the β-1-conjugate, confirmed the low field NMR data and provided a standard to compare the NMR spectra of the isomeric conjugates. Also the expected (81) one ppm downfield shift for the β-1-proton relative to glucuronic acid agrees with that reported by Yoshimoto et al. (82) for mono-O-acylglycopyranoses.

The isomeric conjugates were produced from the biosynthetically obtained ZG which itself was purified from human urine. Cleavage of the isomeric conjugates was not possible with β-glucuronidase, but they did yield Z upon alkaline hydrolysis. In addition to acyl migration of ZG to form positional isomers, anomerization of the sugar is possible, which Hignite et al. (66) hypothesized to occur without prior acyl migration. Caldwell et al. (64) have also suggested that conversion of glucuronic acid to furanose forms or formation of a 3,6-lactone are

possible. Thus, these reactions may be confounding absolute identification of the products formed from the β-1-conjugate under mild alkaline conditions. Although there may be a large number of possible isomers if all of the above reactions take place, the identification of the products formed should provide support for the possible mechanism resulting in the observed loss of the β-1-ZG.

Mass spectrometry of the labile underivatized ZG and isomeric conjugates using a FAB ion source did provide excellent data, as exemplified by Figure II-4, which established that the fractions from preparative HPLC were indeed isomers of ZG. However, all of the isomers as well as ZG yielded qualitatively similar mass spectra and positional information was lacking using the presently employed FAB-MS method. Whether the linkage of glucuronic acid to the aglycone is through a C-, N-, S-, phenol, alcohol or acyl functional group can often be distinguished based upon fragmentation obtained by either electron impact-MS (87), FAB-MS (88), or laser desorption time-of-flight-MS (89). However, the isomeric conjugates of ZG all contain an acyl linkage and would be expected to produce similar fragmentation even if the glucuronide were derivatized with trimethylsilyl groups (87). The more labile nature of the 1-O-acyl isomer compared to the other positional isomers, due to the influence of the anomeric oxygen of the sugar, might be expected to cause a qualitative difference in the extent of cleavage occurring at the ester linkage of the sugar and aglycone. In practice, no difference in the FAB-MS was seen between ZG and its isomeric conjugates. Proton NMR with homonuclear decoupling provided the desired information on isomeric position of the acyl group, but relatively large quantities of the conjugates, about 1 mg each, were needed to do successive decoupling

experiments on NMR within a reasonable length of time. Two-dimensional NMR which can also provide information on coupling constants and connectivities of complex sugar proton spin systems (85) was not required for this structural analysis.

Acyl migration has only been recognized recently by investigators in the area of drug metabolism and biology. In the literature the proof or support for acyl migration of glucuronic acid metabolites of drugs or endogenous compounds varies considerably, from full isolation and identification of the isomeric products formed, as presented here for ZG, to the simple observation of time dependent formation of β-glucuronidase resistant conjugates in urine. Compernolle et al. were first to unequivocally document the occurrence of acyl migration by identifying the isomeric products of bilirubin-IXα monoglucuronide using a complex scheme of chemical derivatization and finally mass spectrometry of the partially methylated alditol acetates (7, 44,61). However, most reports of acyl migration have not included such a rigorous experimental proof. The experiments carried out by most investigators attempting to validate acyl migration include: i) observation of unidentified peaks (TLC, HPLC or GC) under mild alkaline conditions when the putative β-1-glucuronide is incubated in urine or perhaps bile; ii) proof of the β-glucuronidase resistance of the putative isomers formed in (i) above; iii) observation of positive colorimetric reactions specific for glucuronic acid by the putative isomers formed in (i) above; and sometimes, iv) mass spectrometry of the putative isomers. Full structural proof of the isomeric conjugates formed, as presented here for ZG, is not always possible, and may not be necessary. An understanding of the phenomenon and the use of procedures (i) through (iv) would be sufficient for most

investigations of drug metabolism of acidic compounds which form acyl glucuronides. These procedures would be adequate to distinguish between products of acyl migration and other possible conjugates such as conjugates of sulfate, glycine, taurine or other sugars (65).

Since migration has been only recently documented as a potential problem for drugs and endogenous compounds metabolized to acyl glucuronides, one may question how many studies in the past literature have been confounded or in error because of this potential problem. Caldwell, who has studied conjugation reactions for many years, discussed a debate in the 1930's which centered around whether benzoic acid produced 1- or 2-O-acyl glucuronides (64). Caldwell et al. also suspect that acyl migration had occurred for many acidic compounds which in previous studies yielded greater parent drug when hydrolyzed by mild base than by β-glucuronidase (64, 65).

Predicting whether acyl migration will occur for a particular compound is not possible at present. From a review of acyl migration in the carbohydrate literature (32), several factors which affect the rate of acyl migration may be summarized. Of course, pH is one critical parameter because the reaction is catalyzed by base or acid. The effect of pH on acyl migration has been recognized in all of the previous reports for drugs and will be discussed for ZG in Chapter IV. At higher pH, acyl migration must compete with direct hydrolysis of the β-1-glucuronide. Once the isomers are formed, they are also cleaved by base, however, at a slower rate (see Chapter IV). With the postulated mechanism for acyl migration through the ortho acid intermediate (Fig. II-2), the cabocation character of the carbonyl should be an important determinant of the rate of migration. Studies have shown that the

relative rate for the migration of benzoylated sugars increases with the electron withdrawing nature of the group, such that 4-nitrobenzoyl migrated 20 fold faster than benzoyl (32). The stereochemistry of the adjacent groups also affects the rate, with a cis acyl group migrating to the neighboring hydroxyls faster than trans isomers. β-1-Acyl glucuronides are trans, however, upon migration and anomerization of the 1-position; the α-2-acyl isomer has the cis configuration. With the exception of probenecid and diflunisal, all of the drugs reported for acyl migration have been alkyl acids which would be expected to have similarly reactive acyl groups. This may simply result from the fact that NSAID's represent a large portion of the research effort presently ongoing for acidic drugs or it may be due to the fact that alkyl acids have the proper electronic environment at the carbonyl which favors acyl migration relative to hydrolysis at physiological pH. At present, most data on the rates of acyl migration as a function of chemical structure are found in the carbohydrate literature. This literature contains little information about acyl migration in aqueous solutions as found in biological systems.

CHAPTER III

SIMULTANEOUS DETERMINATIONS OF ZOMEPIRAC AND ZOMEPIRAC GLUCURONIDE IN PLASMA AND URINE USING LIQUID CHROMATOGRAPHY

A. INTRODUCTION

Zomepirac (Z) is a nonsteroidal anti-inflammatory drug (NSAID) with analgesic effects (90). Its major metabolite in man is the glucuronic acid conjugate, zomepirac glucuronide (ZG) (26, 49) (Fig. III-1). Previous reports have shown that ZG is present in human plasma at concentrations similar to the parent drug after a dose of Z (25, 26). Our interest in studying the disposition of ZG in human and animals led to the development of an analytical method for simultaneous direct measurement of intact ZG and Z in plasma and urine.

Several methods have been published for the determination of Z alone in plasma and urine. Normal phase (91), reversed phase (92, 93, 94, 95), TLC followed by GC-MS (96), and GC with electron capture detection of Z-pentafluorobenzylester (97) have been employed for analysis of Z. Only one method included a direct analysis of ZG, however, this was limited to urine measurements only (95). Following oral dosing of ^{14}C-labeled Z, ZG has been measured in plasma and urine following TLC separation (25, 26). None of the above methods considered the possibility of ZG degradation which was found by us to be significant in urine and plasma even at physiological pH (98) (Chapter IV). Not only do all except one of the previous methods not measure ZG, but because of ZG instability, they may lead to overestimats of free Z concentrations.

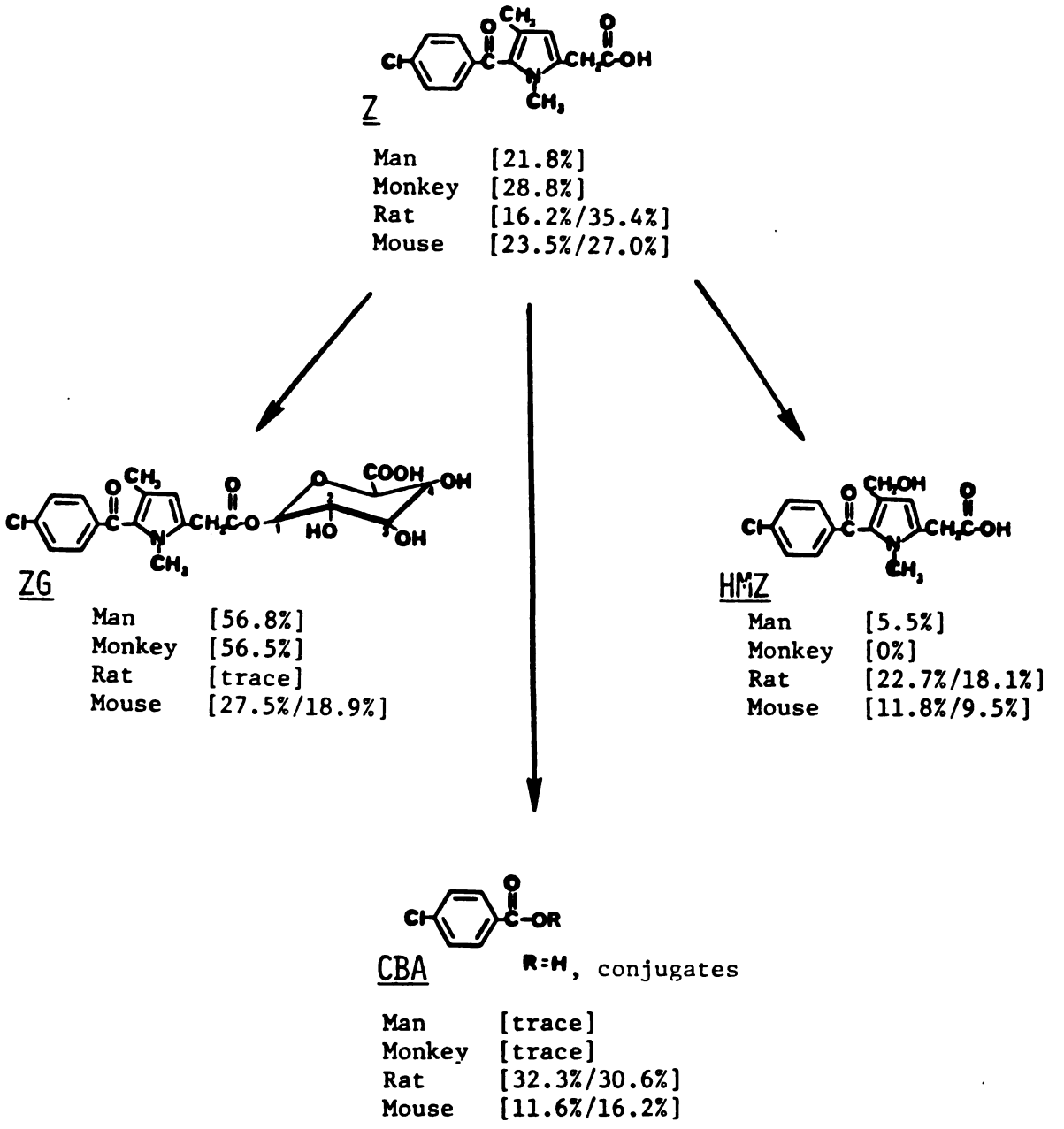


Figure III-1. Summary of known biotransformation products of zomepirac (Z) in human, rhesus monkey, rat and mouse. Reproduced from a review by Muschek and Grindel (Ref. 99). Values in brackets indicate the percent of radiolabelled compound excreted in the urine. (Rat and mouse values are male/female). Abbreviations: zomepirac glucuronide, ZG; hydroxyzomepirac, HMZ; p-chlorobenzoic acid, CBA.

In addition to simple hydrolytic cleavage of ZG to Z, Chapter II documents the problem of intramolecular acyl migration of ZG; the kinetics of which will be discussed in Chapter IV. The phenomenon of acyl migration has only been recognized as a problem for acyl glucuronides since the first report by Compernolle *et al.* (44, 61) in 1978 for bilirubin glucuronide. Subsequently, studies have suggested or proven acyl migration for clofibric acid (64), probenecid (67), isoxepac (68), an investigational NSAID from Wyeth (69), diflunisal (70, 71), and valproic acid (72). The isomeric products formed by acyl migration can have different LC retention times as shown for ZG in Chapter II and as observed for the compounds listed above. No specific attempt was made to resolve the isomeric conjugates during the development of the present LC method, although partial resolution occurred. An objective for analysis of human plasma is to attain as sensitive an assay as possible. Resolution of all of the individual isomers as was done in Chapter II would decrease the sensitivity of the assay. For the present application, ZG and its isomers are simply combined as the total conjugates in our analysis.

The disposition of labile acyl glucuronides was of interest and ZG appeared to be a good model compound as it does reach appreciable levels in human plasma (25, 26). Also, sufficient quantities of biosynthetically derived ZG could be isolated and purified from human urine (Chapter II) for *in vitro* and *in vivo* animal studies. None of the known metabolites of Z (Fig. III-1), including ZG, have been reported to be pharmacologically active or more toxic than the parent drug. Since levels of ZG were similar to those for Z in human, the assay for ZG should be as sensitive as the previous assays reported for Z. A minimal detect-

able concentration of 50 ng/ml would allow detection of Z and ZG in the plasma for up to 24 hours following a normal therapeutic dose (100 mg). This measurement time is greater than 3 half-lives of the drug (2B2, 2B3) and therefore should provide accurate measurement of greater than 90% of the total area-under-the-plasma-concentration vs. time curve (AUC).

Although glucuronidation is the major metabolic route for Z in human (25, 26, 50) and rhesus monkey (26), with subsequent excretion of most of the dose as ZG in urine; other animal species show much different metabolic profiles for Z metabolism (26, 49). A summary of the metabolic pathways for Z in animals and human is presented in Figure III-1 which was taken from the work of Muschek and Grindel (99). Not included in the figure are the rabbit and hamster data from Grindel et al. (26) which found little ZG in the urine of either animal species. Rabbit and hamster excreted a ^{14}C-labelled dose of Z into the urine primarily as free Z and hydroxyzomepirac (HMZ), respectively. The measurement of ZG or Z in both plasma and urine using the analytical methods reported previously is now in doubt, because few of those original experiments included adequate precautions to prevent loss of ZG by either hydrolysis or acyl migration. The stability problem for ZG will be discussed in this Chapter with regard to analytical development; in Chapter IV the stability of ZG in biological fluids and in tissue preparations will be characterized.

B. MATERIALS AND METHODS

Chemicals

Zomepirac sodium $\cdot 2H_2O$ and 5-(4-methoxybenzoyl)-1,4 dimethyl-1-H-pyrrole-2-acetic acid, the methoxy analog of zomepirac used as the internal standard (IS) were kindly supplied by McNeil Pharmaceutical (Springhouse, PA, USA). Desmethylindomethacin (DMI) was obtained synthetically from indomethacin (100) using the method developed by Witzel (101, 102). ZG was obtained by extraction and purification from human urine as described in Chapter II (28). Methanol, acetone, acetonitrile, tetrahydrofuran (THF), ethyl acetate and dichloromethane were HPLC grade from J.T. Baker Chemical Co. (Phillipsburg, New Jersey). All other chemicals were reagent grade. Distilled water was purified by charcoal/ion/cation exchange (Nanopure®, Barnstead, Boston, MA) prior to use.

Developmental studies

Reversed phase HPLC was the method of choice for the analysis of Z and the labile ZG for several reasons: 1) Z absorbs in the UV with ε=11,700 at 313 nm (91) (Fig. III-2); 2) sample preparation for HPLC can be optimized to minimize loss of labile ZG; 3) sample preparation for HPLC is often easier and faster than with other methods; and 4) Z and ZG can be analyzed simultaneously by HPLC. Methanol, acetonitrile and tetrahydrofuran (THF), which are considered as the organic modifiers of first choice for reversed phase HPLC (103, 104), were used for the initial screening of Z and ZG chromatography. Preliminary tests with varying solvent composition and pH of the mobile phase indicated that only methanol provided suitable selectivity between Z, ZG and the

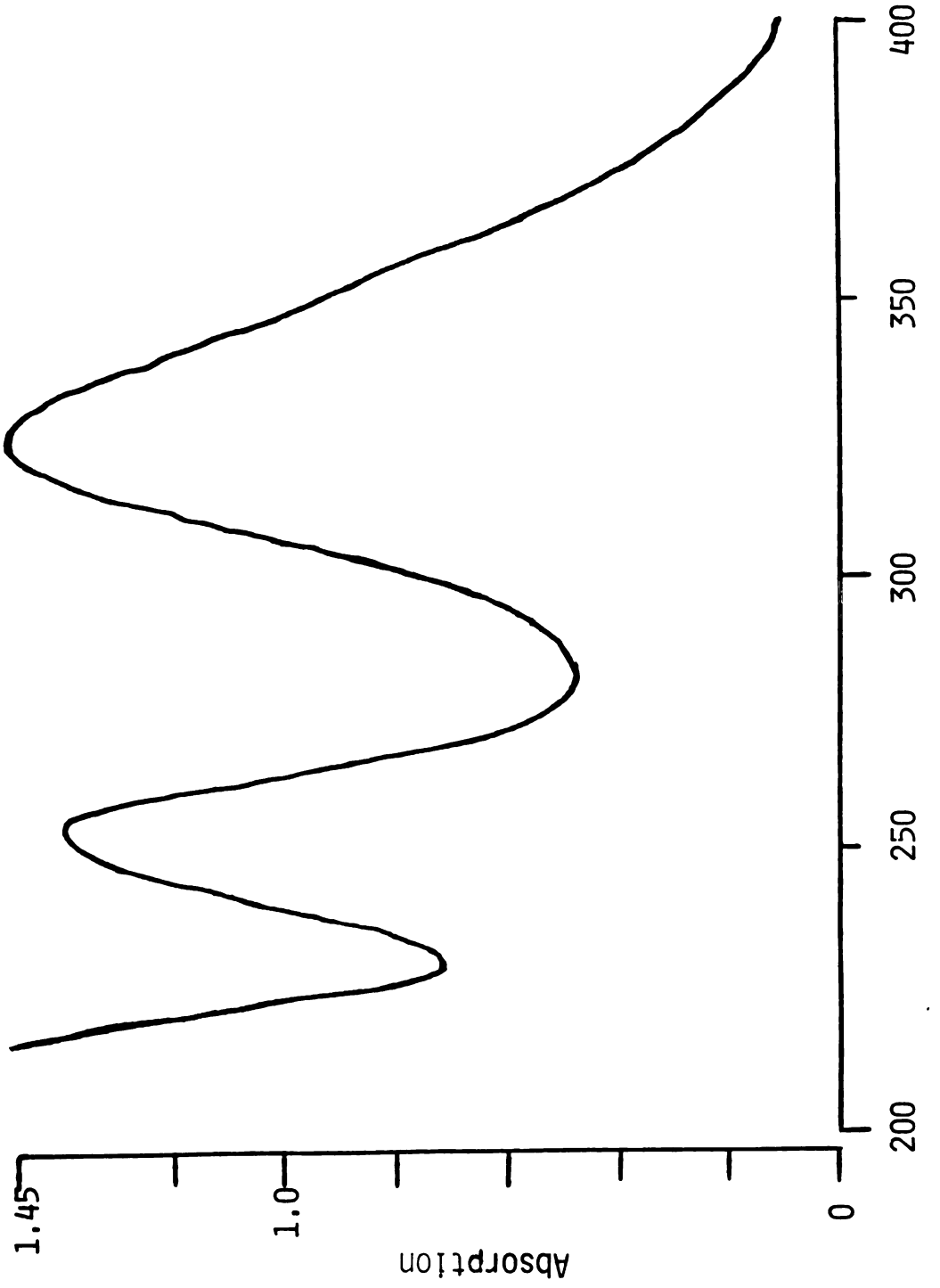


Figure III-2. UV spectrum of zomepirac in methanol. Concentration is 10^{-4} M.

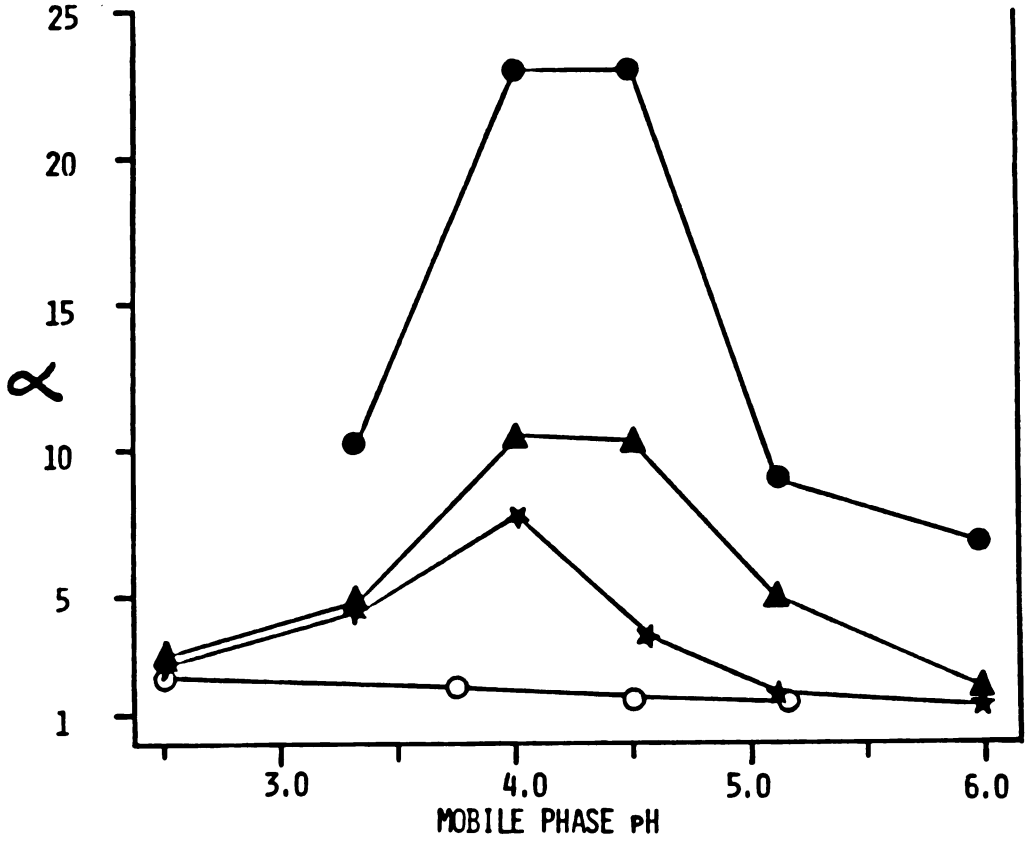


Figure III-3. Selectivity, α, (Z/ZG) as a function of pH and mobile phase organic modifier. Mobile phases used were 40% acetonitrile, ● ; 30% tetrahydrofuran, ▲; 50% methanol, ★; and 58% methanol-ion pair, O . Ion pair reagent was 0.01 M tetrabutylammonium sulfate/0.05 M acetate buffer. All other buffers were 0.01 M acetate. Column conditions for HPLC are described in Methods.

internal standard (Fig. III-3), where selectivity, α, is defined (105) as the ratio of the capacity factors (K') for Z to ZG. Further tests were carried out with methanol combined with ion-pair reagents to obtain the most optimal mobile phase. Previous investigations in our laboratory (28) (Chapter IV), showed that ZG was most stable at pH's between 2 and 5. Because of this and the more favorable selectivity found with the internal standards available, the mobile phase was chosen to be 58% methanol/0.01 M tetrabutylammonium hydrogensulfate and 0.05 M sodium acetate at pH = 4.5. Since acetonitrile/water enhanced the stability of ZG while methanol/water caused loss of ZG to yield Z methyl ester (28) (Chapter IV), acetonitrile/water was used as the solvent in which samples were reconstituted prior to injection. The short retention time on the column (< 10 min) when using the methanol/ion-pair mobile phase caused no significant loss of ZG due to the formation of the methyl ester.

In previous assays for Z in which the unchanged drug was extracted from biological fluids prior to analysis (91, 94, 96, 97), no mention was made of the stability of ZG or its extraction efficiency during the procedures. For the analysis of ZG in plasma following a ^{14}C-labelled Z dose the extraction efficiency with ethyl acetate was only 50% (25). The extraction efficiency of Z and ZG from water, human urine and plasma was examined using dichloromethane, ethyl acetate and ether. To a 10 ml screw top tube was added: 0.2 ml buffer (for urine or water, 0.5 M KH_2PO_4, pH 2.25; for plasma, 1.0 M H_3PO_4) followed by water, urine or plasma (0.4 ml), and finally ca. 4 μg Z or ZG was added. Five ml of organic solvent was then added, the tube was capped and shaken for 15 min on a flatbed shaker (ca. 60 strokes/min). After centrifugation

(2250 x g) for 10 min, 4.0 ml of the organic phase was removed, evaporated to dryness with under a nitrogen stream at 30-40 °C, then 4 µg DMI in 0.5 ml 50% acetonitrile/0.01 M H_3PO_4, pH 2.5, was added as an external standard. After vortexing, aliquots of the extract were injected onto the HPLC where Z and ZG were quantitated by peak area relative to DMI. Samples of Z and ZG served as the controls; i.e. representing 100% extracted. The results of the trial extractions were never satisfactory for both Z and ZG from plasma and urine for any of the extraction solvents tested (Table III-1). The criterion used was a minimum extraction efficiency of 70% for both Z and ZG in both plasma and urine with variability (coefficient of variation, CV) below 10%. Because the extraction methods were considered unacceptable, direct precipitation of the proteins from the plasma was attempted with several organic solvents (106). Satisfactory results were obtained when the protein pellet from the first precipitation of plasma by acetone or acetonitrile was washed a second time. The final method employed (107) is described below.

Sample preparation

A stock solution of zomepirac sodium $\cdot 2H_2O$ was prepared by dissolving 100 mg into 100 ml methanol. By appropriate dilution with methanol, standard solutions of 100 µg/ml and 10 µg/ml were also obtained. A stock solution of ZG was prepared by dissolving 25 mg of this compound into 25 ml 50% acetonitrile/0.01 M phosphate, pH 2. By appropriate dilution with 50% acetonitrile/0.01 M phosphate, pH 2, standard solutions of 100 µg/ml and 10 µg/ml were prepared. A stock solution of IS was made by dissolving 50 mg of this compound into 100 ml

Table III-1. Extraction efficiency of zomepirac (Z) and zomepirac glucuronide (ZG) from water, human urine and human plasma using the solvents ethyl acetate, ether and dichloromethane.

	Extraction Efficiency[a] (%)					
	Ethyl acetate		Ether		Dichloromethane	
	Z	ZG	Z	ZG	Z	ZG
Water	81 (6.8)	77 (7.1)	101 (6.7)	84 (3.6)	85 (12)	8.1 (70)
Urine	87 (4.3)	88 (3.3)	92 (0.7)	76 (2.5)	88 (2.8)	14 (2.2)
Plasma	50 (7.2)	65 (10.6)	88 (4.3)	42 (5.4)	85 (8.3)	ND[b]

[a]Mean ($n \geq 4$), coefficient of variation (CV) are in parentheses.

[b]ND = none detectable.

acetonitrile. All stock and standard solutions were stored at -20 °C. Standard curve samples at concentrations of 0.1, 0.3, 0.6, 1.0, 5.0, and 10.0 μg/ml for both Z and ZG with a concentration of 3.0 μg/ml for IS were prepared in human plasma. Standard curve samples with concentrations of 0.1, 0.3, 0.6, 1.0, 5.0 and 10.0 μg/ml for Z, 1.0, 5.0, 10.0, 50.0, 100.0, and 200.0 μg/ml for ZG and a concentration of 10.0 μg/ml for IS were prepared in human urine. The standard curve samples were prepared with blank human plasma or urine, buffered to pH 2-4 with phosphoric acid and spiked with a small volume (2.5-100 μl) of the appropriate dilution of the standard solutions.

Each aliquot of 0.5 ml plasma or urine to be analyzed was spiked with the internal standard (plasma: 3 μg/ml IS; urine: 10 μg/ml). Protein precipitation was done by addition of 1.0 ml acetone or acetonitrile followed by 30 sec of mixing on a vortex mixer. The precipitate was separated by centrifugation (2250 x g) for 10 min. Supernatant was removed into a clean tube and evaporated under a gentle stream of nitrogen at 30 °C. In the case of plasma, the protein pellet from the first precipitation was not discarded, but was once more treated with 1.0 ml acetonitrile or acetone, vortexed and centrifuged. The second supernatant was combined with that from the first sample treatment. Residue upon evaporation was reconstituted in 0.25-1.0 ml 25% acetonitrile/0.5 M acetate, pH = 4.5; then 50-100 μl was injected onto the HPLC column. Sample preparation and chromatography were done on the same day.

Special precautions in sample handling were taken because of the fast degradation of ZG at higher temperatures and pH (28, 98) (Chapter IV). During clinical trials, blood samples were immediately cooled in

ice after collection, red blood cells were separated from plasma in 10 min using a refrigerated centrifuge (0 - 2 °C) and plasma was then transferrred into a vial containing 10 µl concentrated phosphoric acid (15 M) per ml plasma and immediately frozen at -20 °C. The elapsed time from blood sampling to buffering the plasma to pH 2-4 was kept to a minimum. In practice this interval was usually less than 15 minutes. Urine upon collection was immediately buffered with 5 µl concentrated phosphoric acid per ml urine and frozen at -20 °C. In addition, to minimize the loss of ZG which can occur in basic urine (108), volunteers drank cranberry juice ad lib. throughout the study in order to maintain urine pH below 6. When the assay was applied to animal studies where volumes of blood collected were less than 2 ml, a rapid, 30 sec centrifugation using an Eppendorf 5412 centrifuge (12,500 x g) was substituted for the cooling and refrigerated centrifugation used for larger blood samples.

Chromatography

The HPLC system used consisted of an Altex model 110A pump, a Waters model 710A Wisp automatic injector, a Waters model 440A fixed wavelength UV detector at 313 nm and an Altex Ultrasphere ODS reversed-phase column (15 cm x 4.0 mm ID, 5 µm particle size). Chromatograms were recorded on a Hewlett Packard (Palo Alto, CA) 3380A integrator programmed for area ratios relative to the internal standard. The mobile phase consisted of ion-pairing reagent with acetate buffer (58% methanol/0.01 M tetrabutylammonium hydrogensulfate and 0.05 M sodium acetate, pH = 4.5) which was prepared fresh daily, filtered and degassed by vacuum filtration through a 0.45 µm filter (Millipore Corp., Bedford,

MA). Column conditioning with approximately 50 ml mobile phase was done prior to sample injection. Mobile phase flow rate was usually 1.3 ml/min with minor adjustments (±0.1 ml/min) as column conditions changed slightly over several weeks of use. The retention times of IS, ZG and Z were approximately 4.5, 6.5 and 8.5 min, respectively (Figs. III-4 and 5).

Extraction studies used the same column as above, with a 40% methanol/0.01 M sodium acetate, pH 5.5 mobile phase similar to that employed in Chapter II. The detector wavelength was set at 340 nm. With a mobile phase flow rate of 1.6 ml/min the retention times of ZG, Z and DMI were 7.7, 9.3 and 13.2 min, respectively. Ambient temperature was used for all HPLC.

Quantitation

Area ratios of Z and ZG to the internal standard were plotted against concentrations of Z and ZG, respectively. A weighted (1/concentration) least square regression analysis was performed using a hand held calculator. Linearity was tested and confirmed in the concentration range of at least 0.05-30 μg/ml for Z and ZG in plasma and 0.1-200.0 μg/ml for Z and ZG in urine.

C. RESULTS AND DISCUSSION

Preliminary studies

Extraction of Z from plasma prior to analysis was done in all of the assays reported previously for Z when no radiolabelled drug was used. It was possible to apply plasma directly to TLC plates without prior extraction in animal studies using ^{14}C-labelled Z (26, 108). The

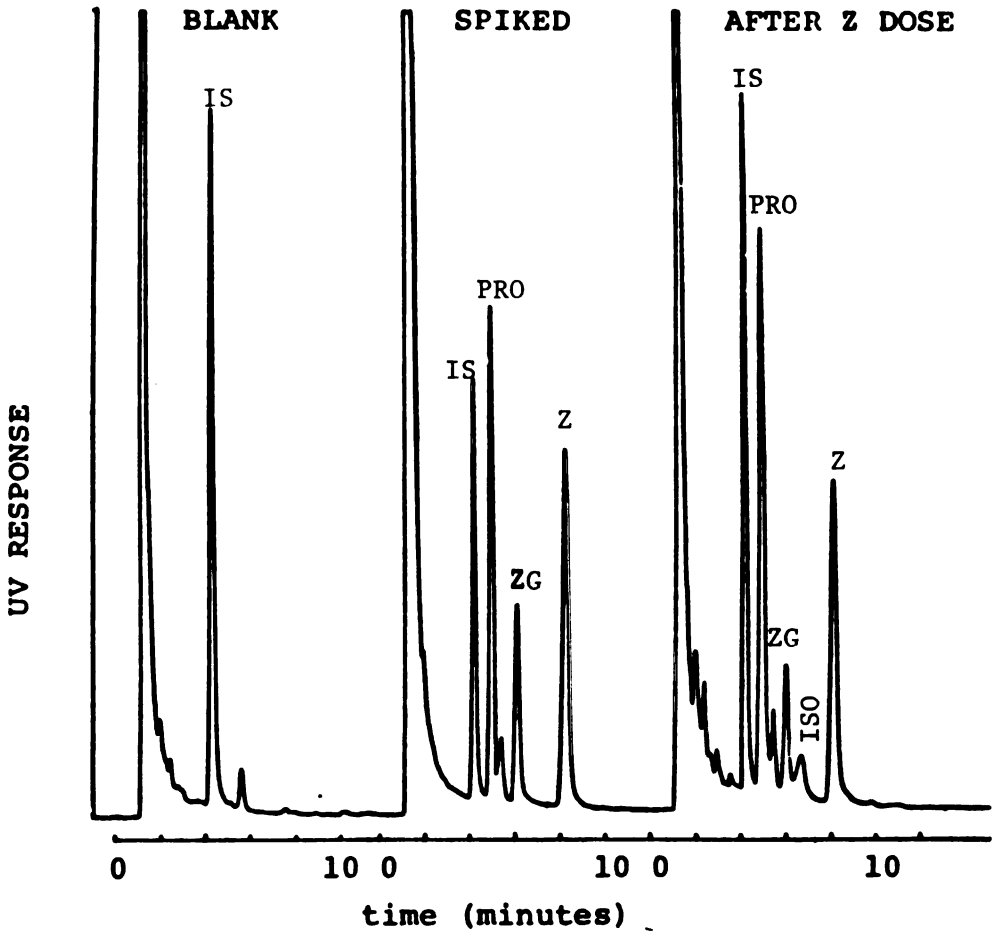


Figure III-4. Chromatograms of blank human plasma spiked with 3 µg/ml internal standard (IS), the methoxy analog of zomepirac; plasma spiked with IS and 1 µg/ml of zomepirac (Z) and zomepirac glucuronide (ZG) with probenecid (PRO); and a plasma sample from a human subject, pretreated with probenecid, one hour after an oral dose of 100 mg of zomepirac as its sodium salt. (a.u.f.s. = 0.016).

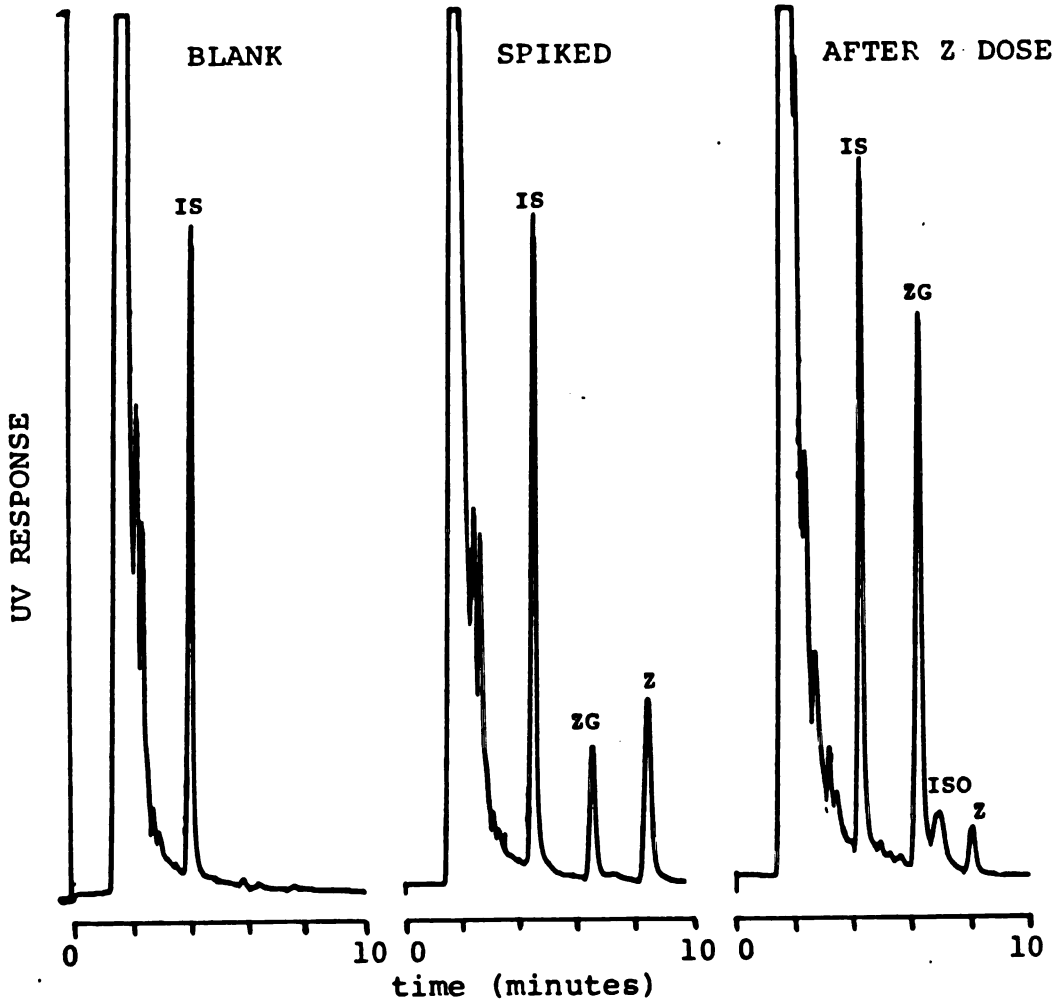


Figure III-5. Chromatograms of blank human urine spiked with 10 µg/ml internal standard (IS), the methoxy analog of zomepirac; urine spiked with IS and 5 µg/ml of zomepirac (Z) and zomepirac glucuronide (ZG) with probenecid (PRO); and a urine sample from a human subject collected between 8 and 12 hours after an oral dose of 100 mg of zomepirac as its sodium salt. (a.u.f.s. = 0.016).

extraction efficiency of ZG from acidified plasma with 10 volumes of ethyl acetate was only 50% (25). We obtained similar results for plasma samples (65%) when extracting ZG with ethyl acetate (Table III-1); however, Z was extracted less efficiently (50%). Ether which has been employed by several laboratories for removing Z from plasma provided 88% extraction efficiency for Z with our method, but a poor recovery for ZG, i.e. 42% efficiency. Both Z and ZG partitioned favorably from water or urine such that the compounds were well extracted using the moderately polar solvents, ether and ethyl acetate (Table III-1). As expected, the less polar solvent dichloromethane, was a poor choice for removing ZG from water, urine and plasma. Using a criterion of at least 70% recovery by extraction for both Z and ZG, none of the three solvents examined was satisfactory for plasma.

Although direct precipitation of proteins from plasma normally does not provide as clean an analytical sample as extraction methods, it is an alternative approach for sample preparation in the analysis of polar compounds which cannot be extracted. Simple removal of plasma proteins prior to direct HPLC analysis has become increasingly popular because of the ease and speed of sample preparation. The many possible methods and their respective efficiencies in removing proteins from plasma were recently reviewed (106). Because ZG can react with methanol to form Z methyl ester (28) (Chapter IV) as was noted for bilirubin acyl glucuronide (47), the aprotic, water miscible solvents, acetone and acetonitrile, were tried first. Acetone has more favorable volatility and did prove to be acceptable for the analysis of Z and ZG, however, it produced a quite flocculant precipitate which did not yield as clean an analytical sample as acetonitrile. With either solvent, a second wash

Table III-2. Intraday variablility of HPLC analysis for zomepirac (Z) and zomepirac glucuronide (ZG) in plasma and urine at low, medium and high concentrations of the standard curves. (n = 6).

	Concentration (μg/ml)	C.V. (%) Z	ZG
Plasma	0.1	10.2	14.8
	1.0	12.4	11.8
	10.0	3.72	2.53
Urine	0.5	2.92	1.50
	5.0	0.45	0.40
	50.0	2.01	1.61

Table III-3. Interday variability of the standard curves for HPLC analysis of zomepirac (Z) and zomepirac glucuronide (ZG). (n = 6).

	Mean slope	S.D.	C.V. (%)
ZG	5.38	0.359	6.67
Z	3.82	0.228	5.98

of the first protein pellet enhanced the recovery slightly and decreased the variability.

The plot of solvent selectivity, α, of Z/ZG separation vs. pH as shown in Figure III-3 yielded a noticable hump at about pH 4.0. The pK_a of Z is 4.7 (108) and estimated to be about 3.5 for ZG (109). The hump results from the pK_a difference between Z and ZG. Use of the ion-pair reagent, tetrabutylammonium chloride, eliminated the rise in selectivity at pH 4.0. A plausible mechanism for this effect is that the higher pK_a compound, Z, which is predominately unionized at pH 4.0, does not form ion pairs; while ZG which is predominantly ionized at this pH forms an ion pair which increases its retention on HPLC (110).

From the preliminary studies, optimal conditions for sample preparation and HPLC were determined. The results obtained with the conditions selected are presented below.

Recovery and variability

Recoveries were estimated by calculating the ratio of the slopes of standard curves obtained by protein precipitation with acetonitrile, to those obtained from untreated aqueous standard curves for three independently prepared sets of standards. Recoveries were 74 ± 5% (mean ± s.d.) and 82 ± 7% for ZG and Z, respectively, in plasma over the concentration range of 0.1 - 10.0 μg/ml. In urine the recoveries were 90 ± 3% and 95 ± 2% for ZG and Z, respectively over the concentration range of the urine standard curves.

Intraday variability was calculated from the area ratios at three different concentrations: 0.1, 1.0 and 10.0 μg/ml in plasma and 0.5, 5.0 and 50.0 μg/ml in urine for both Z and ZG. The results are shown in Table III-2.

Interday variability is given in Table III-3, where the mean slopes of six, independently prepared, standard curves for Z and ZG in plasma are given together with the standard deviation and variability. These standard curves were prepared over a two month time period.

Degradation and stability

Precautions undertaken to maintain an acidic urine pH, and the immediate further acidification of plasma and urine samples together with the work-up procedure minimized the degradation of ZG to less than 4% in spiked samples due to sample handling, storage and work-up. However, analysis of clinical samples from human subjects receiving Z orally (Chapter VI) yielded greater fractions of the degradation products from ZG. This could only result from degradation of ZG to the acyl migration products under the apparent *in vivo* physiological conditions. This degradation was accounted for by measuring the peaks of isomers (ISO, Figs. III-4 and 5) and ZG as the total conjugates. Degradation of acyl glucuronides is not an unusual phenomenon among NSAID's (6, 68, 69, 71), nor among other drugs that are extensively conjugated to acyl glucuronides (44, 64, 67 72). However, degradation of acyl glucuronides *in vivo* has not been extensively documented, though Faed and McQueen (22) did find β-glucuronidase resistant conjugates of clofibric acid in human plasma and recently Meffin et al. (111) found that clofibric acid acyl glucuronide was rapidly cleaved *in vivo* when given intravenously to the rabbit. There is also evidence that acyl migration of bilirubin glucuronide (44, 61) occurs in the plasma of cholestatic patients as shown by the appearance of β-glucuronidase resistant bilirubin conjugates in plasma (112).

No noticeable degradation of Z occurred during sample handling, storage and sample preparation. In addition, the stock solutions of Z did not show significant changes in content over a period of five months. A small loss of ZG did occur in the stock solutions (3.2%) over a three month period. A correction for this loss was made in each batch of samples from the human clinical study that were analyzed (Chapter VI). The stock solution of the internal standard, IS, also showed a slight degradation of approximately 2% per month. This, however, did not influence the assay since a fresh internal standard solution was prepared for each set of human plasma samples that were analyzed.

Application

This assay has been used successfully in a clinical study that investigated the influence of a steady state drug level of probenecid on Z pharmacokinetics (113) (Chapter VI). Probenecid did not interfere with the plasma assay and could be measured together with Z and ZG (Figs. III-4 and 5). Plasma levels of probenecid were in the range of 20-100 μg/ml, much higher than Z or ZG. Fortunately, because of a significantly lower molar extinction coefficient for probenecid at 313 nm as compared to Z and ZG, the probenecid peak heights were of the same order of magnitude as those for Z and ZG. No interference peaks due to endogenous compounds were observed with the clinical samples. In Figure III-4 typical plasma concentration time profiles of Z and ZG with concomitant probenecid dosing are illustrated for one human subject. Plasma concentration time profiles of Z and ZG after ingestion of 100 mg Z with and without probenecid could be followed up to 25 hr after Z administration. In all cases this part of the AUC accounted for more than 95% of the total.

Probenecid did cause problems with the analysis of urine. Putative metabolites of probenecid in urine were eluted before probenecid and interfered with the IS measurement. When this occurred, quantitation was done by normalizing peak areas with constant injection volumes. When probenecid metabolites did not interfere with the IS, the use of constant injection volumes produced reproducible IS areas sufficient for quantitation. The simplicity of the sample preparation for urine and the absence of protein in urine probably account for the reliability of this method of quantitation.

Application of the assay method to plasma samples from guinea pig, rabbit and rat produced similar results as those observed for human plasma samples. The assay was adjusted for the use of smaller sample sizes, 0.1 and 0.2 ml. Analytical sensitivity was less with decreased sample size, however, this was not problematic as animals received higher Z doses (when normalized to body weight) than did human subjects.

Acyl migration

This direct injection assay method for Z and ZG has great advantages over previous methods. Although in a recent publication ZG and Z were analyzed simultaneously in urine, no special precautions were taken prior to analysis to prevent loss of ZG (95). These authors suggested that an anomolous peak in urine might be the putative hydroxylated metabolite of Z. We believe it likely that this peak results from an isomeric conjugate of ZG due to acyl migration. The fact that ZG is degrading, forming other isomers (not susceptible to β-glucuronidase) prior to liberation of the Z (28), means that estimation of the glucuronide loss simply by following formation of the free Z will

result in error. To what extent the degradation of acyl glucuronides is affecting the results of clinical studies with drugs metabolized to such labile compounds is not known, because in most cases no detailed information is given about sample handling or stability of the metabolite prior to analysis. The importance of this information is emphasized in this assay and should be considered for further assays of other NSAID's, since degradation of acyl glucuronides is probably a common occurrence among these drugs.

CHAPTER IV

STABILITY OF ZOMEPIRAC GLUCURONIDE AND ITS ISOMERIC CONJUGATES IN BIOLOGICAL FLUIDS AND TISSUE HOMOGENATES

A. INTRODUCTION

Early studies from our laboratory documented the instability of the acyl glucuronide of zomepirac (Z), 5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid, in urine and water when the solution pH was greater than 5 (28). In solutions with a pH between 5 and 9, degradation of zomepirac glucuronide (ZG) is primarily due to intramolecular acyl migration which produces isomeric products which exhibit different retention times upon liquid chromatographic separation (28) (Chapter II). Acyl migration of ZG which was addressed in Chapter II has now been reported for many acidic drugs: clofibric acid (62, 64), isoxepac (68), probenecid (67), Wyeth #18,251 (69), diflunisal (70, 71), valproic acid (72), and also for the endogenous conjugate of bilirubin (7, 44, 61) In order to study ZG disposition it is necessary to develop a method to minimize conjugate degradation during the processing of biological fluids prior to analysis. The analytical method has been presented in Chapter III. For the determination of acyl glucuronides of naproxen and ketoprofen in urine, Upton et al. (6) recommended that urine should be analyzed immediately after collection, but even then, a small fraction of unconjugated drug in the urine was found and was probably due to conjugate hydrolysis. The isomerization of bilirubin glucuronide in bile at pH 7.4 was somewhat decreased by reducing the temperature, but for complete stabilization it was necessary to lower

the pH to 3 (44). Bilirubin monoglucuronide is believed to isomerize to β-glucuronidase resistant conjugates in the blood of patients with cholestasis (112). Faed and McQueen (22) found high concentrations of clofibric acid glucuronide in human plasma which was unstable unless the plasma was cooled and assayed promptly. These investigators recommended care be taken when handling samples containing acyl glucuronides, however, no controlled or systematic studies on the stability of labile acyl glucuronides in plasma or blood were conducted. We have investigated methods to maintain the integrity of ZG in biological fluids prior to and during analysis. Based upon these studies, a sample handling procedure for blood was developed. The analytical results were then compared between blood samples with and without precautions to maintain glucuronide stability.

Loss of acyl glucuronides in biological fluids in vitro, which is primarily due to acyl migration, can be measured and possibly minimized by controlling the temperature, pH and solvent composition. It is more difficult to assess the loss of the β-1 conjugate in vivo. Initial studies found that ZG was eliminated rapidly when given intravenously to rabbits and guinea pigs (Chapter V). The loss of ZG in vivo was accompanied by rapid and substantial formation of Z. The rates of ZG loss from blood in vitro could not fully explain its rapid hydrolysis in vivo. This suggested that much of the ZG elimination in vivo was possibly due to enzymatic hydrolysis of the ester glucuronide. The possible hydrolysis of ZG by enzymes located in the tissues of animals was therefore examined. β-Glucuronidase which is present in many mammalian tissues (114, 115) might be responsible for the cleavage of Z. A less apparent route for the hydrolysis of acyl glucuronides in

vivo is by the ubiquitous nonspecific esterases which have broad substrate specificity and are present in many mammalian tissues, especially the liver (116, 117). Acyl glucuronides are unique among glucuronide conjugates in that they are esters and may be succeptible to hydrolysis by esterases. If esterases, and not β-glucuronidase, are the enzymes responsible for the cleavage of acyl glucuronides *in vivo*, this may explain the observation that drugs which are metabolized to ester glucuronides, such as clofibric acid (29, 30), diflunisal (21, 118) and ketoprofen (8, 31), exhibit decreased clearances in renal failure. In contrast, some drugs which form more stable phenolic glucuronides, for example acetaminophen (17) and oxazepam (19, 119), yield no noticable change in clearance when renal function is decreased.

Acyl migration is a physical, nonenzymic phenomenon which should occur in blood *in vivo* if the β-1 acyl conjugate has sufficient residence time *in vivo* to allow migration to occur. The migration may have significant effects on the disposition of the conjugates because isomers of the β-1 conjugate are not cleaved by β-glucuronidase and these isomers may exhibit different clearances, protein binding characteristics and succeptibilies to esterases. If cleavage by β-glucuronidase is a prerequisite for reabsorption during enterohepatic recycling (120), then the isomeric conjugates would not be able to complete the cycle and would instead be eliminated in the feces. As will be described in Chapter VII, the isomeric conjugates also lead to a covalently bound Z-protein adduct in plasma. The ability to unambiguously measure acyl glucuronides and their isomers in biological fluids is necessary when studying the disposition of compounds metabolized to acyl glucuronides in humans and animals. Knowledge of the factors affecting the

hydrolysis or acyl migration of the conjugates in vitro and in vivo is therefore essential and was obtained from studies carried out in biological fluids and animal tissue preparations.

B. MATERIALS AND METHODS

Chemicals

Zomepirac·Na·$2H_2O$ and 1,4-dimethyl-1H-5-(4-methoxybenzoyl)-pyrrole-2-acetic acid, the internal standard (IS), were graciously supplied by McNeil Pharmaceuticals (Springhouse, PA). Zomepirac glucuronide (ZG) and its isomeric conjugates were isolated and purified from human urine as previously described (28) (Chapter II). Bovine liver β-glucuronidase (Type B-10), phenylmethylsulfonyl fluoride (PMSF), physostigmine (eserine) and phenolphthalein glucuronide were purchased from Sigma Chemical (St. Louis, MO). D-saccharic acid 1,4-lactone was obtained from Aldrich (Milwaukee, WI). Solvents used for analytical procedures were HPLC grade. All other chemicals were reagent grade.

Stability studies in buffer, organic solvents, plasma and blood

The stability of ZG and its purified isomeric conjugates was evaluated at 37 °C by reducing to dryness methanolic solutions of about 400 µg of ZG, or its respective isomer, then dissolving the residues in 4.0 ml of 0.1 M sodium phosphate buffer at the desired pH. Aliquots were taken over time and adjusted to pH 2 to 5 by the addition of dilute HCl, if necessary, and assayed within several hours. Because of the instability of fraction 2 obtained from the preparative HPLC separation, the eluted peak of fraction 2 from HPLC was used directly for stability studies after the removal of methanol from the mobile phase by

evaporation under a stream of nitrogen. The final concentration of fraction 2 was about 1 μg/ml. Time-dependent peak-height ratios were determined relative to IS using the analytical method described in Chapter II. Incubations of ZG and the other isomers with β-glucuronidase, 3,000 U/ml, were done at pH 5.0, 37 °C. Complete hydrolysis of ZG by the enzyme was achieved in 30 min. Saccharic acid 1,4-lactone (50 mM), a competitive inhibitor of β-glucuronidase (78), was used to evaluate the specificity of the enzymatic hydrolysis.

The stability of ZG in the reconstituted solution prior to HPLC analysis was evaluated in 50% methanol and 50% acetonitrile in aqueous solutions at pH from 2 to 8. The analysis was done as described above. When ZG was dissolved in 50% methanolic solutions at pH greater than 5 the loss of ZG yielded little formation of the isomeric conjugates or free Z. The methanolic solutions were then adjusted to pH 2 and extracted with several volumes of hexane. The hexane extract was analyzed by capillary GC and low resolution mass spectrometry and compared to synthetic zomepirac methyl ester. Capillary GC was done with flame ionization detection on a Packard 429 instrument (Downers Grove, IL). A 0.25 mm ID fused silica column, coated with 0.25 μ film thickness SE-30 from J & W Scientific (Rancho Cordova, CA) which was 22 m long was used with helium carrier gas at a flow rate of 40 cm/sec. Both the detector and injector were heated to 325 °C with nitrogen make-up flow of 30 ml/min to the detector. After injection the oven was programmed from 50 to 200 °C at 30 °C/min, then at 10 °C/min to 300 °C. Split injection of about 40:1 with appropriate dilution of the samples provided a single peak at 232 °C with the temperature program. Electron impact mass spectrometry was done with direct probe inlet on an

MS-25 spectrometer, Kratos (San Diego, CA). Zomepirac methyl ester was prepared synthetically with diazomethane (121).

In separate experiments examining the effects of temperature and pH on the stability of an acyl glucuronide, ZG was added to 0.15 M sodium phosphate buffer, plasma or blood to provide concentrations of 10 to 50 µg/ml. The solutions were then adjusted to the desired pH by the addition of phosphoric acid or sodium hydroxide. Aliquots were taken over time and the concentration of ZG was determined by HPLC as previously described in Chapter II (28). Stability was examined at 37, 22 and 4 °C. The pH values investigated for plasma were 7.4 and 3.0. It was not possible to carry out studies in blood at pH 3.0 since coagulation occurred. Alternatively, small amounts of citric acid were added to blood (5,10 and 25 mM solutions) to lower its pH slightly, (i.e. pH = 6.9, 6.6 and 5.3, respectively).

The above studies established a method for the proper processing of blood samples which was applied in the analysis of Z and ZG in plasma as described in Chapter III. Blood drawn into a syringe was immediately transferred to a heparinized tube (about 10 units/ml blood) cooled on ice. Within 10 minutes the blood was centrifuged at 0 to 4 °C, and the plasma was pipetted into a vial containing 10 microliters of concentrated (14.7 M) phosphoric acid per ml of plasma. The plasma, now at pH 2 to 4 was then frozen at -20 °C until analysis. For the sampling procedure where no precaution was taken, control blood samples were drawn, centrifuged at room temperature and the plasma was then frozen without the addition of acid. To compare the two blood handling methods, zomepirac (5 mg/ml) was given by oral gavage (20 mg/kg) to five male rhesus monkeys (3.6-4.4 kg) (122). Twenty ml samples of blood were

taken at one and four hours after the dose. Blood obtained at each sampling time was pooled then divided; the untreated half served as the control while the remainder was treated as described above. Replicate control and treated samples of the plasma were analyzed by HPLC for ZG, its isomeric conjugates and zomepirac using the method described in Chapter III (107). Briefly, plasma was analyzed by direct precipitation of the proteins with two parts of acetonitrile, followed by centrifugation and a second wash of the protein pellet with acetonitrile. The combined supernatants were evaporated at 35° C under a stream of nitrogen, then the residue was dissolved in 25% acetonitrile/0.5 M sodium acetate, pH 4.5 prior to injection onto a C18 Ultrasphere® (Altex, Berkeley, CA) reversed phase LC column, 15 cm X 4 mm ID. The mobile phase was 58% methanol/ 0.01 M tetrabutyl ammonium sulfate and 0.05 M sodium acetate, pH = 4.5 at a flow rate of 1.3 ml/min. Detection was by UV at 313 nm using a Waters 440 detector (Waters, Milford, MA).

Stability studies in tissue preparations

Following an overnight fast, adult male guinea pigs (EZH, Williams, CA) were decapitated, the liver and small intestine were removed, washed twice in cold 0.15 M sodium phosphate buffer, pH 7.4, and homogenized with 5 parts buffer using a ground glass motar and pestle. The small intestinal segments utilized were from the duodenal and jejunal regions. These segments were flushed well with about 40 ml buffer to remove any intestinal contents prior to homogenization. The 10,000 x g supernatant was collected for use in subsequent incubations. Most incubations were done on the same day as the tissue preparation was obtained, however, storage of the homogenate at -80 °C for several days did not produce

noticeable changes in the activity for the homogenate. Incubations were done at 37 °C in 0.15 M sodium phosphate buffer at protein concentrations of about 2-4 mg/ml as determined by the Bio-Rad (Richmond, CA) protein assay method (123). Physostigmine, 1 mM; PMSF, 1 mM; and saccharic acid 1,4-lactone, 20 mM, were tested for the inhibition of ZG hydrolysis in the liver and small intestinal tissue homogenates. Stock solutions of PMSF (50 mM in ethanol), physostigmine (20 mM in 0.15 M phosphate buffer, pH 7.4) and saccharic acid 1,4-lactone (400 mM in 0.15 M phosphate buffer, pH 7.4) were made daily. A 30 min preincubation with the inhibitor was carried out prior to the addition of ZG to maximize the interaction between the inhibitors and the enzymatic systems.

Incubations were started by the addition of ZG dissolved in ethanol. Ethanol was also added to the control incubations and was never more than 5% of the total volume. Aliquots of the incubation mixture were taken over 30 to 60 minutes and the reaction was stopped by the addition of 2 parts acetonitrile, and 0.02 parts concentrated H_3PO_4 to adjust the pH to 2 to 4. The sample was then immediately cooled on ice. Assay of Z, ZG and isomeric conjugates of ZG was done by HPLC as described in Chapter III (107). Results were analyzed by determining the first order loss of ZG which was corrected for the loss occurring in buffer. The rate of ZG loss was then normalized to protein concentration in the incubation. Control incubations were done with ZG in buffer alone, buffer with 1000 U/ml β-glucuronidase, buffer with the respective inhibitor added, and buffer with 1000 U/ml β-glucuronidase and 20 mM saccharic acid 1,4-lactone. Phenolphthalein glucuronide was also employed as a positive control for the activity of β-glucuronidase

and the tissue homogenates. The rapid hydrolysis of phenolphthalein glucuronide by 1000 U/ml β-glucuronidase was completely inhibited by 20 mM saccharic acid 1,4-lactone.

C. RESULTS

A qualitative time-dependent profile of ZG rearrangment to the other isomeric conjugates in buffer at pH 7.4 is illustrated in Figure IV-1. The final product eventually formed after many hours is Z, though the other isomers were initially formed at a faster rate. Thus, the rate of loss of ZG did not equal the rate of Z formation. Semilogrithmic plots of concentration of isomer after incubation at time t, A (t), relative to the initial isomer concentration, A (0), are presented in Figures IV-2 and IV-3. The pH-dependent loss of ZG in buffer (Fig. IV-2) shows that the rate of ZG loss was not appreciable unless the pH was greater than 5. Table IV-1 documents the considerable change in half-lives of ZG loss over the pH range studied. At pH 7.4 the half-life of ZG in buffer was 27 min, whereas at pH 2.0 it was 160 hours.

When the purified isomers of ZG were incubated individually at pH 7.4 they rearranged to form all of the other isomers as well as the presently unidentified and unusually unstable fraction 2. However, none of the isomers of ZG, when incubated in buffer at pH 7.4, formed fraction 3, ZG. The purified isomers, as opposed to ZG, gave apparent first order decomposition only for the early time points as shown in Figure IV-3. This reflects the reversible nature of acyl migration, which ZG (fraction 3) did not experience. All purified isomers were stable in a pH range of 2 to 5. Fraction 2 was so labile at pH 7.4 that only a single measurement could be made and therefore no rate constant

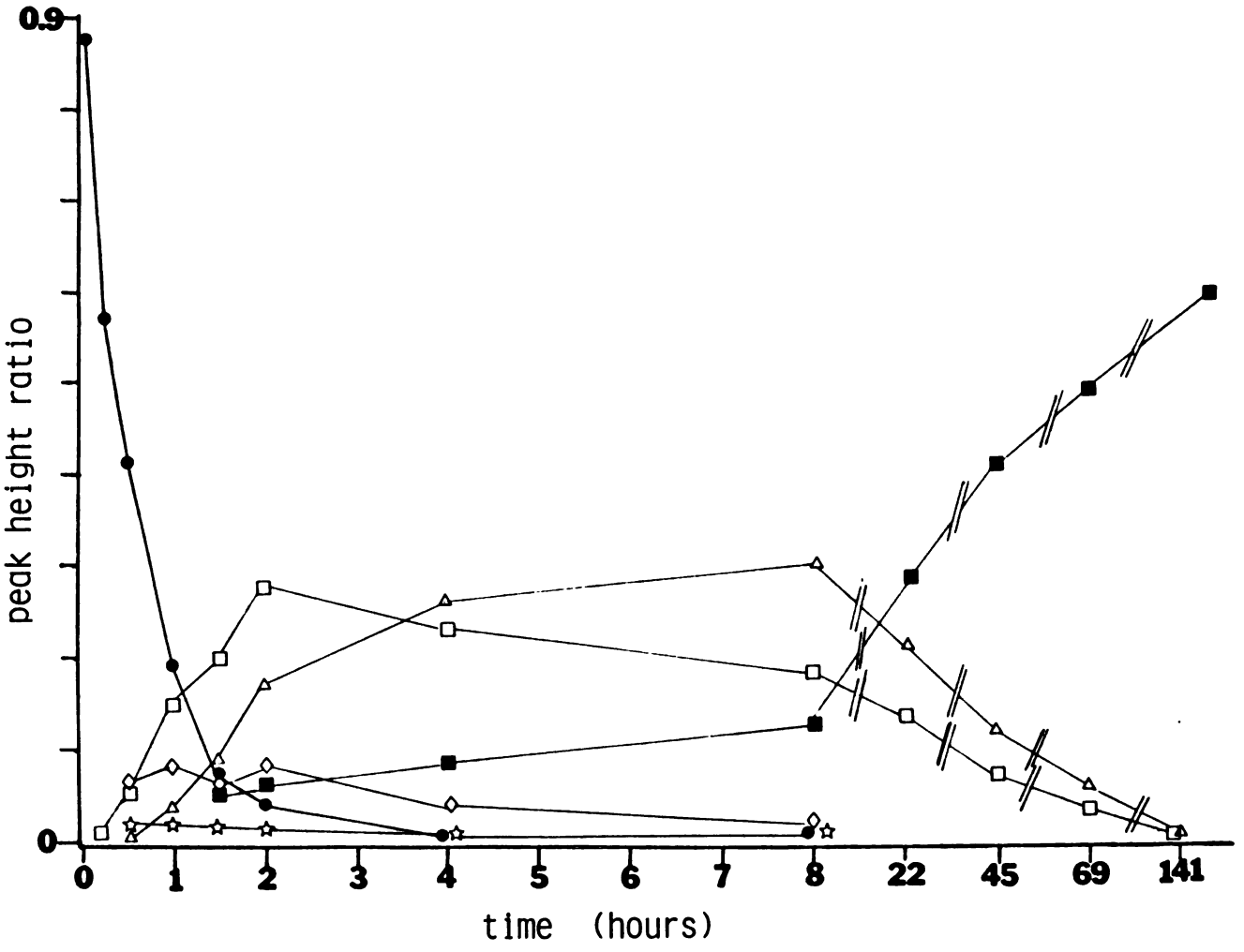


Figure IV-1. Time-dependent degradation of zomepirac glucuronide to its isomeric conjugates and hydrolysis to Z in 0.1 M phosphate buffer, pH 7.4, 37 °C.
Key: ● ,zomepirac glucuronide; ■, Z; Δ ,α/β-4-O-acyl isomer; □, α/β-3-O-acyl isomer; ◇ , α/β-2-O-acyl isomer; ☆, fraction 2 from HPLC.

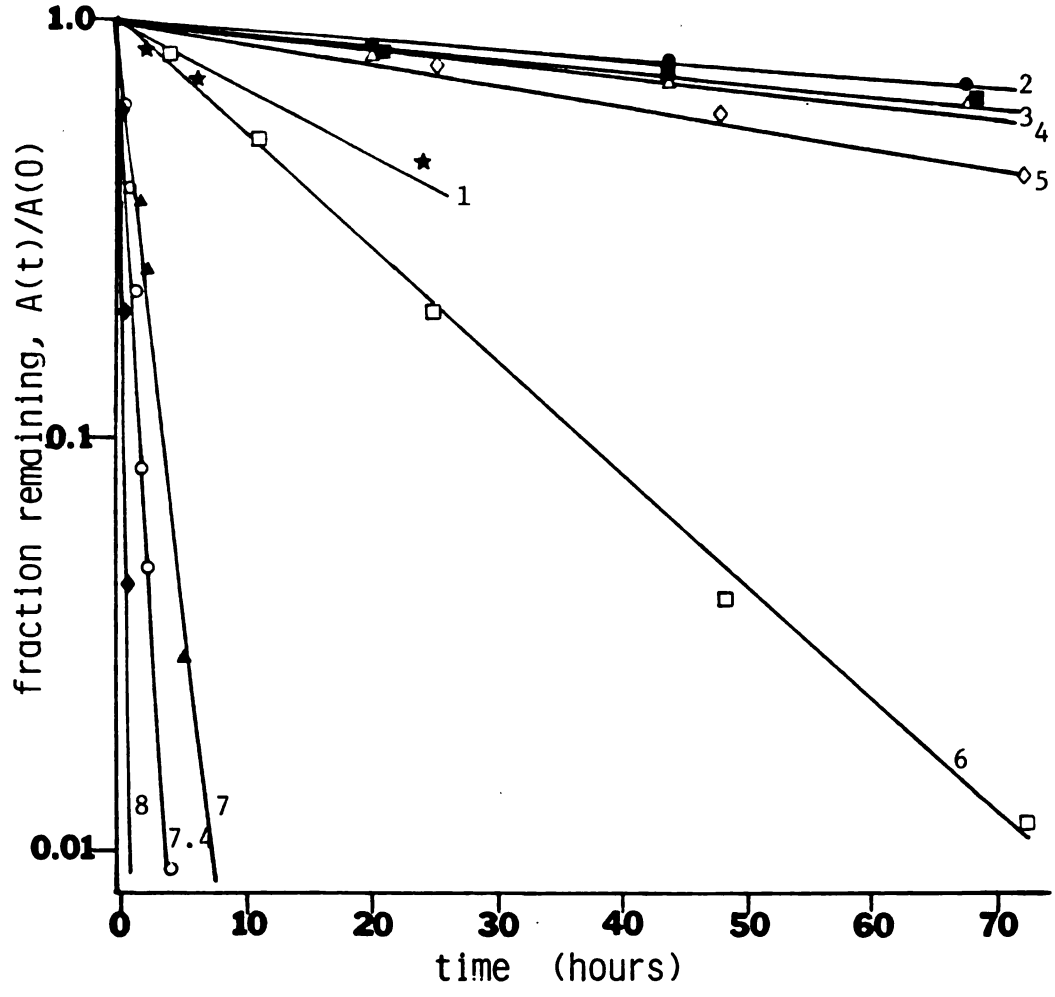


Figure IV-2. Time-dependent degradation of zomepirac glucuronide in 0.1 M phosphate buffer at various pH values. Number indicates the pH of the incubation. Temperature is 37 °C.
Key: ★, pH 1.0; ●, pH 2.0; ■, pH 3.0; △, pH 4.0; ◇, pH 5.0; □, pH 6.0; ▲, pH 7.0; ○, pH 7.4; ◆, pH 8.0.

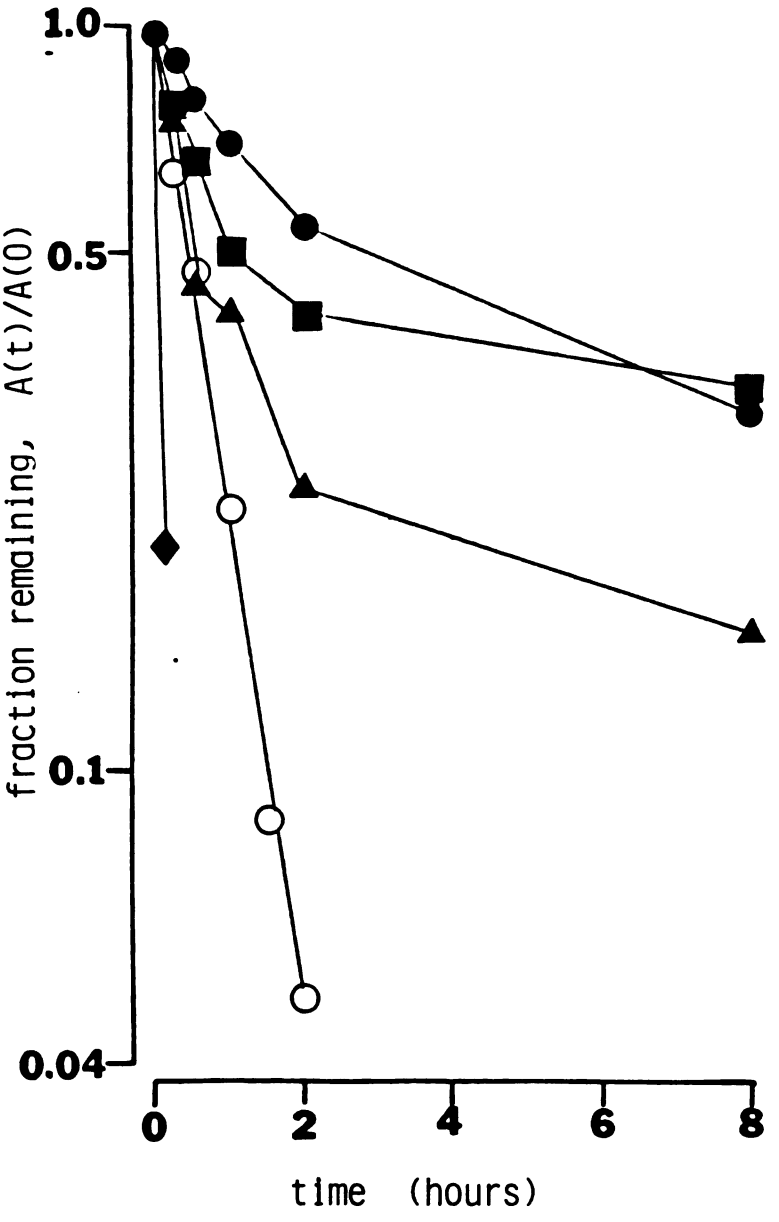


Figure IV-3. Time-dependent hydrolysis and rearrangement of of zomepirac glucuronide and its purified isomeric conjugates in 0.1 M phosphate buffer, pH 7.4, 37 °C.
Key: ○, zomepirac glucuronide; ●, α/β-4-O-acyl isomer; ■, α/β-3-O-acyl isomer; ▲, α/β-2-O-acyl isomer; ◆, fraction 2 from HPLC.

Table IV-1. pH-Dependent degradation of zomepirac glucuronide and its purified positional isomers in 0.1 M phosphate buffer at 37 °C.

HPLC fraction	Positional[a] isomer	pH	K_{app} (hrs^{-1})	Half-life (hrs)
3	β-1	1.0	0.0300	23.1
3	β-1	2.0	0.00429	161
3	β-1	3.0	0.00645	107
3	β-1	4.0	0.00676	103
3	β-1	5.0	0.0117	59.
3	β-1	6.0	0.0638	10.9
3	β-1	7.0	0.702	0.99
3	β-1	7.4	1.54	0.45
3	β-1	8.0	6.30	0.11
1	α/β-4	7.4	0.304	2.27
2	U[b]	7.4	rapid decomposition	
4	α/β-3	7.4	0.670	1.03
5	α/β-2	7.4	0.670	1.03

[a]Position based on data in Chapter II.
[b]Unidentified zomepirac conjugate.

Table IV-2. The effect of organic solvents and pH on the degradation of zomepirac glucuronide in 0.1 M phosphate buffers at 37 °C.[a]

Solvent	Half-life (hr)
Buffer A, pH 2.0	190
50% CH_3OH/Buffer A	300
50% CH_3CN/Buffer A	2300
Buffer B, pH 5.0	65
50% CH_3OH/Buffer B	36
50% CH_3CN/Buffer B	220
Buffer C, pH 6.0	12
Buffer D, pH 7.4	0.45
50% CH_3OH/Buffer D	0.16
50% CH_3CN/Buffer D	1.0
Buffer E, pH 8.0	0.12

[a]Initial ZG concentration was 100 μg/ml. Incubations were up to 140 hours.

was reported in Table IV-1, although the half-life was estimated to be less than 5 minutes.

ZG was more stable in buffered acetonitrile/water solution than in aqueous buffer alone, often by an order of magnitude. The loss of ZG in the HPLC mobile phase (a methanolic solution) was not fast enough to alter the HPLC analysis, but it did preclude the use of the mobile phase as the solvent for sample reconstitution and storage prior to HPLC analysis since this time is often many hours. The loss of ZG from methanolic solutions at pH greater than 5 was confirmed to occur by the formation of Z methyl ester. The hexane extract of the methanolic solutions of ZG produced a GC peak with a retention time identical to that for the synthetic Z methyl ester. Hydrolysis of the hexane extract with aqueous base led to liberation of Z as determined by HPLC. The mass spectra for both the extracted and synthetic methyl ester were identical with ions at m/z 305, 246 and 139. These correspond, respectively, to the parent ion, the loss of 59 which is characteristic of methyl esters and the p-chlorobenzoyl fragment of Z methyl ester. Table IV-2 summarizes the results found for the addition of methanol or acetonitrile to a buffered ZG solution at several pH values. Acetonitrile enhanced the stability of ZG; whereas methanol at a pH greater than 5 led to increased degradation of ZG both through acyl migration and methanolysis to form zomepirac methyl ester. This phenomenon has been documented for bilirubin glucuronide (47) and is probably a problem with any nucleophilic solvent since acyl glucuronides have also been reported to react with sulfhydryl groups (48).

The degradation of ZG in plasma and blood appears to follow first order kinetics as was shown for the buffered solutions above (28). The

Table IV-3. The effect of temperature upon the apparent first-order degradation half-life of zomepirac glucuronide.[a]

SOLVENT	Half-Life (minutes) 37 °C	22 °C	4 °C
PHOSPHATE BUFFER[b], pH 7.4	31	150	>1000
BLOOD, pH 7.3 - 7.5[c]	17	59	285
PLASMA, pH 7.3 - 7.5[c]	10	47	200

[a]All samples were incubated for 120 minutes.
[b]Buffer was 0.15 M potassium phosphate, pH 7.4.
[c]Initial pH of blood and plasma was 7.3 to 7.5.

Table IV-4. The effect of citric acid upon the pH of blood and the apparent first order degradation half-life of zomepirac glucuronide.[a]

Citrate conc. (mM)	0	5	10	25
pH	7.3	6.9	6.6	5.3
Temperature °C	Half-Life (min)			
22	59	140	780	>1000
4	285	>1000	>1000	>1000

[a]All samples were incubated 180 minutes.

apparent half-lives for the loss of ZG are presented in Table IV-3. Cooling the sample, as expected, does decrease the rate of ZG loss; however, it does not completely prevent ZG degradation in plasma or blood. The conjugate degraded faster in plasma than in blood and was most stable in buffer at all three temperatures studied. Decreasing the pH of the solution to 3.0 is a more effective means of preventing degradation of ZG, but this cannot be applied to whole blood. Addition of citric acid to blood modified the pH enough to enhance the stability of ZG significantly without causing blood coagulation (Table IV-4). A small degree of hemolysis did occur with the addition of citric acid to blood. When cooling the blood (0 to 4°C) was combined with acidification of the plasma with phosphoric acid, as described above for samples from the monkeys, the loss of ZG from spiked blood samples was less than 4%. Most of this loss was due to rearrangement to isomeric conjugates of ZG; there was little hydrolysis to Z.

The comparison of sample handling procedures for blood obtained from the monkeys after a Z dose revealed large differences in the plasma concentrations of ZG and Z between the control and treated samples. Untreated blood showed a total loss of ZG to form isomeric conjugates (ISO) as well as free Z. Representative chromatograms comparing an untreated, control sample (A) to a treated sample which was cooled and acidified (B) are shown in Figure IV-4; the HPLC data obtained are presented in Table IV-5. The samples which were cooled and buffered (Fig. IV-4) have only about 60% the Z level of the untreated samples. The artifactually elevated Z levels in the untreated samples are due to the hydrolysis of ZG or ISO prior to sample preparation for LC.

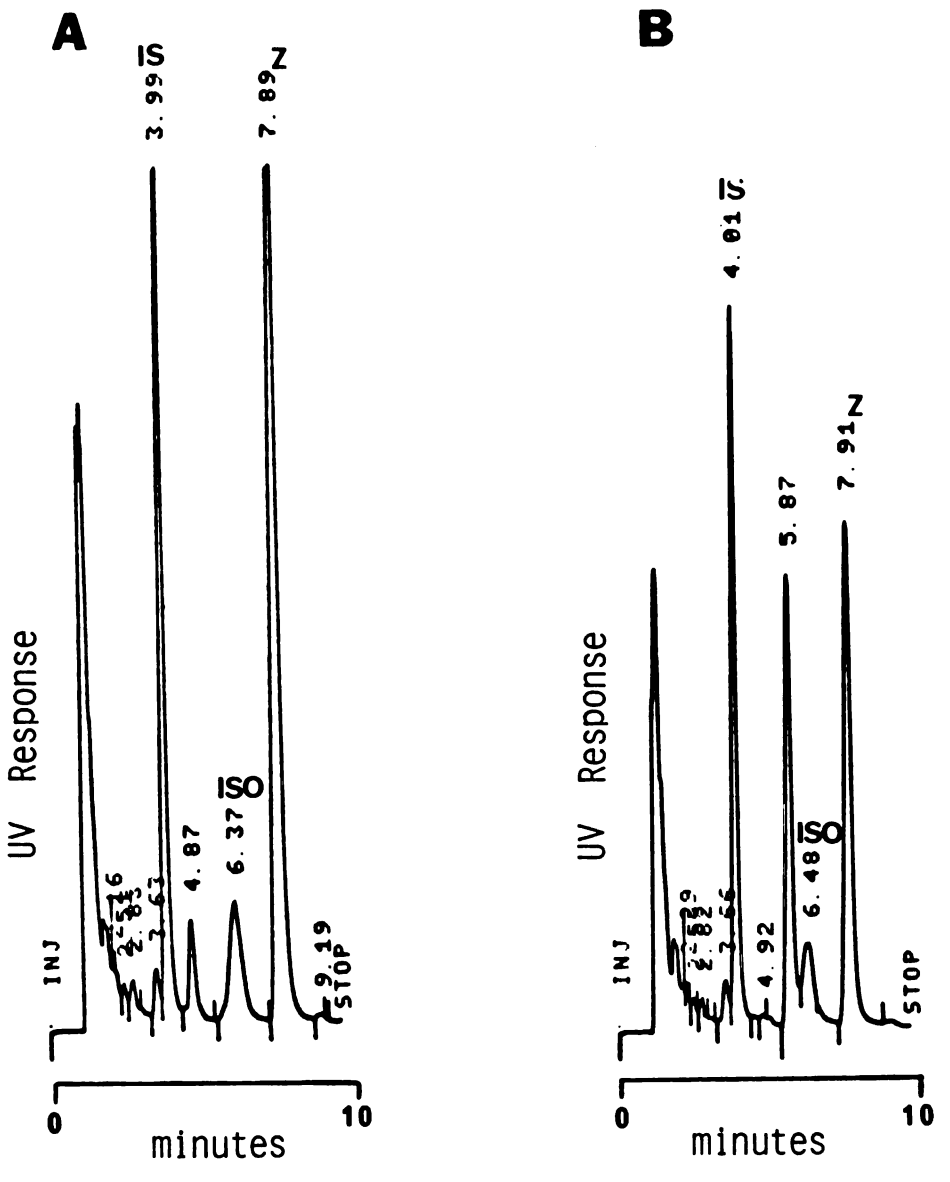


Figure IV-4. Representative chromatograms for an untreated control plasma sample (A) and for acidified plasma obtained from a cooled blood sample (B). The sample is from rhesus monkeys one hour after a 20 mg/kg oral dose of zomepirac (Z). HPLC conditions are described in the text (Chapter III). In sample A no ZG peak is noted, while areas under the Z and ISO peaks are greater in A relative to the internal standard (IS) peak as compared to sample B. Abbreviations are zomepirac, Z; internal standard, IS; and isomeric conjugates of ZG, ISO.

Table IV-5. Concentrations of zomepirac glucuronide (ZG), its isomeric conjugates (ISO), and zomepirac (Z) in plasma from treated and control blood samples after a 20 mg/kg oral dose of zomepirac to five rhesus monkeys.

	Treated 0 °C, pH 2-4			Control 22 °C, pH 7.4		
	ZG	ISO	Z	ZG	ISO	Z
Time (hr)		(µg/ml)			(µg/ml)	
1	4.82[a] ±0.25	1.93 ±0.23	3.92 ±0.12	ND[b]	1.68 ±0.32	6.30 ±0.20
4	1.41 ±0.12	0.67 ±0.03	1.14 ±0.02	ND	0.56 ±0.19	1.88 ±0.16

[a]Mean ± s.d.
[b]Not Detectable.

Incubations of ZG with tissue homogenates caused an increase in the rate of ZG loss as compared to buffer solutions at the same pH. Liver homogenates had much greater activity for ZG hydrolysis, when normalized to protein content, than did small intestinal homogenates. The increased loss of ZG in tissue homogenate relative to that for buffer was due to enzymatic hydrolysis; there was no observable increase in acyl migration products. As compared to small intestinal homogenate, the liver homogenates had 18.2 ± 3.5 (n=3) fold higher rates of ZG hydrolysis. A representative plot of ZG loss vs. time for the incubations with tissue homogenates is shown in Figure IV-5. Saccharic acid 1,4-lactone (20 mM) which completely inhibited hydrolysis of ZG by 1000 U/ml β-glucuronidase, had no noticeable effect on the ability of liver homogenate to cleave ZG. However, both esterase inhibitors, PMSF and physostigmine, inhibited the activity of liver homogenate such that the rate of hydrolysis of ZG was indistinguishable from that in buffer solution.

D. DISCUSSION

Conducting stability studies of the conjugate, ZG, and its isomers in buffer provided valuable information which was then successfully applied to biological fluids. The loss of ZG is markedly pH-dependent with maximum stability at pH 2-4 (Fig. IV-2, Table IV-1). Between pH 6 and 8 the rate constant for ZG loss, K_{app} (Table IV-1), increases 100 fold. This linearity between log K_{app} and pH with a slope of 1.0 suggests that in this range the reaction is specific base catalyzed (124). The enhanced migration due to acid (pH 1) and base agrees with previous data for the catalysis of this type of reaction (32). The isomers of ZG are more stable than ZG, and once formed at pH 7.4 the

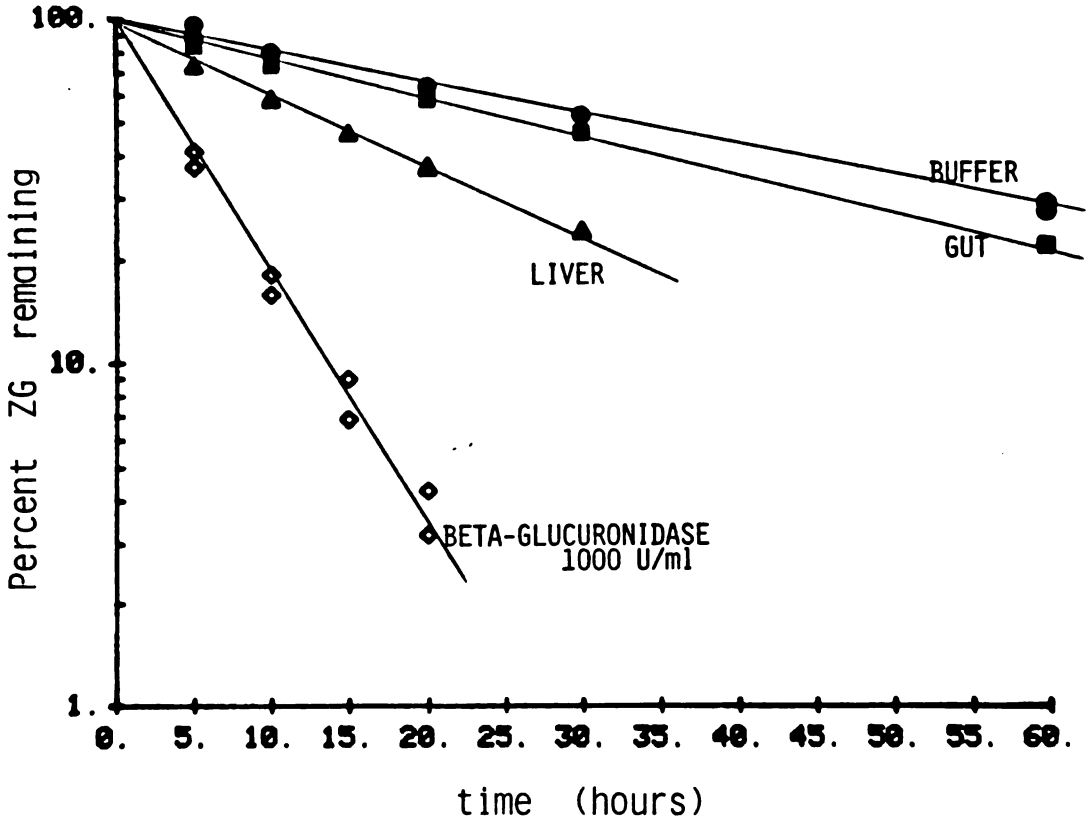


Figure IV-5. Representative profile of hydrolysis of zomepirac glucuronide (ZG) when incubated with tissue homogenates of the guinea pig and with β-glucuronidase at 37 °C. Each time point has replicate measurements. Other conditions are described in the text.

isomers very slowly hydrolyze to yield Z (Fig. IV-1). The greater stability of the isomers relative to ZG is to be expected, as ZG has the anomeric oxygen contributing to its instability. Figure IV-3 also shows that the purified isomers rapidly equilibrate with the other isomers, however, at equilibrium no ZG was present. Fraction 2 from HPLC, which was never identified, was unusually labile (Fig. IV-3). We speculate that fraction 2 might be the α-1-isomer of ZG, which as a cis isomer should transfer the acyl much more rapidly than the trans or β-isomer (32). The choice of organic solvents to use with acyl glucuronides requires careful consideration. Properly selected aprotic solvents such as acetonitrile appear to enhance stability of the conjugates, perhaps by decreasing the base catalyzed reactions of acyl migration and hydrolysis. However, a solvent such as methanol which is routinely utilized by many labs, resulted in total loss of the conjugate with formation of a methyl ester. It is difficult to believe that the only previous occurance of this problem was the report of artifactual formation of bilirubin methyl esters in 1974 (47).

Few pharmacokinetic and metabolism studies of compounds which form labile acyl glucuronides have considered the problem of degradation of the β-1 conjugate in biological fluids prior to analysis. As a result, an even smaller number of investigators have made attempts to control and prevent the loss of the conjugate in the biological fluid before analysis. This is especially true for the analyses of blood or plasma samples. With the exception of the present study, the report by Faed and McQueen for clofibric acid (22) and the studies with bilirubin (44), all of the previous reports of acyl migration have been obtained in urine or in bile. A recent publication studying the disposition of

clofibric acid glucuronide in rabbits reported control of the conjugate stability in blood by rapid cooling and acidification as we have recommended here (111).

It is possible to prevent the loss of an acyl glucuronide either by hydrolysis or acyl migration once a biological fluid sample has been collected. However, it is not always possible to prevent such changes from occurring before sample collection. Urine often resides in the bladder, and bile in the gallbladder, for extended periods of time prior to voiding of the organ. Thus, what is collected from the urine or bile may not reflect what the kidney, or liver respectively, produced. The pH of both urine (125) and bile (126) are often high and both organs are known to accumulate high concentrations of glucuronide conjugates (127, 128). As a result, the possibility of observing acyl migration of drug conjugates in urine or bile is much higher than for plasma and indeed most of the reports of acyl migration have dealt with urine or bile.

Once isomeric conjugates are formed in a biological sample they can make identification of the conjugate more difficult. Contributing to the difficulty are the variable amounts of isomers which may be present in each sample depending upon how the individual sample was handled prior to analysis. The isomers thus formed often have different chromatographic retention times, and most importantly, are not cleaved by β-glucuronidase (28, 62). Problems with acyl migration led to the confusion between several labs in determining the metabolism of clofibric acid (62, 64) confounded the identification of conjugates of bilirubin (61, 129), probably resulted in lower yields of conjugates for many xenobiotics when Caldwell *et al.* compared β-glucuronidase hydrolysis with aqueous base treatment (65) and may explain the

unidentified metabolite of a flufenamic acid derivative in the urine of cynomolgus monkeys which was not seen with rhesus monkeys (130). Urine from cynomolgus monkeys on average is about one pH unit higher than urine from rhesus monkeys (130). Our comparison between Z and conjugate levels in monkey plasma treated by cooling and acidification vs. control plasma exemplifies the types of problems presented by acyl migration if the handling of the biological fluid is not controlled. Control of temperature and pH after collection of a blood sample along with proper selection of the solvents employed in the analysis minimize the degradation of ZG (Table IV-5, Fig.IV-4). We feel that in the future this potential problem must be considered when developing an analytical method for drugs metabolized to acyl glucuronides.

Although ZG was less stable in blood than in buffer (Table IV-3) its loss in vivo after an IV dose to animals (Chapter V) was even more rapid which suggests hydrolysis of ZG by well perfused tissues. Liver tissue which has high concentrations of both enzymes likely to cleave ZG, β-glucuronidase (114) and esterases (116, 120), proved to have a high capacity to hydrolyze ZG in vitro. The hydrolysis of ZG by liver homogenate was inhibited by PMSF and physostigmine, but was not affected by saccharic acid 1,4-lactone. Thus, most of the hydrolysis was due to nonspecific or presently unidentified esterases, not β-glucuronidase. Preliminary studies also indicate that the isomers of ZG which are resistant to β-glucuronidase are also cleaved by liver homogenates in vitro although at variable rates.

Hydrolysis of acyl glucuronides by esterases, though not unexpected, has only recently been considered. Previously, hydrolysis of glucuronides in vivo was discussed primarily in relation to entero-

hepatic cycling and glucuronides were considered as a class for which β-glucuronidase was the primary enzyme suspected to cleave the conjugates *in vivo* (131, 132). However, Meffin *et al.* (111) recently suggested that the decreased clearance of clofibric acid in renal dysfunction was due to hydrolysis of the conjugate by esterases rather than by β-glucuronidase. The rationale for the exclusion of β-glucuronidase is that drugs which form phenolic glucuronides, such as acetaminophen (17) and oxazepam (19, 119), have clearances near normal in renal deficiency even though the glucuronide conjugates accumulate. A later report supported this hypothesis by showing that the irreversible esterase inhibitor, diisopropylfluorophosphate, increased the clearance of clofibric acid by decreasing the rate of hydrolysis of its glucuronide *in vivo* in the rabbit (9). Hydrolyis of the glucuronide, whether it is chemical or enzymic, results in an effective recycling or reversible metabolism. The hydrolysis makes it difficult to determine the metabolic clearance of the drug, especially in renal failure when more conjugate is available for hydrolysis. The *in vitro* results presented here support the hypothesis of participation of esterases in the hydrolysis of ZG *in vivo* and *in vivo* experimental data to substantiate this hypothesis will be presented in Chapter V.

CHAPTER V

DISPOSITION OF ZOMEPIRAC AND ZOMEPIRAC GLUCURONIDE IN ANIMALS: THE EFFECT OF ESTERASE INHIBITION BY PHENYLMETHYLSULFONYL FLUORIDE

A. INTRODUCTION

The disposition of drugs metabolized to acyl glucuronides in vivo is believed to involve isomerization of the glucuronide by acyl migration and also regeneration of the parent drug from the conjugates by hydrolytic cleavage. The potential of a futile cycle existing for the disposition of acyl glucuronides (Fig. V-1) was discussed briefly in Chapter IV. Based on this rationale, we carried out studies of zomepirac (Z) and zomepirac glucuronide (ZG) disposition in small laboratory animals, in which the experiments were designed to resolve whether the hydrolysis of ZG occurs in vivo and to delineate the mechanism for this hydrolysis. Another objective for performing these animal experiments was to develop a small animal model which would provide substantial concentrations of the acyl glucuronide in vivo after a dose of the parent drug. This model was needed to study the disposition and accumulation of acyl glucuronides in vivo in an attempt to correlate exposure to the reactive conjugate with the extent of irreversible binding of the parent drug to plasma proteins in vivo. This irreversible binding will be discussed in greater detail in a subsequent chapter.

Several reasons influenced our selection of Z as the model compound for use in our studies of acyl glucuronide disposition in animals. Previous studies in our laboratory have documented the instability and

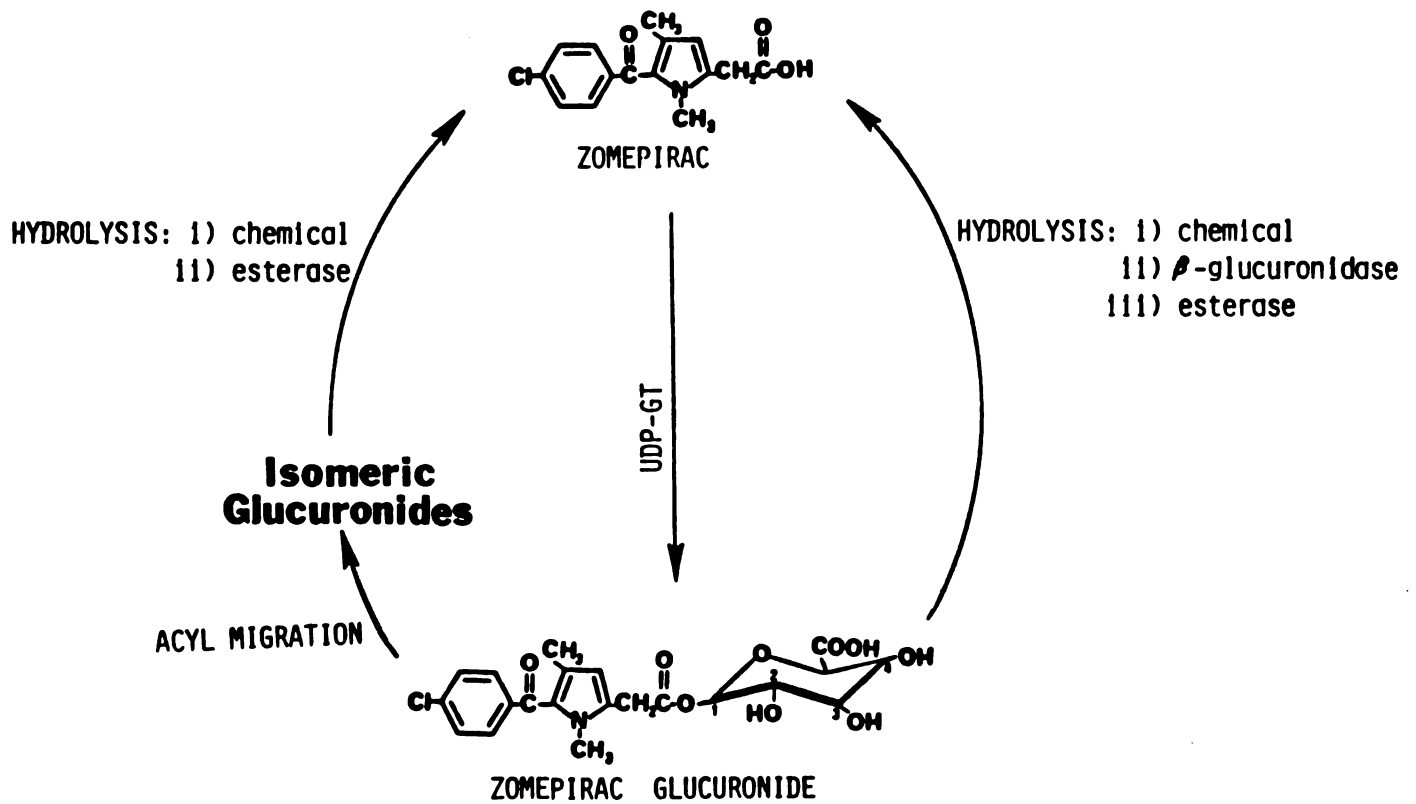


Figure V-1. Scheme for the futile cycle of zomepirac and zomepirac glucuronide in vivo. The potential routes for the hydrolysis of zomepirac glucuronide and its isomers are presented.
UDP-GT = Uridine diphosphate glucuronyltransferase.

acyl migration of ZG (28) and provided a knowledge base for the handling and analysis of Z and ZG. An especially important factor was that sufficient quantities of ZG could be isolated and purified in our laboratory from human urine, which could then be dosed to the animals.

Glucuronidation is a major Phase II metabolic pathway for many drugs and xenobiotics (51, 53, 54) which results in the addition of glucuronic acid to the compound (Fig. II-1). Glucuronides are frequently inactive pharmacologically or less active than the parent compound (133), although there are examples, such as morphine-6-glucuronide (134), where the glucuronide does possess pharmacological activity (2). There have been no reports documenting any pharmacological activity for ZG. Glucuronidation is most often a detoxification mechanism for xenobiotics (2, 54). However, the N-hydroxy-glucuronides of phenacetin (45) and N-acetylaminofluorene (46) are precursors which lead to covalent binding and probable toxicity of these xenobiotic (14). Glucuronide conjugates once formed are more polar and water soluble than the parent compound and hence have greater ability to be excreted in the urine (109) and the bile (128, 135). Because of their enhanced ability to be excreted, glucuronides are usually eliminated rapidly without appreciable accumulation in the blood of healthy humans or animals (136). However, impaired excretory function, as in renal failure or cholestatic liver diseases, can result in accumulation of glucuronides and other drug metabolites (15, 16, 137, 138). In contrast, Z was found to yield significant levels of its conjugate, ZG, in plasma of healthy human volunteers after an oral dose of Z (25, 26, 113) (Chapter VI). ZG was also present in the plasma of rhesus monkeys at concentrations similar to Z after an oral dose of Z (98) (Chapter IV).

ZG has not yet been reported to reach appreciable concentrations in plasma of other animals after a dose of Z. The elimination of Z in most other animals reported thus far was primarily by oxidative metabolism to yield hydroxyzomepirac (HMZ) and p-chlorobenzoic acid (CBA) (26) (Fig. III-1). An exception was the rabbit which excreted 3.5% of a ^{14}C-labelled Z dose in the urine as ZG and 66% as Z (26). Based on our previous stability studies conducted with ZG (Chapter IV), it was not certain whether the Z reported to be in the rabbit urine was excreted as such or formed from the hydrolysis of ZG in the urine due to the high pH of urine among herbivores. Preliminary experiments were performed with the rabbit to determine its suitability as an animal model simulating Z and ZG disposition in human. Subsequently, the guinea pig was examined as a potential animal model, since no previous reports had studied the disposition of Z in the guinea pig and the animal is known to have high UDP-glucuronyltransferase levels and to glucuronidate many compounds efficiently (139).

As previously discussed in Chapter IV, acyl glucuornides are known to be unstable in buffer and in biological fluids at physiological pH (98). Drugs which form ester glucuronides have decreased clearance in renal failure, e.g. clofibric acid (29, 30), diflunisal (21, 118) and ketoprofen (8, 31, 140). In contrast, acetaminophen (17) and oxazepam (19, 119) which form phenolic glucuronides exhibit no change in their clearances in patients with renal failure even though the conjugates accumulate to high concentrations during renal failure. Gugler first proposed a futile cycle scheme (141) to explain the disposition of clofibric acid in renal failure (30). A similar futile cycle is depicted for Z and ZG in Figure V-1. The possiblilty of the hydrolysis

of acyl glucuronides *in vivo* has now been discussed by several investigators as a mechanism to explain the decreased clearances of acidic drugs in renal failure when the drug is eliminated almost exclusively by glucuronidation (8, 24, 29, 111, 140). Initially, the rationale proposed to explain the discrepancy observed between phenolic and ester glucuronides was that the labile ester conjugate was chemically hydrolyzed *in vivo*; while the phenolic conjugate was stable. However, Meffin *et al.* (111) found that elimination and cleavage of clofibric acid glucuronide by the rabbit *in vivo* was extremely rapid with a half-life about 2 min. He proposed that this rapid elimination was due to hydrolysis of the ester glucuronide by esterases (111). Recently, after the studies presented here had been completed, Rowe and Meffin (9) also showed that an irreversible inhibitor of esterases, diisopropylfluorophosphate (DFP), caused an increase in clofibric acid clearance by the rabbit. The increased clearance of the parent drug was presumably caused by DFP inhibiting the hydrolysis of clofibric acid acyl glucuronide by esterases. This report by Meffin *et al.* (9), however, only provides indirect evidence to support their hypothesis because the authors did not measure the plasma concentrations of the glucuronide. Moreover, in their experiments only clofibric acid, not the acyl glucuronide, was administered intravenously with concurrent DFP. This is unfortunate, as Meffin *et al.* (111) have previously documented the isolation of clofibric acid glucuronide and utilized the conjugate in animal studies.

To examine the disposition of ZG *in vivo* it was necessary to find an appropriate small animal in which plasma levels of the conjugate are measurable. In an effort to enhance plasma concentrations of ZG and to

reduce the nonlinearities in the plasma profiles of Z caused by irregular emptying of the gallbladder and subsequent enterohepatic cycling (142, 143), the bile ducts of the guinea pigs were ligated during surgical preparation. The ability to inhibit esterases *in vivo* was also desired in order to test the hypothesis that nonspecific esterases were involved in hydrolyzing ZG. Rowe and Meffin (9) used intraveneous administration of DFP to inhibit esterases in the rabbit *in vivo*. However, in their experiments it was necessary to titrate the rabbit with atropine and to artificially ventilate the animal because DFP also irreversibly inhibits acetylcholinesterase (144). Prior to the publication of Rowe and Meffin (9), we had selected phenylmethylsulfonyl fluoride (PMSF) as the esterase inhibitor for our *in vivo* studies. PMSF was found in our *in vitro* studies to inhibit the esterases responsible for the cleavage of ZG in guinea pig liver homogenate (Chapter IV), and more importantly, PMSF is much less toxic than DFP. During investigations directed toward finding possible inhibitors of esterases for treating pancreatitis Turini, Singer *et al.* (145) found that PMSF was not fatally toxic to mice or rats until a dose of 200 mg/kg was administered. We could therefore dose PMSF to the guinea pigs without other life supportive treatments during the experiment.

B. METHODS AND MATERIALS

Chemicals

Zomepirac·Na·$2H_2O$ and 5-(4-methoxybenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid, the internal standard (IS), were gifts from McNeil Pharmaceuticals (Springhouse, PA). Zomepirac glucuronide (ZG) was purified from human urine as previously described (28) (Chapter II).

Phenylmethylsulfonyl fluoride (PMSF), mannitol and sodium taurocholate (40%, ox bile) were obtained from Sigma (St. Louis, MO). The anesthetics, ketamine (Vetalar®, Parke-Davis, Morris Plains, NJ) and acepromazine maleate (Med-Tech, Elwood, KS) were purchased through the UCSF Animal Care Facility. Solvents used for analysis were HPLC grade. All other chemicals were reagent grade.

Analytical methods

For the analysis of Z, ZG and isomers of ZG, blood samples from animals were rapidly centrifuged at 12,500 x g using an Eppendorf 5412 centrifuge. Then the plasma was separated and buffered to pH 2-4 with 0.01 parts concentrated phosphoric acid. The time from blood sampling to pH adjustment was less than 90 seconds. After buffering, plasma samples were frozen at -20 °C. Plasma samples were assayed by HPLC (107) using the method described in Chapter III which was further modified for smaller sample sizes (0.1 - 0.2 ml). Because of the limited availability of ZG, standard curve samples were not routinely prepared for the conjugate. Instead, since the ratio of the standard curve slopes (ZG/Z) was similar to the ratio of molecular weights (ZG/Z), the standard curve for Z was used to estimate ZG concentrations (in Z equivalents).

Experimental protocol for the rabbit

Trial studies of Z and ZG disposition in the rabbit were conducted with healthy, male New Zealand White rabbits (Simonson, Gilroy, CA) which weighed about 3 kg. Rabbits were fed a standard laboratory diet, then fasted overnight with water ad lib. prior to drug administration.

Following containment of the animal in a clear plastic restraining device (Plas-Labs, Lansing, MI), the marginal ear vein was cannulated by inserting an Intracath® catheter (Deseret, Sandy, UT) about two inches into the vein until blood flowed readily. The catheter was kept patent by flushing it periodically with a solution of 10 U heparin/ml normal saline. Urine was collected by catheterization of the urinary bladder with a size 7F Swan-Ganz® catheter (Edwards Labs, Santa Ana, CA). Solutions of Z (20 mg/ml), or ZG (dose in 1 ml), in 40% propylene glycol were administered I.V. through a marginal ear vein of the ear not cannulated for blood sampling. After adminstration of Z or ZG, blood samples (ca. 1.5 ml) were collected periodically at intervals up to 180 minutes. The initial studies of Z and ZG disposition in rabbit included three individual doses of Z and two doses of ZG using three rabbits.

Experimental protocol for the guinea pig

Studies of Z and ZG disposition were conducted using adult, male guinea pigs (500-1000 gm) (EZH, Williams, CA). Guinea pigs were fed a standard laboratory diet, but were fasted overnight prior to surgical preparation. Initial studies were done to determine the pharmacokinetics of Z and ZG in guinea pigs without bile duct cannulation or ligation. Then the effects of bile ligation and cannulation were examined. Finally, the effects of a concurrent dose of PMSF on the pharmacokinetics of Z and ZG were studied in bile ligated guinea pigs.

Surgical preparation of the guinea pigs was necessary in order to cannulate or ligate the bile duct, collect urine at designated intervals and to provide the ability to collect blood samples rapidly. A conscious, post surgical preparation was not considered, as guinea pigs

do not tolerate ligation of the bile duct and often die within 24 hours after such ligation (146). Anesthesia was initiated with intramuscular (IM) injection of ketamine:acepromazine (75:2 mg/kg) into the thigh. Induction of anesthesia required about 15 minutes, after which surgical manipulation could begin. The animal was restrained after anesthesia with its ventral side up on a 4 x 8 inch test tube rack with rubber bands employed to retract the limbs of the animal. Body temperature of the animal was maintained by using a heat lamp (250 watts) at a distance of about 12 inches. Areas of the animals body to be surgically manipulated were shaven and cleansed with Betadine® (povidone-iodine). To assist anesthesia, intradermal injections of a 2% lidocaine solution were made along incision sites prior to surgery. To prevent loss of urine, the glans penis was tied with suture material. A 2 cm abdominal incision was cut through the skin anterior to the penis; fat, muscle and connective tissue were dissected to expose and withdraw the urinary bladder. After drainage with a syringe, a small incision was made in the urinary bladder for insertion of a catheter of PE-90 tubing (polyethylene, Clay Adams, Parisippany, NJ) with a flame flanged tip. The catheter was tied in place at the lower, ureter end of the bladder to minimize bladder void volume.

Catheters were placed in the jugular vein and carotid arteries to infuse solutions and withdraw blood, respectively. A 4 cm incision was made along the left side of the neck; fat and tissue were dissected clear to expose the swollen external jugular vein which was then cannulated with PE-50 tubing and flushed with normal saline. The carotid artery was then exteriorized, cannulated and kept patent with a solution of 10 U/ml heparin in normal saline. All animals recieved a 5

cm midline incision along the linea alba below the sternum. The gallbladder was emptied using a syringe. After being isolated and cleared, the bile duct was either ligated, cannulated (PE-90) or sham manipulated. The cystic duct was ligated just below the gallbladder to prevent hepatic bile from entering the gallbladder, though this was not done for sham treated, control animals.

Surgical manipulation usually took about 60 minutes after induction of anesthesia. Anesthesia was maintained with the same ratio of ketamine:acepromazine as used for induction, but only one third the induction dose was needed. The time interval required for dosing of maintenance anesthetic varied considerably between animals and was monitored by the withdrawal response of the guinea pig to a pinching stimulus to the paw and/or the gnawing reflexes of the jaw. Animals with ligated bile ducts required less frequent dosing of maintenance anesthetic, which was presumably due to reduced biliary excretion of the anesthetic. Anesthesia was usually maintained for about seven to eight hours, since six hours was the normal length of time for blood sampling after a dose was given.

Bile duct ligated guinea pigs treated with PMSF were given a 50 mg dose of PMSF intraperitoneally (IP) as a suspension in 1 ml 3% Lo-CMC (carboxymethyl-cellulose). This was given after surgical preparation, 30 minutes prior to the Z or ZG dose. Controls animals received only the suspending agent.

Solutions of Z were prepared for I.V. administration by dissolving $Z \cdot Na \cdot 2H_2O$ in sterile normal saline to yield a concentration of 5 mg/ml. The solution was sterilized by passing it through a 0.22 μ pore size Milex® filter (Millipore, Bedford, MA). Solutions of Z were stable

for several months after preparation. A 3 or 5 mg Z dose was given to each animal when the disposition of Z was studied. ZG is less stable than Z, and was not available as its sodium salt. The poor solubility of ZG in water precluded the use of an aqueous solution for injection of ZG. Instead, about 5 mg (Z equivalents) of ZG were weighed individually when needed, dissolved in 0.5 ml ethanol and injected I.V. over one minute. Several microliters of the ZG solution was dissolved in 30% acetonitrile/acetate buffer, pH 2.5, for later determination of purity and content by HPLC.

Following I.V. injection of Z to the guinea pig, serial blood samples (0.5 ml) were collected for up to six hours. The elimination half-life for Z was about 2 hours in guinea pigs with patent bile ducts, so only a few samples were obtained in the first hour. When examing for the evidence of irreversible binding of Z to plasma proteins (Chapter VII), 2 ml blood samples were obtained at 10 minutes, 2, 4 and 6 hours. Even when these larger quantities of blood were obtained, the total volume of blood withdrawn from the animal was only 15 ml over the course of the study. Because of the rapid loss of ZG *in vivo*, blood samples (0.5 ml) were obtained frequently during the first 30 minutes after an I.V. dose of ZG. Afterward, sampling frequency was reduced to examine the disposition of Z liberated from ZG by hydrolysis.

Urine samples were collected at half hour intervals in early experiments, then at one hour or two hour intervals in latter studies. The urinary bladder was flushed with a sterile solution of 0.15 M sodium acetate, pH 4.0, to assure complete collection of urine and also to decrease the urine pH so that hydrolysis or acyl migration of ZG was minimized. Once collected, urine was adjusted to pH 2-3 with phosphoric

acid then stored at -20 °C prior to analysis. In an effort to maintain urine flow, 5% mannitol in normal saline was infused through the jugular vein using a Harvard Pump. Several flow rates (5-20 ml/hr) were tried, however, none appeared to enhance the urine flow. It appeared that surgical manipulation and anesthesia adversely affected renal function and urine output. This was more evident when the urinary excretion data of Z and ZG were analyzed later.

When bile was collected it was allowed to drain into a vial containing acetonitrile and small amounts of phosphoric acid to prevent loss of ZG through its rearrangement to other isomers. Initial studies where bile acids lost in the collected bile were replaced with an infusion of taurocholate produced unsatisfactory results. The crude taurocholate from ox bile may be causing an immune response in the guinea pig, as death due to respiratory difficulty occurred several hours after the start of the taurocholate infusion.

Protein binding

The reversible protein binding of Z in guinea pig plasma was determined by equilibrium dialysis. Blank plasma obtained from a guinea pig after surgical preparation was added to 5-10 U/ml of heparin. The same animal was then given 50 mg PMSF IP and the "PMSF treated" plasma was collected 30 minutes after the PMSF dose. Besides possible effects of PMSF on the plasma protein binding of Z, the duration of bile ligation or anesthesia may also affect the protein binding due to an accumulation of endogenous compounds as seen in renal failure (137, 147). To examine the possible effect of bile ligation on protein binding, plasma was harvested from two different animals which had

completed experiments with ligation of the bile ducts for longer than five hours. One animal had received PMSF, the other was a control animal which had not been given PMSF. Both animals had received a ZG dose. Equilibrium dialysis was done using 0.5 ml half cells separated by a Spectrapor® dialysis membrane (Spectrum Medical Ind., Los Angeles, CA) which had a molecular weight cutoff of 12-14K. The membrane was delipidated with 15% ethanol, washed with water, then allowed to soak overnight in 0.13 M sodium phosphate buffer, pH 7.40 prior to use. ^{14}C-Zomepirac·Na·H_2O with specific activity of 11.7 μCi/mg (322 μCi/mmole), and purity >98% by HPLC was diluted with unlabelled Z to provide about 80,000 dpm/ml at the desired concentration of 20 μg/ml in buffer. Plasma was dialyzed against 0.13 M phosphate buffer, pH 7.4, at 37 °C with the ^{14}C-labelled Z initially added to the buffer side of the dialysis cell. Pilot studies indicated that dialysis equilibrium was attained in less than three hours, which agreed with the previous report of rapid dialysis of Z (148, 149). Previous studies had also indicated that Z binding to plasma proteins was not concentration dependent from 0.1 to 10 μg/ml in plasma samples from human, rat, mouse and monkey (148). In plasma from rat and monkey, Z binding at 20 μg/ml was similar to that at 10 μg/ml, but at 50 μg/ml in monkey plasma, Z did start to exhibit an increase in free fraction as compared to that at lower concentrations (148). The subsequent dialysis experiments were therefore carried out by incubating the samples for six hours with 20 μg/ml Z initially on the buffer side of the dialysis cell. Aliquots (0.25 ml) taken from each side of the dialysis cell were digested with 0.25 ml of 1 M sodium hydroxide overnight, neutralized with 0.075 ml glacial acetic acid, then mixed with 5 ml scintillant (Aquasol®, New

England Nuclear, Boston, MA). Counting to 1% precision was done using a scintillation counter (Beckman, Mountain View, CA). The free, unbound fraction (α) of Z in plasma was calculated as:

$$\alpha = \frac{^{14}C \text{ CPM Buffer}}{^{14}C \text{ CPM Plasma}}$$

The protein binding of ZG was not determined, as ZG is unstable in plasma and would degrade during the time required for equilibrium dialysis.

Data analysis

When analyzing the data obtained from plasma and urine, Z and ZG are both expressed in Z equivalents. Area under the plasma concentration vs time curve (AUC) for the animal data was estimated using the linear trapezoidal approximation (150). The concentration at zero time was extrapolated assuming log-linearity of the plasma concentration vs time plot during the first 30 minutes. The terminal AUC from the last data point extrapolated to infinite time was estimated by $C_{p,last}/\beta$, where β is the terminal log-linear slope of the plasma concentration vs time curve estimated by use of the last two or three data points. Total plasma clearance, CL, was determined as CL=Dose/AUC, where both AUC and Dose were expressed in Z equivalents. Where indicated, AUC were normalized to a 5mg/kg dose and CL was normalized to body weight and expressed as such.

Because ZG may hydrolyze to Z *in vivo* and Z is then metabolized to ZG again *in vivo*, the dispositional relationship between Z and ZG can be described as reversible metabolism. Figure V-1 depicts the futile

cycle between Z and ZG which can be simplified to the following pharmacokinetic model:

Figure V-2. $\xleftarrow{CL_{10}}$ Z $\underset{CL_{21}}{\overset{CL_{12}}{\rightleftarrows}}$ ZG $\xrightarrow{CL_{20}}$

The clearance terms are expressed with numbers to provide more clarity to the equations derived from the model and also to conform with the notation employed by Hwang et al. (151) in an article describing and deriving equations for reversible metabolism. Equations defining reversible metabolism which have been described by other authors (152, 153, 154) will not be derived here. By administration of drug and metabolite on separate occassions with measurement of both compounds in plasma, the individual clearance terms described by the model in Figure V-2 can be determined (151):

V-i. $CL_{10} = (AUC_2^M * D - AUC_2^D * M)/DENOM$

V-ii. $CL_{20} = (AUC_1^D * M - AUC_1^M * D)/DENOM$

V-iii. $CL_{12} = M * AUC_2^D/DENOM$

V-iv. $CL_{21} = D * AUC_1^M/DENOM$

where, D = dose of the parent drug

M = dose of the metabolite (in units of D equivalents)

AUC_1^D = AUC of the parent drug when dosing the parent drug

AUC_2^D = AUC of the metabolite when dosing the parent drug

AUC_2^M = AUC of the metabolite when dosing the metabolite

AUC_1^M = AUC of the parent drug when dosing the metabolite

$DENOM = AUC_1^D * AUC_2^M - AUC_2^D * AUC_1^M$

The fraction of an administered ZG dose which is eventually hydrolyzed to Z ,f_H, is determined by:

$$f_H = (CL_z * AUC'_z)/Dose'_{zg}$$

where the dose of ZG, $Dose'_{zg}$, is expressed in Z equivalents, CL_z is the apparent total clearance of Z and is determined from previous administration of Z to other animals, and AUC'_z is the AUC of the parent drug produced after a dose of the acyl glucuronide, ZG (Appendix V-1).

Comparison of data from control vs treated animals, where treatments were the effect of bile duct ligation or concurrent administration of PMSF, was done using a t-test (155). The use of equations V-i through V-iv to determine clearances in a reversible system is more complex and estimates of error in the use of these equations will be included in the discussion (Appendix V-2).

C. RESULTS

Disposition of Z and ZG in the rabbit

When Z was administered to rabbits at doses of 2.5 and 5.0 mg/kg, the only compound detectable in plasma was Z. There was no measurable ZG present in the plasma. The clearance of Z from plasma was 0.78 ± 0.13 ml/min/kg (mean ± s.d., n=3) and the average half-life of Z varied from 71 to 115 minutes. When I.V. doses of ZG (4.99 and 7.88 mg of Z equivalents) were given to two rabbits the conjugate was cleared very rapidly such that the half-lives observed were 3.7 and 8.7 minutes, respectively (Fig. V-3). The total plasma clearance of ZG averaged 34.1 ml/min/kg (n=2) which was 44 fold greater than the average clearance of Z in the rabbit. The AUC_z's after ZG dosing to the rabbits were 550 and 1386 µg-min/ml, respectively, for the two doses given. Using the plasma

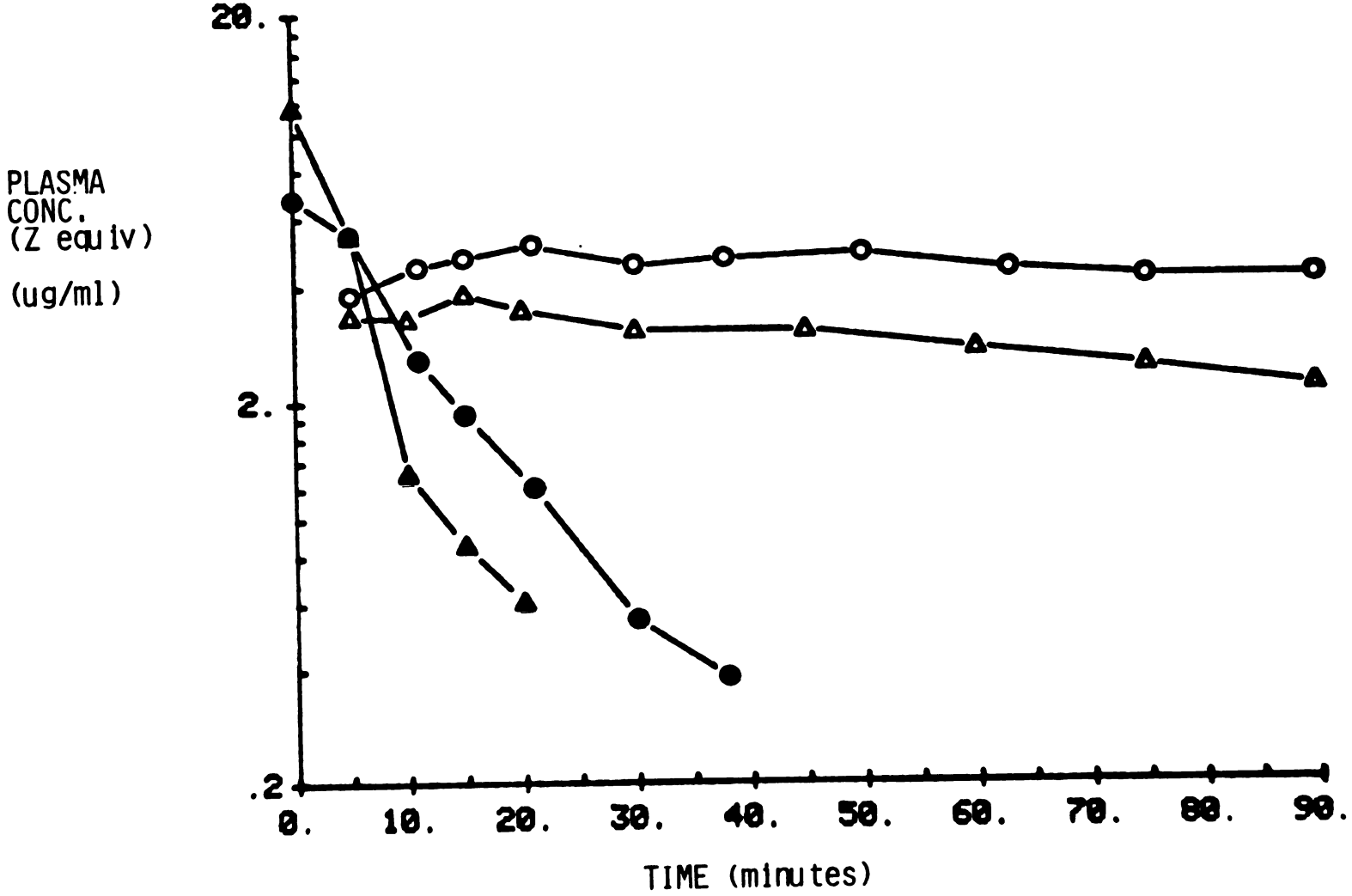


Figure V-3. Disposition of zomepirac glucuronide (closed symbols) after an intravenous dose to two rabbits, (dose normalized to 5 mg zomepirac). Open symbols = zomepirac; circles = rabbit #2; triangles = rabbit #3.

clearance of Z which had been previously determined in the same rabbit, the fractions of ZG dose hydrolyzed, f_H, were 22 and 41%, respectively. Although ZG was not detected in plasma after an intravenous dose of Z, a small fraction (<10%) of the Z dose excreted in the urine was present as ZG. Thus, the urinary excretion data of Z and ZG obtained for the rabbit does agree with that previously published (26).

Disposition of Z and ZG in the anesthesized guinea pig

The data for the disposition of Z in guinea pigs which did not have ligated or cannulated bile ducts are presented in Table V-1. The plasma clearance of Z after a 3 mg dose was 1.47 ± 0.45 ml/min/kg (mean ± s.d., n=5). As with the rabbits, there was no ZG observed in the plasma of guinea pigs after IV dosing of Z. The urinary excretion of the Z dose by the guinea pig was almost entirely present as its conjugate, ZG. The absolute amount of ZG excreted into the urine varied considerably between animals and the excretion rate was usually lower during the first hour of urine collection than during subsequent intervals. The variablility of urinary excretion was much greater than the measured plasma clearance which suggests that the surgical preparation of the animal had an adverse effect on renal function. As a result, urinary excretion data was of little value and will not be reported here.

Taurocholate obtained from ox bile was infused into several guinea pigs as a supplement for bile acids lost when the bile duct was cannulated for bile collection. The taurocholate was apparently toxic to the guinea pigs, causing respiratory distress and eventual death one to three hours after the start of the infusion. However, it was possible to collect bile for one to several hours from four animals

Table V-1. The plasma clearance of zomepirac (Z) in guinea pigs with patent bile ducts after a 3 mg I.V. dose of zomepirac.

Animal	Wt. (kg)	AUC[a](0-∞) (μg-min/ml)	CL_z (ml/min)	CL_z (ml/min/kg)
10-4A	0.500[b]	3280	0.862	1.72
10-7B	0.500[b]	5842	0.515	1.03
12-9B	0.517	3891	0.771	1.49
12-14A	0.512	2825	1.06	2.07
12-16A	0.561	5080	0.509	1.05
mean	0.518	4224	0.743	1.47
±s.d.	0.025	1222	0.236	0.45

[a]Dose and AUC are expressed in Z equivalents.
[b]Weights for animals 10-4A and 10-7B are estimates.

given an I.V. dose of Z with supplementation of bile acids. The fraction of administered Z dose excreted into the bile during the first hour was 36% ± 20% (mean ± s.d.) with a range from 16 to 61% of the dose. The bile collected contained almost equal concentrations of Z and its conjugates, with a significant fraction of the conjugates present as the isomeric conjugates of ZG. Thus, it appears that a large fraction of Z and its conjugates are rapidly excreted into the bile of guinea pigs which do not have the bile duct ligated.

ZG was administered on two occasions to guinea pigs which had not had their bile ducts ligated. Similar to that shown in Figure V-3 for the rabbit, ZG was rapidly cleaved in vivo by the guinea pig with liberation of free Z. The clearances of ZG were 38 and 79 ml/min/kg, respectively for the two animals; much higher than the clearance of Z. There was no ZG measurable in plasma after the initial ZG dose had been cleared even though Z plasma concentrations remained elevated for several hours.

Since ZG was undetectable in plasma of guinea pigs after an I.V. dose of Z and a large fraction of the dose was excreted in the bile as ZG and its conjugates, the bile ducts of guinea pigs were ligated in an effort to achieve measurable concentrations of ZG in plasma and also to reduce nonlinearities in the plasma profiles caused by enterohepatic cycling. As shown in Figure V-4 and in the 100 to 360 minutes portion of Figure V-6, ZG levels were detectable in plasma of bile duct ligated guinea pigs when Z concentrations were high. After an I.V. dose of Z was given to the bile duct ligated guinea pigs without PMSF the AUC_{zg} was only about 6% of that observed for AUC_z (Table V-2). When ZG was given I.V. to bile duct ligated guinea pigs, it was rapidly cleaved in

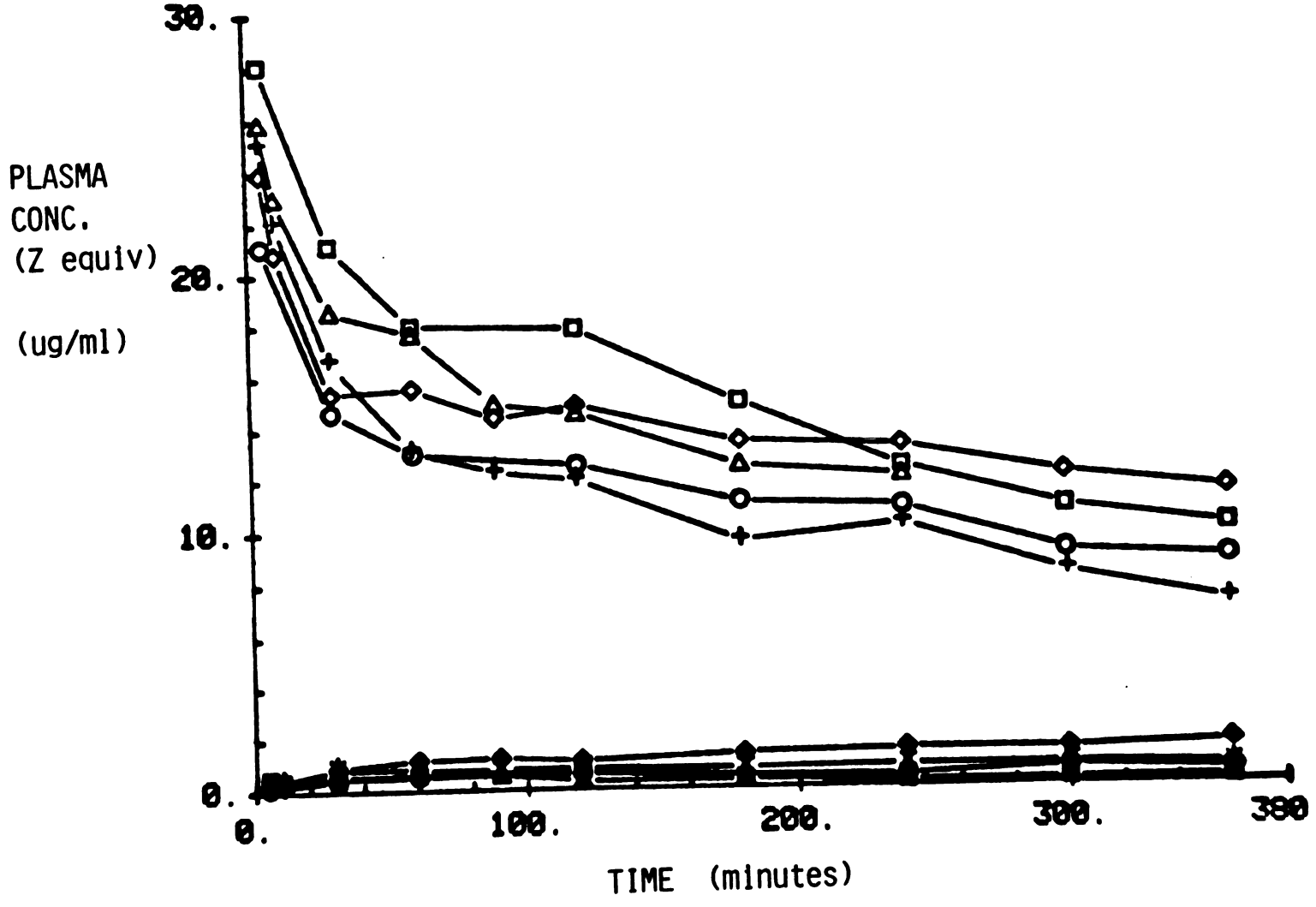


Figure V-4. The disposition of zomepirac (open symbols) and zomepirac glucuronide (closed symbols) in the guinea pig after an intravenous dose of zomepirac to five animals not given PMSF. Data are normalized to a 5 mg/kg dose.

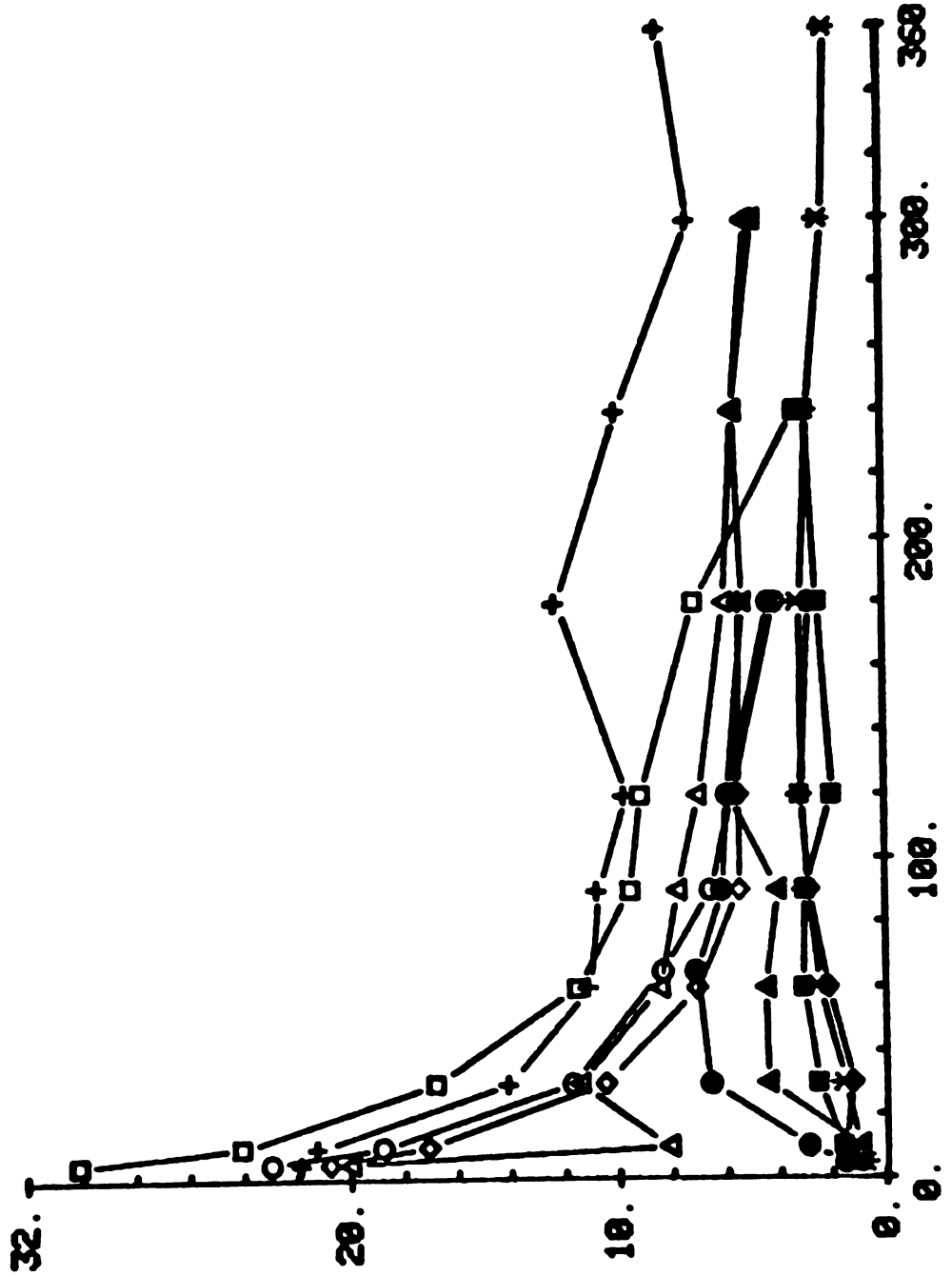


Figure V-5. The disposition of zomepirac (open symbols) and zomepirac glucuronide (closed symbols) in the guinea pig after an intravenous dose of zomepirac to five animals given PMSF concurrently. Data is normalized to a 5 mg/kg dose.

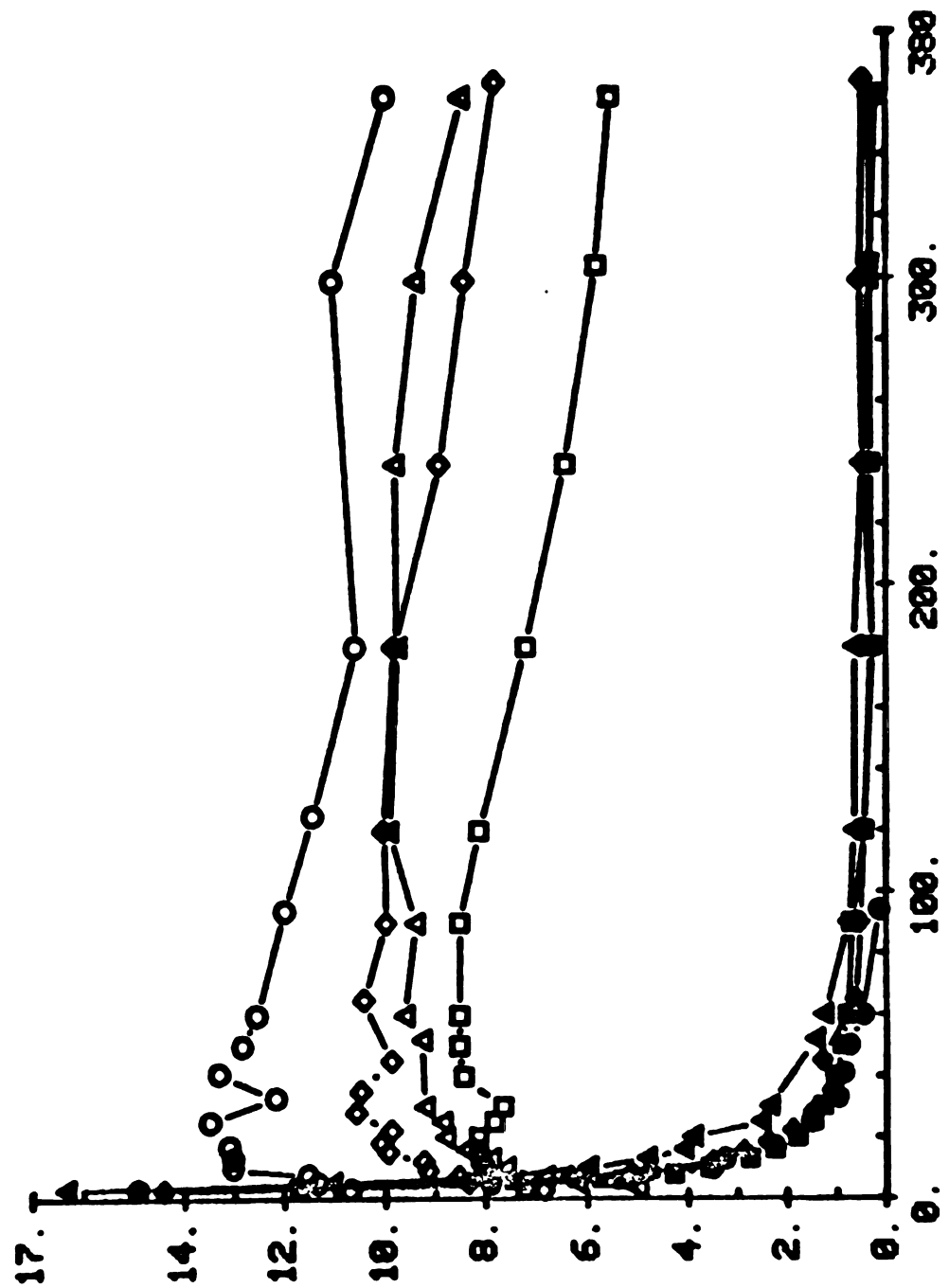


Figure V-6. The disposition of zomepirac glucuronide (closed symbols) and zomepirac (open symbols) in the guinea pig after an intravenous dose of zomepirac glucuronide to animals not given PMSF. Data is normalized to a 5 mg/kg dose of zomepirac glucuronide when expressed in zomepirac equivalents.

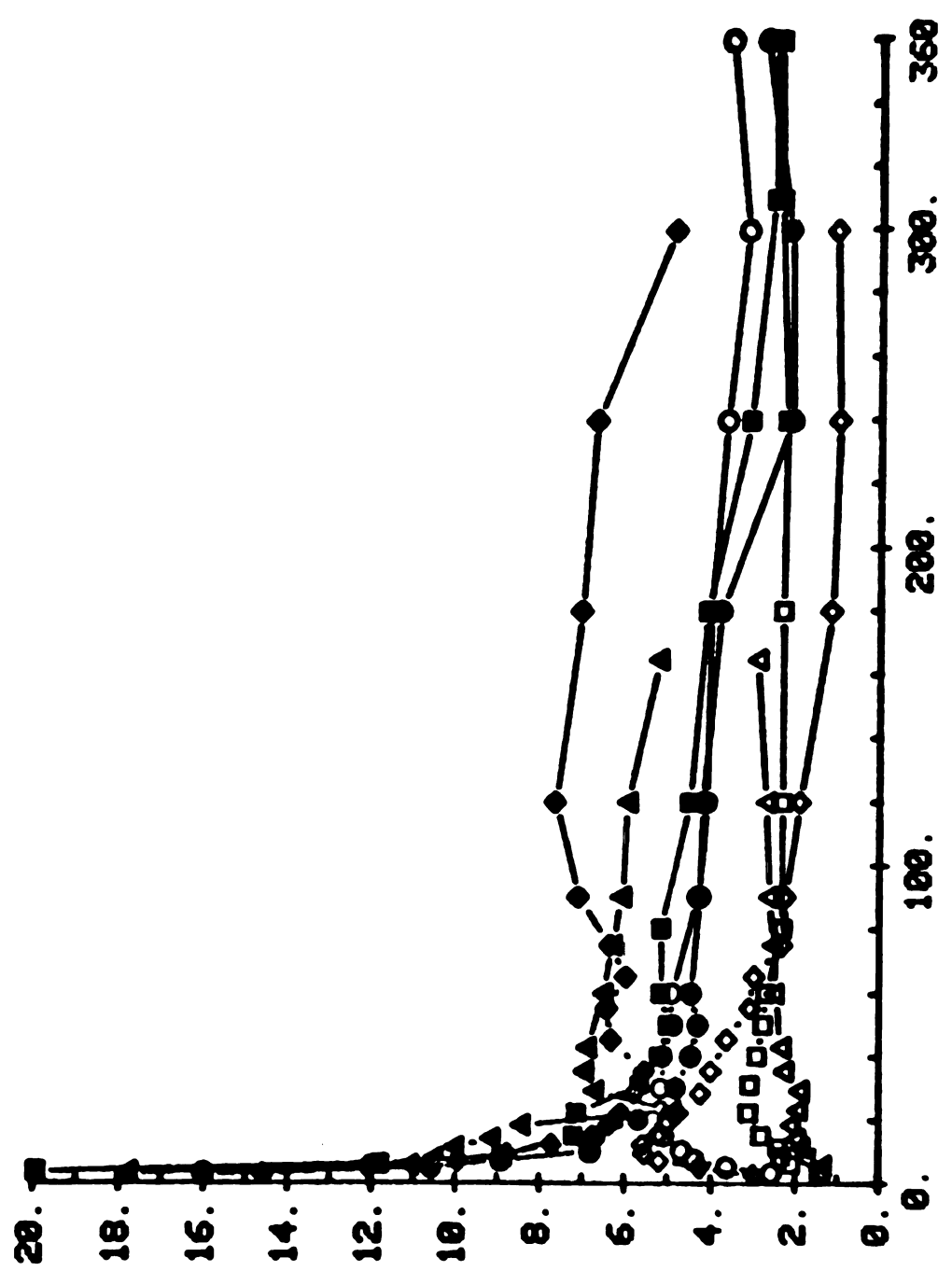


Figure V-7. The disposition of zomepirac glucuronide (closed symbols) and zomepirac (open symbols) in the guinea pig after an intravenous dose of zomepirac glucuronide to animals given PMSF concurrently. Data are normalized to a 5 mg/kg dose of zomepirac glucuronide when expressed in zomepirac equivalents.

Table V-2. The plasma clearance of zomepirac (Z) and the formation of zomepirac glucuronide (ZG) in guinea pigs with ligated bile ducts after a 5 mg intraveneous dose of zomepirac.

Animal	PMSF	Wt. (kg)	AUC[a](0-∞) (μg-min/ml) Z	ZG	AUC ratio ZG/Z	CL_z (ml/min)	CL_z (ml/min/kg)
8-4A	-	0.772	11,936	598	0.050	0.419	0.543
8-4B	-	0.882	12,745	236	0.019	0.392	0.445
8-14A	-	0.875	12,910	269	0.021	0.387	0.442
8-14B	-	0.961	14,788	1,769	0.120	0.338	0.352
8-24B	-	0.812	7,916	573	0.072	0.632	0.778
mean		0.860	12,059	689	0.056[b]	0.434	0.512[b]
±s.d.		0.072	2,541	626	0.042	0.115	0.163
8-11A	+	0.931	2,355	1,945	0.826	2.12	2.28
8-11B	+	0.930	2,949	992	0.336	1.70	1.82
8-17A	+	0.725	5,473	4,218	0.771	0.914	1.26
8-17B	+	0.929	3,478	1,455	0.418	1.44	1.55
8-24A	+	0.878	7,558	1,750	0.232	0.662	0.753
mean		0.879	4,363	2,072	0.517[b]	1.37	1.53[b]
±s.d.		0.089	2,136	1,252	0.226	0.589	0.58

[a]Dose and AUC are expressed in Z equivalents.
[b]Statistically different between control and treated, $p < 0.005$.

Table V-3. The plasma clearance of zomepirac glucuronide (ZG) and the formation of zomepirac (Z) in guinea pigs with ligated bile ducts after an intraveneous dose of zomepirac glucuronide.

Animal	PMSF	Wt. (kg)	Dose[a] (mg)	AUC[a](0-∞) (μg-min/ml) Z	AUC (μg-min/ml) ZG	AUC ratio Z/ZG	CL_{zg} (ml/min)	CL_{zg} (ml/min/kg)
9-1B	-	0.589	3.14	10,885	203	0.019	15.5	26.2
9-21A	-	0.423	3.81	12,501	775	0.062	4.92	11.6
9-21B	-	0.475	4.67	16,392	1137	0.069	4.11	8.65
9-22A	-	0.630	6.28	21,522	1438	0.067	4.37	6.93
mean		0.529	4.48	15,325	888	0.054[b]	7.23	13.3[b]
±s.d.		0.097	1.36	4,734	531	0.024	5.53	8.8
9-1A	+	0.585	3.91	3494	2997	0.858	1.30	2.23
9-12A	+	0.637	4.56	2163	3203	1.48	1.42	2.23
9-18B	+	0.463	4.31	2561	5344	2.09	0.807	1.74
9-22B	+	0.645	5.77	1656	6500	3.93	0.888	1.38
mean		0.583	4.64	2469	4511	2.09[b]	1.11	1.90[b]
±s.d.		0.084	0.80	778	1698	1.33	0.30	0.41

[a]Dose and AUC are expressed in Z equivalents.
[b]Statistically diffferent between control and treated, $p < 0.025$.

vivo to yield free Z (Fig. V-6) as was previously observed for the guinea pigs with patent bile ducts. The apparent plasma clearance of ZG in the bile duct ligated group was 13.3 ± 8.8 ml/min/kg, much higher than that of Z (0.512 ± 0.163 ml/min/kg). Both the apparent plasma clearance of Z (Table V-2) and ZG (Table V-3) decreased in bile duct ligated guinea pigs as compared to the animals without ligation (Table V-1). Apparent clearance of Z decreased 65% with bile duct ligation from 1.47 ± 0.45 to 0.512 ± 0.163 ml/min/kg. The apparent plasma clearance of ZG appeared to decline even further, from 38 and 79 ml/min/kg in the two animals examined with patent bile ducts, to 13.3 ± 8.8 ml/min/kg (n=4) in those animals with ligated bile ducts. Although the small sample size makes comparison difficult, the ratio of ZG clearance to that of Z remained very high in animals with bile duct ligation.

The IP administration of the esterase inhibitor, PMSF, caused a marked alteration upon the disposition of both Z and ZG. Comparing Figures V-4 and V-5 it is evident that PMSF increased the rate of Z loss after an I.V. dose of Z. The apparent plasma clearance of Z increased 3 fold by concurrent PMSF, from 0.51 to 1.53 ml/min/kg (Table V-2) for bile duct ligated guinea pigs. The increased rate of loss of Z due to PMSF was accompanied by a large increase in the concentrations of ZG detected in the plasma. With concurrent dosing of PMSF the concentrations of Z and ZG are similar at 200 to 300 minutes after the Z dose (Fig. V-5). When comparing AUC_{zg} between control and PMSF treated experiments after an I.V. dose of Z, the AUC_{zg} increased 3 fold when the esterase inhibitor was added (Table V-2).

Table V-4. The fraction of an intraveneous dose of zomepirac glucuronide (ZG) hydrolyzed to yield Z after administered to the bile ligated guinea pig.

Animal	PMSF	Wt. (kg)	ZG Dose[b] (mg)	CL_z[c] (ml/min/kg)	AUC_z[b] (μg-min/ml)	f_H[a]
9-1B	-	0.589	3.14	0.512	10,885	1.04
9-21A	-	0.423	3.81	0.512	12,501	0.711
9-21B	-	0.475	4.67	0.512	16,392	0.854
9-22A	-	0.630	6.28	0.512	21,522	1.11
mean ± s.d.						0.929 ± 0.181[d]
9-1A	+	0.585	3.91	1.53	3494	0.799
9-12A	+	0.637	4.56	1.53	2163	0.462
9-18B	+	0.463	4.31	1.53	2561	0.421
9-22B	+	0.645	5.77	1.53	1656	0.283
mean ± s.d.						0.492 ± 0.219[d]

[a]Fraction hydrolyzed, f_H, is described in the text and derived in Appendix V-1.
[b]Dose of ZG and AUC_z are expressed in Z equivalents.
[c]CL_z is the mean value obtained from the Z dose study (Table V-2).
[d]Statistically different from control animals, $p < 0.01$.

Table V-5. Plasma protein binding of zomepirac (Z) in control and PMSF treated guinea pigs and the effect of duration of bile ligation and anesthesia on the protein binding.

	Z free fraction[a], α	
Treatment	0.5 - 1.0 hr post ligation	5.5 - 6.5 hr post ligation
Without PMSF	0.0655 ± 0.0041	0.224 ± 0.008
With PMSF	0.0804 ± 0.0024	0.159 ± 0.016

[a]n=5, mean ± s.d.

To determine the effect of PMSF on the disposition of ZG, more information can be obtained by administration of ZG itself than by simply following ZG formed after a dose of Z. The contrast between Figures V-6 and V-7 indicates the decreased rate of ZG hydrolysis when PMSF is given prior to an I.V. dose of ZG. In Figure V-7 the treatment with PMSF resulted in ZG concentrations remaining higher than those of Z during most of the experiment. The apparent plasma clearance of ZG, CL_{zg}, decreased 86% by concurrent PMSF administration (Table V-3). The fraction of an I.V. dose of ZG which was eventually cleaved *in vivo* to yield Z was decreased to almost half of its control value by giving PMSF concurrently (Table V-4). Without PMSF 93 ± 18% of the ZG dose was hydrolyzed *in vivo*; while with PMSF the fraction hydrolyzed was reduced to 49 ± 22% of the dose.

Anesthesia or bile duct ligation may have an effect on the protein binding of Z and subsequently the elimination of Z. These possible effects and the effect of PMSF on the reversible binding of Z to guinea pig plasma proteins were examined and the results are summarized in Table V-5. The duration of bile duct ligation or anesthesia did increase the free fraction of Z, but PMSF had no consistent effect on Z protein binding at either the early or later times after ligation of the bile duct and induction of anesthesia (Table V-5).

D. DISCUSSION

Based upon the initial studies, the rabbit was not considered as a potential animal model for simulating Z and ZG disposition in human since no ZG was observed in rabbit plasma after a 5 mg/kg dose of Z to male rabbits. The clearance of Z obtained for the rabbit, 0.78 ± 0.13

ml/min/kg, was higher than that reported by Grindel *et al.* (26) which was 0.36 ± 0.04 ml/min/kg. Grindel *et al.* used female rabbits given a 1 mg/kg dose of Z. Although there have been no reports of dose dependence clearance of Z, the former studies in animals did find that female rats had a slightly but statistically significant lower clearance of Z as compared to male rats (26). Conscious rabbits hydrolyzed ZG rapidly to Z after an I.V. dose, which was similar to the finding for clofibric acid glucuronide in rabbits (111). Estimates for the fraction of ZG dose hydrolyzed *in vivo*, f_H, were 22 and 41% in the two rabbits indicating that much of the conjugate dose has been rapidly eliminated by other routes besides hydrolysis to Z. This may be due to biliary excretion of ZG in rabbits since the route was found to be appreciable in the guinea pig.

Guinea pigs with patent bile ducts also did not produce measurable ZG levels in plasma after dosing of Z. Neither animal species investigated was completely satisfactory as a model for simulating Z and ZG disposition in human. The disposition of Z in primates such as the rhesus monkey seems to be most similar to that in human as indicated by the published data (26) and our findings that ZG levels in monkey are comparable to those of Z (Chapter IV). However, a small and inexpensive animal such as the guinea pig was necessary for our studies. The guinea pig offered several advantages over the rabbit. Being much smaller, the guinea pig requires a lesser dose of ZG which was available only in limited quantities. It is difficult to purify ZG from human urine and Zomax® has been withdrawn from the U.S. market since March, 1983. The guinea pig tolerates anesthesia and surgery better than the rabbit, which allows surgical intervention to ligate or cannulate the bile

duct. Bile duct ligation was required in order to elevate the plasma concentrations of ZG to measurable levels in the guinea pig after an I.V. dose of Z or ZG. Ligation of the bile duct decreased the clearance of both Z and ZG presumably because much of the drug and conjugate was excreted in the bile. The reduced clearance of ZG increased ZG plasma levels in vivo improving our ability to accurately measure this metabolite. The decrease in Z clearance, however, lengthened the half-life of Z to such an extent that the six hours of blood sampling was not long enough. Therefore, almost half of the total AUC estimated was based upon the assumption of a continued log-linear decline in Z concentration after six hours. Maintaining anesthesisa longer than eight hours was not feasible, although some animals did last longer.

Addition of the esterase inhibitor, PMSF, markedly reduced the apparent plasma clearance of ZG (Table V-3) but increased the apparent clearance of Z (Table V-2). These results support the hypothesis that esterases are primarily responsible for the hydrolysis of ZG in vivo and also verify the existance of a futile cycle between Z and ZG (Fig. V-1). When the data are analyzed further to determine the individual component of the clearance for Z and ZG based upon the reversible metabolism model (Fig. V-2), it is shown that the only dramatic change in the disposition of Z and ZG with concurrent PMSF is due to the inhibition of ZG hydrolyis (Tables V-6 and V-7). The experimental design did not allow dosing of Z and ZG separately to the same animal on different occassions nor was it possible to give tracer doses of a radiolabelled Z along with cold ZG to determine the clearance of both compounds simultaneously in one animal. Instead, the mean data (Table V-6) obtained from each group of animals were used for the analysis

Table V-6. Mean AUC's of zomepirac (Z) and zomepirac glucuronide (ZG) normalized to a 5 mg dose and 1 kg body weight.

Dose[a]	PMSF	n	Normalized AUC[a] (μg-min/ml) Z	ZG
Z	-	4[b]	9544 ± 2293	342 ± 140
ZG	-	4	9069 ± 1765	480 ± 227
Z	+	5	3754 ± 1738	1736 ± 806
ZG	+	4	1607 ± 717	2746 ± 662

[a]Dose and AUC are expressed in Z equivalents.
[b]For purposes of determining reversible clearances (Table V-9) an outlier from this group (8-14B) was deleted.

Table V-7. The effect of PMSF on the plasma clearance of zomepirac (Z) and zomepirac glucuronide (ZG) in the bile duct ligated guinea pigs.

Parameter[b]	Clearance (ml/min/kg) Without PMSF	With PMSF
CL_{10} ($CL_{z\rightarrow other}$)	0.47	0.67
CL_{20} ($CL_{zg\rightarrow other}$)	1.6	1.4
CL_{12} ($CL_{z\rightarrow zg}$)	1.2	1.2
CL_{21} ($CL_{zg\rightarrow z}$)	31	1.1

[a]Model is described in the text (Figure V-2), and clearance terms are calculated with equations V-1 through V-4 (Ref. 151).
[b]Parameters are those defined by equations V-i through V-iv. The equivalent term relative to Z and ZG disposition is given in parentheses.

using equations V-i through V-iv. Calculation of the possible error involved in the individual clearance parameter was estimated by error analysis (Appendix V-2). Although the errors are substantial using this method of analysis and experimental design, the clearance term for ZG hydrolysis in vivo in bile duct ligated guinea pigs is 31 ml/min/kg in control group and is reduced to 1.1 ml/min/kg with concurrent PMSF. The other clearance terms were not noticably altered by PMSF administration (Table V-7).

The magnitude of the effect of PMSF on the rate and extent of ZG hydrolysis is not fully appreciated if only the fraction of the ZG dose hydrolyzed, f_H, is used as a measure for the capability of the animal to hydrolyze the conjugate in vivo. The value of f_H was reduced to 53% of its original control value, from 0.93 to 0.49, by treatment with PMSF (Table V-4). However, the estimate for hydrolytic clearance of ZG, $CL_{zg \rightarrow z}$, was reduced even more by PMSF to 4% of its initial value (Table V-7). If clearance of the conjugate by routes other than hydrolysis is relatively small, then even substantial changes in hydrolytic clearance of the conjugate will not appreciably alter the fraction hydrolyzed, which approaches unity for both cases. Therefore, the fraction hydrolyzed may be a poor measure for comparing hydrolytic ability in vivo. One should determine the hydrolytic clearance simultaneously to make the comparison. Meffin et al. (111) employed the value of the fraction hydrolyzed to compare the efficiency by which rabbits hydrolyzed clofibric acid glucuronide in vivo. The equation used by these authors to determine f_H was incorrect, but fortuitously their estimates were close to what would have been obtained with the correct equation (Appendix V-1).

The study of clofibric acid disposition in rabbits conducted by Rowe and Meffin (9) which employed the esterase inhibitor, DFP, found results that are somewhat in agreement with our results here for Z. DFP increased the total and unbound plasma clearance of clofibric acid in anesthesized rabbits by 3.0 and 2.8 fold, respectively. An unexplainable observation in their study was that no change in either clearance value occurred in conscious rabbits when DFP was added. The DFP treated animals also received the anticholinergic, atropine, which may have decreased biliary excretion (156) complicating the comparisons between DFP treated and control animals. Anesthesia caused a marked increase in unbound and total clearance of clofibric acid in the rabbit treated with DFP; while in control animals anesthesia decreased both measures of clearance. The complexity of the experimental model and the inconsistency of the results obtained by Rowe and Meffin (9) make interpretation of their results difficult.

The guinea pig studies with PMSF as the esterase inhibitor reported here have several advantages over the rabbit studies published by Meffin *et al.* (9) with DFP as the inhibitor. Our model did not require atropine administration for life support as PMSF administration at 50 mg/kg was not fatal. Any possible effect of PMSF on biliary excretion was eliminated by ligation of the bile duct. Most importantly, we administered both Z and ZG separately, rather than relying upon inferences of an inhibitory effect on ZG disposition by following Z disposition *in vivo*. We have also confirmed that PMSF inhibits ZG hydrolysis in liver homogenates *in vitro*, whereas the inhibitor of β-glucuronidase, 1,4-saccharolactone, had no effect on ZG cleavage by liver homogenates *in vitro* (Chapter IV). The possible effect of PMSF on

the reversible protein binding of Z which may affect Z elimination was shown to be unimportant when interpreting our results for Z disposition (Table V-5). The possiblility that PMSF might affect protein binding of ZG cannot be ruled out. Most previous data indicate that glucuronide conjugates of drugs, being more polar than the respective parent drug, bind to protein less avidly than the parent drug (157). Since we would not expect the protein binding of ZG to be high, any effect of PMSF on ZG binding to protein would have little effect on its clearance (158).

The acyl glucuronide, ZG, was efficiently hydrolyzed *in vivo* by the guinea pig as well as the rabbit. The hydrolysis is not a physical process, instead it occurs rapidly by the action of nonspecific, unidentified esterases. It is suggested in Chapter IV on the basis of *in vitro* data, that the liver may be a major site for the hydrolyis of ZG *in vivo* in guinea pig. The futile cycling of acyl glucuronides by esterases *in vivo* (Fig. V-1) may have significant consequences. As first proposed by Gugler (141), the futile cycle explains why drugs, which are completely metabolized with acyl glucuronides as the major metabolite, exhibit reduced parent drug clearances when the excretion of the conjugates are reduced. Excretion of conjugate may be reduced by several different ways. Renal failure (15, 16, 137), hepatic disease with cholestasis (138) or decreased renal function with age may compromise potential routes of elimination for acyl glucuronides. Inhibitors of renal excretion of organic acids, such as probenecid (159), could also decrease the active renal secretion of the acyl glucuronide with subsequent increase in the hydrolysis of the conjugate (Chapter VI). In contrast, stable conjugates such as acetaminophen glucuronide (17) and oxazepam glucuronide (19, 119) would not experience

rapid hydrolysis because as ethers they are not substrates for esterases. If clearance of the conjugates is reduced they may accumulate *in vivo* if they are not cleaved rapidly *in vivo*. This may be the case for ZG in primates (Chapter IV and VI). Recently, it has been found that acyl glucuronides are reactive, leading to the formation of covalent adducts of the parent compound with albumin *in vitro* and *in vivo* (10, 11). As mentioned in the introduction, one reason to study Z and ZG disposition in a small animal was to develop methods to examine the covalent binding of Z to proteins *in vivo*. The covalent binding of ZG will not be discussed in depth here, however, knowledge of the elimination of ZG *in vivo* has provided invaluable information when studying the covalent binding *in vivo* (Chapter VI).

When tested for an animal model simulating Z and ZG disposition in human, neither rabbit, guinea pig nor rat (preliminary data not presented here) was satisfactory. Therefore, it was necessary to manipulate the small animal by surgically ligating the bile duct in order to increase ZG levels in plasma. Even bile duct ligation did not result in plasma concentrations of ZG similar to those of Z as was seen in primates. In order to achieve a ZG/Z plasma concentration ratio approaching one, an inhibitor of nonspecific esterases, PMSF,which prevents the hydrolysis of ZG was required. The rather heroic measures needed to elevate ZG levels in the guinea pig raise the question of what species differences exist between primates and the small animals studied. Do small animals have lower formation rates of ZG, higher esterase levels *in vivo*, more efficient biliary excretion of the conjugate once formed in the liver, or perhaps higher renal clearance of the glucuronide than primates? However, in addition to all the

possibilities mentioned above, the conjugates may bind to plasma protein to a greater extent in primates, resulting in a smaller volumes of distribution for the conjugate.

E. Appendix V-1. Derivation of the fraction of the acyl glucuronide dose which is hydrolyzed in vivo when the dose is not pure.

The fraction of an acyl glucuronide dose which is hydrolyzed in vivo has been used by Meffin *et al.* (111) as a measure to compare the ability of an animal to hydrolyze the conjugate. Besides the inherent limitations of using f_H as a measure for hydrolytic ability which has been discussed in the text, the equation presented by Meffin *et al.* (111). (Equation (a) below) is incorrect unless the fractional purity of the dose, P, is 1.0. A correct equation derived using mass balance considerations is presented here. The data of Meffin *et al.* (111) for f_H calculation were reanalyzed using the corrected equation. Meffin's equation considered radiochemical purity, however, the equation derived here can also be applied to unlabelled compounds if the impurity is due to the parent drug.

(a) $f_H = CL * AUC' * P / D'$ (Reported by Meffin *et al.*, Ref. 111)

Where, CL = clearance of the parent drug determined in previous experiments, or simultaneously, if possible.

D' = dose of the conjugate; either radiolabel or cold compound based on the same molar equivalents as AUC'.

P = fractional purity of the conjugate dose where impurities are due to the parent compound.

AUC' = AUC of the parent compound when dosing the acyl glucuronide metabolite.

At time zero all that is present after the dose of ZG is ZG and Z, where Z represents the impurity. After administration some ZG is hydrolyzed to form Z_H, and the original impurity Z also contributes to the AUC'_Z. Additional terms are:

$$\text{Dose} = ZG + Z \qquad P = ZG/(ZG + Z)$$

then,

(b) $f_H = Z_H/ZG$

(c) $Z_H = (Z_H + Z) - Z = CL_z{*}AUC'_z - Z$

(d) $Z = (1 - P){*}\text{Dose}$

Combining (c) and (d),

(e) $Z_H = CL_z{*}AUC'_z - (1 - P){*}\text{Dose}$

(f) $ZG = \text{Dose}{*}P$

Substituting (e) and (f) into (b) equation (g) is obtained,

(g) $f_H = Z_H/ZG = [CL_z{*}AUC'_z - (1 - P){*}\text{Dose}]/\text{Dose}{*}P$

Table V-8. Data from Meffin et al. (111) for fraction of a clofibric acid glucuronide dose given to rabbits which is hydrolyzed in vivo. A comparison of two methods to determine f_H.

Rabbit	Wt. (kg)	Dose ($dpmx10^{-6}$)	f_H (Eq.a)	CL_z (ml/min/kg)	P	AUC[a] (dpm-min/ml)	f_H (Eq.g)
1	3.4	10.33	0.60	1.33	0.85	1.61×10^6	0.65
2	3.2	10.33	0.37	0.47	0.84	3.02 "	0.33
3	3.5	10.33	0.50	0.38	0.84	4.64 "	0.52
4	3.1	9.87	0.40	0.39	0.84	3.89 "	0.38
5	3.8	1.62	0.71	0.31	0.99	0.99 "	0.71

[a]Derived from $AUC = f_H{*}\text{Dose}/CL{*}Wt{*}P$

In this instance the purity was high and the fraction hydrolyzed was large so that the two methods agreed quite closely. Differences between equations (a) and (g) become most apparent when impurities are larger or f_H is low.

E. Appendix V-2. Error analysis of reversible metabolism model.

An estimate for the possible error involved in a measurement of a parameter which is a function of several variables can be determined by the use of partial differential equations (160, 161). Standard deviation of the measured individual variable was used for the estimating the error (Table V-9). Equation V-i can be rewritten as equation (a):

V-i. $CL_{10} = (AUC_2^M*D - AUC_2^D*M)/(AUC_1^D*AUC_2^M - AUC_2^D*AUC_1^M)$

(a) $f(CL_{10}) = (a*w - b*x)/ (w*y - x*z)$

Where, $w = AUC_2^M$ $y = AUC_1^D$ $a = D = 5000$ µg

$x = AUC_2^D$ $z = AUC_1^M$ $b = M = 5000$ µg

Equations V-ii, V-iii and V-iv can also be rewritten as equations (b), (c) and (d), respectively:

(b) $f(CL_{20}) = (b*y - a*z)/(w*y - x*z)$

(c) $f(CL_{12}) = b*x/(w*y - x*z)$

(d) $f(CL_{21}) = a*z/(w*y - x*z)$

An example of the partial differential for $f(CL_1)$ is:

$$(e)\quad df(CL_{10}) = \frac{\delta f(CL_{10})}{\delta w} dw + \frac{\delta f(CL_{10})}{\delta x} dx + \frac{\delta f(CL_{10})}{\delta y} dy + \frac{\delta f(CL_{10})}{\delta z} dz$$

$$= \frac{DEN*a - NUM*y}{DEN^2} dw + \frac{DEN*(-b) - NUM*(-z)}{DEN^2} dx$$

$$+ \frac{-NUM*w}{DEN^2} dy + \frac{-NUM*(-x)}{DEN^2} dz$$

Where, DEN = wy-xz and NUM = aw-bx.

Similar expressions can be derived for the other clearance terms. Summation of the square of the partial derivatives with respect to each variable provides an estimate for the square of the error (5AA, 5AA2). From the following equations, estimates for the variablity in clearance parameters were determined and are presented in Table V-10 below.

Table V-9. Summary of values used for estimating clearance errors.

Variable[a]		$AUC^b(0-\infty)$ Without PMSF	With PMSF
$w \pm dw$	AUC_2^M	480 ± 227	2746 ± 662
$x \pm dx$	AUC_2^D	342 ± 140	1736 ± 806
$y \pm dy$	AUC_1^D	9544 ± 2293	3754 ± 1738
$z \pm dz$	AUC_1^M	9069 ± 1765	1607 ± 717

[a]Variables are from equations V-i through V-iv.
[b]Normalized AUC data are from Table V-6.

Table V-10. Derived estimates for the errors in clearance terms for reversible metabolism.

Parameter		Clearance Error (ml/min/kg) Without PMSF	With PMSF
dCL_1	$(dCL_{z\rightarrow other})$[a]	0.41	0.65
dCL_2	$(dCL_{zg\rightarrow other})$	8.9	0.64
dCL_{12}	$(dCL_{z\rightarrow zg})$	2.4	1.6
dCL_{21}	$(dCL_{zg\rightarrow z})$	60	1.0

[a]Parameter in parentheses are equivalent terms relative to Z and ZG.

CHAPTER VI

THE DISPOSITION OF ZOMEPIRAC AND ZOMEPIRAC GLUCURONIDE IN HUMANS AND THE EFFECTS OF CONCURRENT PROBENECID ADMINISTRATION

A. INTRODUCTION

Zomepirac (Z), (5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid), is a nonsteroidal, antiinflammatory analgesic (NSAID) which was previously marketed as the dihydrate of the sodium salt in the U.S. under the tradename Zomax® (McNeil Pharmaceutical, Springhouse, PA). It was voluntarily withdrawn from distribution in the U.S. in March, 1983, because of the occurrance of unexplained anaphylactic reactions which in some cases resulted in death (33, 34). In humans, Z is primarily eliminated by metabolic conjugation to the acyl glucuronide (ZG) which is subsequently excreted in the urine (25, 26, 50, 162). A minor oxidative metabolite, hydroxyzomepirac (HMZ, Fig. III-1), has also been reported in humans and this too is also excreted in the urine (25, 26). Recent studies from our laboratory found that ZG, which is present in the plasma of humans (25, 26), is unstable in biological fluids at physiological pH (98) (Chapter IV). It is possible that previous pharmacokinetic data for Z disposition in humans may have been in error because of the hydrolysis of ZG to Z in blood and plasma prior to analysis. Therefore, we examined the disposition of Z and ZG in humans using an analytical method and sample handling procedure which prevented artifactual loss of ZG.

Probenecid, (4-[dipropylaminosulfonyl]benzoic acid), is often used concurrently with NSAID's for the treatment of gout (159, 163). Since

probenecid can alter renal excretion of acids (159, 163), it was of interest to determine whether it would affect the disposition of Z and ZG in human. It has been documented that probenecid interacts to varying degrees with all of the NSAID's of the aryl-alkanoic class which have been examined for such a possible interaction. These include indomethacin (23, 165, 166), naproxen (166), ketoprofen (24) and carprofen (167), all of which are metabolized in humans to acyl glucuronides to a significant extent. A similar interaction exists with clofibric acid which is also metabolized to an acyl glucuronide (168, 169). The mechanism for probenecid's effect on the disposition of NSAID's and other acidic compounds which are eliminated by formation of acyl glucuronides and then excreted in the urine has not been resolved. Probenecid may competitively inhibit formation of the glucuronide, or it may block renal or biliary excretion of the conjugate with subsequent chemical or enzymic hydrolysis of the labile conjugate in vivo. The present study characterizes the disposition of Z and ZG in humans and investigates the possible basis for the interaction between NSAID's and probenecid.

B. METHODS

Study design

Six male human volunteers were given 100 mg of Z as a tablet (Zomax®) orally on two separate occassions. On one occassion Z was given at 8 AM without concurrent probenecid, the other Z dose was given after previous oral administration of 500 mg probenecid (Benemid®) twice daily for 3 days prior to the Z dose and continued during the blood sampling period. On the day of the Z dose the morning probenecid dose

was taken one half hour prior to the adminsitration of Z. The two experiments were separated by 7 to 10 days and volunteers were randomly assigned to a particular dosing regimen.

All subjects participating in the study were healthy, male volunteers between the ages of 21 and 30, and weighed from 68 to 84 kg. A consent form describing the study protocol which was approved by the University Human Experimentation Committee was read and signed by each volunteer prior to participation in the experiment. Prior to and after the study the subjects were determined to be healthy by physical examination, blood and urine analysis. None of the subjects were smokers. Other drugs and alcohol were excluded for 14 days prior to and during the study. At 8 a.m. a 100 mg tablet of Z was taken orally with 180 ml of water. A light liquid lunch was served after collection of the blood sample at 4 hours and dinner was ad lib. after the blood sample was taken at 10 hours. Water and cranberry juice (Ocean Spray®, Plymouth, MA) were taken ad lib. throughout the study. The cranberry juice was provided to acidify the urine and the volunteers were instructed to avoid foods which might increase urine pH, such as cabbage, banana, cantaloupe and large amounts of vegatables (170). Subjects were ambulatory during the study, and were instructed not to engage in strenuous excercise.

Sample collection

An indwelling heparin lock was inserted into the forearm of each subject to allow for blood sample collection during the part of the study when samples were taken frequently. Between samples the catheter was flushed with a solution of 10 U heparin/ml normal saline. This

heparin solution was discarded before blood was sampled. Blood samples (ca. 5 ml) once obtained were immediately put into an ice cooled tube (Vacutainer®) containing 143 U heparin, then mixed. The green rubber closure of the Vacutainer® tube caused an interfering peak upon HPLC analysis of the plasma. To prevent contamination of plasma, the rubber closure was discarded and the tubes containing blood were gently mixed after being sealed with Parafilm®. Within 15 minutes the cooled blood was centrifuged for 10 minutes at 0-4°C to separate plasma, then the plasma (ca. 2.5 ml) was transferred to a vial containing 25µl of 86% phosphoric acid which decreased the plasma pH to 2 to 4. This rapid handling of blood and plasma at reduced temperature minimized the loss of ZG by hydrolysis and acyl migration which is appreciable at room temperature and physiological pH (28, 98) (Chapters III and IV). By using this procedure, ZG loss was kept to less than 4% when control spiked blood samples were analyzed. Plasma once buffered to pH 2 to 4 was frozen at -20 °C until analyzed by HPLC within several weeks. Blood was sampled prior to the Z dose and at 10, 20, 30, 40, 60, 90, minutes, 2, 3, 4, 5, 6, 8, 10, 12 and 24 hours after the dose. When probenecid was given concurrently, additional blood samples were obtained at 28, 32, 34, 36 and 48 hours.

Precautions for ZG stability in urine were also implemented using the best possible methods short of urinary bladder catheterization. Since the stability of ZG improves quite dramatically with a decrease of pH from 7.4 to 5.0 (28) (Chapter IV), diet was modified as described above to maintain urine pH below 6, if possible. For the short intervals of urine collection between 0 and 8 hours after the dose, urine was collected, the pH and volume were recorded, then a 15 ml

aliquot was saved for analysis. Urine was processed upon collection in order to minimize artifactual loss of ZG. The urine saved for analysis was added to a vial containing 75 µl of 86% phophoric acid which reduced the pH to 2 to 4. Urine was then frozen at -20 °C prior to analysis for Z and ZG which was done within one month. Over long collection intervals, which necessitated voiding during the interval, urine was collected into a container with 1 ml of 86% phosphoric acid and then kept refrigerated until the end of the collection interval. The combined urine from an interval was then adjusted to pH 2 to 4 with phosphoric acid prior to volume measurement and a 15 ml aliquot was frozen at -20 °C. Urine collection intervals were 0-1, 1-2, 2-3, 3-4, 4-6, 6-8, 8-12, 12-24 and 24-48 hours. When probenecid was given concurrently, additional urine collections were made from 24-36, 36-48 and 48-72 hours.

Analytical methods

Plasma and urine were assayed for Z, ZG (directly) and probenecid using HPLC methods developed in our laboratory (107) (Chapter III). This method minimizes ZG loss due to hydrolysis and acyl migration which may be a significant problem with other previous assays for Z in the literature. There were detectable concentrations of isomeric conjugates of ZG in plasma and urine. The concentrations were often more than what could be accounted for due to acyl migration during sample handling prior to analysis. ZG and its isomers are not completely resolved with the analytical method employed here and were combined together as the total conjugate for the purpose of this study.

Data analysis

Pharmacokinetic analysis was done using model independent equations. Absorption was assumed to be complete as was previously reported for Z (25, 50). Area-under-the-plasma-concentration vs time curve (AUC) was calculated by the linear trapezoidal method (150) from time zero to the time of the last peak observed, subsequently the log-linear trapezoidal method (171, 173) was used. The extrapolated AUC was estimated by $C_p(last)/\beta$, where C_p is the plasma concentration and β was the terminal rate constant as determined by the last 2 or 3 data points which appeared to be log-linear. Calculation of pharmacokinetic parameters was performed using methods previously employed for Z (25). These pharmacokinetic equations assume complete absorption of an oral Z dose and that all metabolism of Z occurs in the liver (158). Using these assumptions, $CL_z/F = CL_{intrinsic,z}$ (158), where F is the availablility of Z after an oral dose. Other terms are defined below. Values for hepatic blood flow and the blood/plasma ratio for Z were those used by O'Neill, et al. (25), 1450 ml/min (173, 174) and 0.52, respectively. The equations used are presented below with three additional equations for estimating metabolic and renal clearances of Z:

1. $CL_{P,Int} = Dose_z \; / \; AUC_z(0-\infty)$
2. $CL_{B,Int} = CL_{P,Int} \; / \; R_{B/P}$
3. $E = CL_{B,\ Int} \; / \; (Q + CL_{B,Int})$
4. $CL_B = Q * E$
5. $CL_P = Q * E * R_{B/P}$
6. $CL_{R,z} = Ae_z(0-\infty) \; / \; AUC_z$
7. $CL_{z\rightarrow zg} = Ae_{zg}(0-\infty) * CL_{P,z} \; / \; Dose_z$
8. $CL_{z\rightarrow other} = CL_{P,z} - CL_{z\rightarrow zg} - CL_{R,z}$

9. $CL_{R,zg} = Ae_{zg}(t_n - t_{n-1}) \; / \; AUC_{zg}(t_n - t_{n-1})$

where, $CL_{P,Int}$ = plasma intrinsic clearance.

$CL_{B,Int}$ = blood intrinsic clearance.

$R_{B/P}$ = ratio of blood to plasma concentrations

Q = hepatic blood flow.

E = hepatic extraction ratio.

Ae = amount excreted in the urine.

CL_B = clearance in terms of blood.

CL_P = clearance in terms of plasma.

CL_R = renal clearance based on plasma concentrations.

$CL_{z \rightarrow zg}$ = apparent metabolic clearance of Z to form ZG.

$CL_{z \rightarrow other}$ = clearance of Z not accounted for by renal clearance or metabolic clearance to form ZG.

It is assumed that all ZG, once formed, is excreted in the urine since a previous report found that greater than 90% of an oral ^{14}C-labelled dose of Z is excreted in the urine (25). The equations used assume that all metabolism of Z occurs in the liver (158) and that no renal metabolism is occurring. The possible hydrolysis of ZG in vivo is not accounted for in equation 7; this apparent clearance term reflects net elimination of Z by metabolism to form ZG which is subsequently excreted in the urine (Appendix VI-1).

Statistical analysis was done using a two-tailed paired Student's t-test comparing control and probenecid treatment for each subject (155). A p-value of less than or equal to 0.05 was considered significant.

C. RESULTS

Analysis of the plasma samples obtained in the control study revealed that ZG levels were higher than the expected levels based upon a previous report (25). The concentrations of ZG in plasma approached and at some times exceeded Z levels even when they were expressed in molar units and when no probenecid was present. In the control experiments the mean AUC_{zg} was slightly higher than AUC_z. When probenecid was added, the mean AUC_z increased by 4.3 fold; while AUC_{zg} increased to a lesser extent, 2.8 fold (Table VI-1). Figures VI-1 and VI-2 show two representative plasma concentration profiles for Z and ZG in the absence and presence of probenecid. The figures also depict the irregular shape of the profiles with the existence of secondary peaks and shoulders which may be indicative of enterohepatic cycling of Z (26). Absorption also seemed irregular, with late peak times for several of the volunteers, as reflected by the wide variability of the peak times as presented in Table VI-1. As a result, the time to peak and the peak concentration attained were not significantly different between control and probenecid treated subjects (Table VI-1). ZG can be hydrolyzed to Z or rearrange to form isomeric conjugates in blood and plasma (98) (Chapters II and IV), but the sample handling procedure minimized this loss to about 4%. As shown previously in Figure III-4, many of the HPLC chromatograms for plasma samples contained isomeric conjugates peaks representing greater than 4% total conjugates. This indicates that acyl migration is occurring in vivo, although the extent of this rearrangement was not fully evaluated due to the limited resolution obtained using this HPLC method. In the analysis used here, ZG and its isomeric conjugates were combined and are referred to simply

Table VI-1. Summary of the pharmacokinetic analysis of a 100 mg oral dose of zomepirac to man with and without concurrent probenecid administration.

Parameter[a]	Zomepirac	Zomepirac + Probenecid	Ratio Probenecid/Control
$C_{p,max,z}$(μg/ml)[b]	1.60 ± 1.74[c]	3.54 ± 1.58	2.21
Peak time$_z$(min)	145 ± 126	101 ± 87	0.70
$C_{p,max,zg}$(μg/ml)[b]	1.09 ± 0.42	1.70 ± 0.72	1.62
Peak time$_{zg}$(min)	138 ± 110	139 ± 78	1.01
CL/F_z(ml/min)[e]	682 ± 246	155 ± 58	0.23[d]
$CL_{p,z}$(ml/min)[e]	348 ± 71	127 ± 40	0.36[d]
$CL_{z\rightarrow zg}$(ml/min)[e]	252 ± 60	74 ± 27	0.29[d]
$CL_{R,z}$(ml/min)	36 ± 10	7.7 ± 2.6	0.21[d]
$CL_{z\rightarrow other}$(ml/min)	60 ± 16	45 ± 14	0.75
$CL_{R,zg}$(ml/min)	406 ± 110	115 ± 32	0.28[d]
Extraction Ratio (E)	0.45 ± 0.10	0.16 ± 0.078	0.36[d]
$Ae_z(0-\infty)$(mg)[b]	5.5 ± 0.98	5.1 ± 0.58	0.93
$Ae_{zg}(0-\infty)$(mg)[b]	72 ± 5.9	58 ± 5.1	0.81[d]
$Ae_{total}(0-\infty)$(mg)[b]	78 ± 5.2	63 ± 5.3	0.81[d]
$AUC_z(0-\infty)$(μg-min/ml)[b]	165 ± 64	708 ± 308	4.29[d]
$AUC_{zg}(0-\infty)$(μg-min/ml)[b]	180 ± 57	499 ± 134	2.77[d]

[a]Parameters are derived by equations described in the text.
[b]All concentrations and amounts are expressed in zomepirac equivalents.
[c]Mean ± s.d., n=6.
[d]Significant differences, $p < 0.05$, paired t-test, n=6.
[e]Possible hydrolysis of ZG to Z in vivo was ignored when determining these clearance values. Therefore, these are not true clearances, but are "apparent" or "net" clearances.

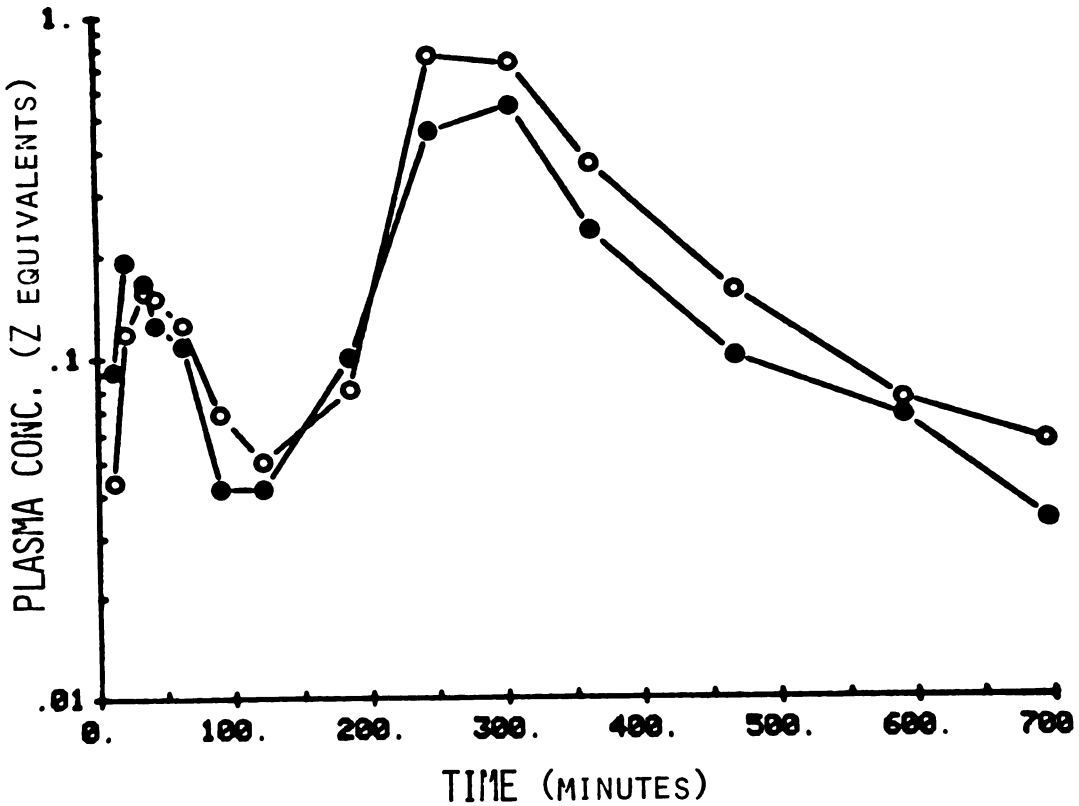


Figure VI-1. Representative plasma concentration profile for a human subject after an oral 100 mg dose of zomepirac. Closed symbols = zomepirac; open symbols = zompepirac glucuronide. (Subject MD-Z).

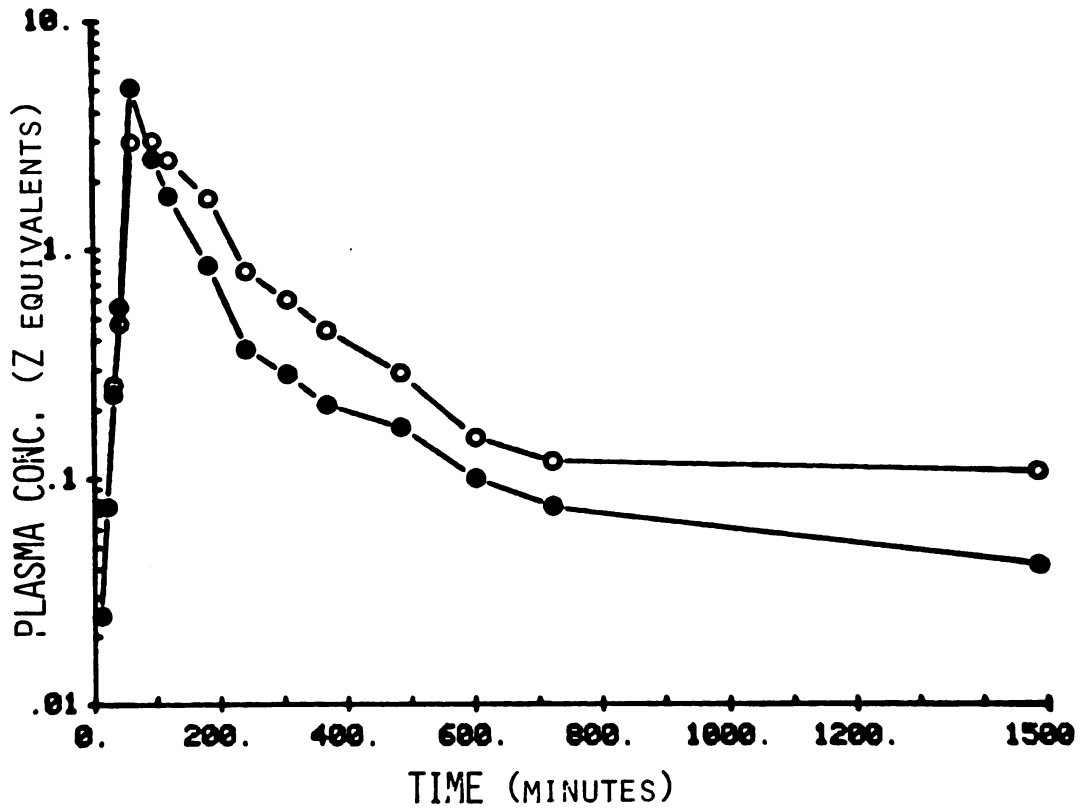


Figure VI-2. Representative plasma concentration profile for a human subject after an oral 100 mg dose of zomepirac when taking probenecid concurrently (500 mg bid). Closed symbols = zomepirac; open symbols = zomepirac glucuronide. (Subject DD-Z/P).

as ZG. In most subjects the concentration-time profile of ZG was nearly parallel to that of Z (Figs. VI-1 and VI-2) which indicates that the half-life of ZG was probably controlled by its rate of formation from Z.

Clearance values for Z and ZG were altered significantly by probenecid administration (Table VI-1). Mean total plasma clearance/F of Z was reduced from 682 to 155 ml/min. When analyzed further, this decline was due to a reduction of plasma clearance of Z and a decrease in the extent of first pass metabolism. Metabolic clearance of Z to form ZG, which is the primary clearance mechanism for Z in humans, decreased 71%, from 252 to 74 ml/min, when probenecid was given concurrently. Renal clearance of Z was much less than metabolic clearance, but it too was decreased 79% by probenecid administration, from 36 to 8 ml/min. However, clearance of Z by all the other unidentified routes was relatively unchanged at 60 and 45 ml/min, respectively, for the control and treated groups. When combined, the total plasma clearance for Z was reduced from 348 to 127 ml/min by probenecid. Adding the effect of an increased availability, F=(1 - E), which went from 0.55 to 0.84, the resultant clearance/F showed an even greater change, which was a 77% reduction, from 682 to 155 ml/min. Finally, the renal clearance of ZG was reduced by probenecid treatment from 406 to 115 ml/min.

Renal excretion of both Z and ZG accounted for 78 and 63% of the total dose in control and probenecid treated experiments, respectively. Almost all of the Z dose eliminated in the urine was present as its glucuronide metabolite, ZG, with only about 5% of the total dose eliminated in the urine as unmetabolized Z in both control

and probenecid treated studies. Even with the precautions taken here to maintain ZG stability in urine, the value of unchanged drug in the urine may be overestimated as was noted for ketoprofen, naproxen and probenecid by Upton et al. (6). Urinary recovery of ZG was significantly decreased, from 72 to 58%, by concurrent probenecid administration (Table VI-1).

Probenecid concentrations in plasma were usually in the range of 20 to 100 μg/ml during the studies when probenecid was dosed concurrently. These levels are about 20 fold higher in molar concentrations than those observed for Z. Urine pH was always 6.5 or lower when measured, with most urine samples at pH 5.5.

D. DISCUSSION

The effect of probenecid on the disposition of Z is qualitatively similar to that seen with the other NSAID's tested for this interaction. Our results for Z disposition in control experiments, however, differ from the previous reports in the literature which did not control for ZG instability in the blood or plasma. Of the previously published reports only the study by O'Neill et al. (25) examined plasma for ZG. When the data of O'Neill et al. are examined closely and adjusted for probable degradation of ZG in the samples prior to analysis there is agreement between their data and the results presented here. When the AUC_{zg} determined by O'Neill et al. using radiochemical TLC analysis is normalized to a 100 mg Z dose, as used in our study, and corrected for molecular weight of Z, a value of 236 ± 47 μg-min/ml is obtained. This AUC_{zg} value is similar to the value of 180 ± 57 μg/-min/ml found in this report (Table VI-1). If we assume that almost all of the ZG in a plasma

sample of O'Neill *et al.* degraded during sample handling prior to and during analysis of Z by HPLC, then the Z concentration and thus the AUC_z would have been overestimated. When we further analyze O'Neill's data by subtracting the AUC_{zg}, as normalized to Z dose and molecular weight, from the AUC_z, as normalized to Z dose, we obtain a corrected AUC_z of 330 μg-min/ml for a 200 mg Z dose. This corrected AUC_z value corresponds to a CL/F value of 606 ml/min. This adjusted clearance value is in reasonably close agreement with our result where CL/F is 682 ± 246 ml/min. We believe that the discrepancy between our results and those of O'Neill and others results from the lack of control for ZG stability in the analytical methods utilized by the other investigators, with most if not all of the ZG degrading to Z prior to or during analysis. Two separate methods were used by O'Neill *et al.* for the analysis of Z and ZG (25), respectively. Plasma was washed with ether at pH 7.4; a pH which would allow ZG to degrade. Plasma was then extracted at low pH and analyzed by HPLC (91). ZG was determined by TLC after extraction of acidified plasma with ethyl acetate (25). Although acyl migration of ZG may have occurred in the plasma sample prior to TLC method, the acid would stabilize the conjugates and ZG. It is doubtful that the isomeric conjugates so formed would be resolved from each other or from ZG by the TLC employed since the compound identified as ZG was reported to have an R_f of about 0.05. As mentioned above, the other previous reports on Z disposition in human did not examine for ZG in the plasma, nor did they control for possible ZG hydrolysis to Z prior to analysis. The CL/F values normalized to a 70 kg man obtained by previous investigators for Z in healthy volunteers are calculated by us to be 297 (26), 200 (50) and 259 ml/min (162), which agree with the

value of 254 ml/min obtained by O'Neill et al. (25). A comparison of our analytical method and that used by O'Neill et al. has shown (98) (Chapter IV) that ZG degrades entirely to Z and isomeric conjugates of ZG when no precautions are taken to maintain the stability of the labile acyl glucuronide, ZG.

Urinary excretion data from the present study are in better agreement with the previously published data. In control experiments almost all Z is eliminated in the urine as ZG (25, 26, 50, 162) and our results show that only about 5% of the dose is excreted in the urine as unmetabolized Z. Although ZG loss does occur at the pH of the urine, 5-7, it is slow and primarily results in the formation of isomeric conjugates (28) which are cleaved by base hydrolysis. The $CL_{R,zg}$ of 406 ml/min in control subjects is much higher than the renal glomerular filtration rate, indicating an extensive renal tubular secretion of ZG. This secretion is decreased dramatically by probenecid, a compound which is known to competitively block active secretion of organic acids (159, 163).

Peak plasma concentrations of Z and the time to reach the peak were quite variable. Thus no significant difference in peak time was observed when probenecid was added, even though AUC_z increased more than four fold. Multiple peaks were observed for many of the plasma concentration-time profiles (Figs. VI-1, VI-2) which may be indicative of either enterohepatic cycling, which has been stated to occur for Z in rats (26), or perhaps variable and unpredictable absorption. The irregular shape of the concentration-time profile was also evident in a recent report on Z disposition in man (162). Likewise, O'Neill et al. stated that their data were not appropriate for individual fitting to

traditional pharmacokinetic models (25).

A direct comparison of our results with previous studies of the probenecid and NSAID interaction yields the same conclusion which Upton *et al.* (24) stated earlier - that the mechanism of the interaction differs more quantitatively than qualitatively when the NSAID's are compared. Naproxen (73), indomethacin (175, 176), carprofen (167), ketoprofen (74) and zomepirac (25, 26) are eliminated to a large extent by conjugation with glucuronic acid with varying degrees of Phase I metabolism. The glucuronides once formed are then primarily excreted in the urine. Our results and analysis show that probenecid appears to be affecting glucuronidation and renal clearance of Z, but has no discernable effect on other metabolic processes. Probenecid, which is given in larger doses, has much higher plasma concentrations than that observed for Z. A major route of elimination for probenecid is conjugation with glucuronic acid (177, 178), and this may competitively decrease ZG formation as shown by the 71% reduction of $CL_{z \to zg}$. *In vitro* data of the inhibition of furosemide glucuronidation by probenecid (179) suggests that this mechanism may be possible for Z. However, this mechanism of inhibition of glucuronidation can not be easily distinguished *in vivo*. The inhibition of renal excretion of ZG by probenecid ($CL_{R,zg}$ decreased 72%) results in an accumulation of ZG in the plasma. The reduced $CL_{z \to zg}$ value may be an artifact due to the *in vivo* hydrolysis of the conjugate to the parent compound, Z. This alternative mechanism which involves a futile cycle for glucuronide conjugates *in vivo* (Fig. V-1) was first proposed by Gugler (141) in order to explain the change in disposition of clofibric acid in renal failure (29, 30). Upton *et al.* (24) discussed the possiblity of a

futile cycle for ketoprofen pharmacokinetics in healthy subjects given probenecid concurrently and Verbeeck (8) suggests that this is the favored explanation for the decrease in clearance seen for ketoprofen in the elderly who normally have reduced renal function. More recently, Meffin and Veendendaal (111) have shown by dosing clofibric acid glucuronide to rabbits that the conjugate is cleaved very rapidly *in vivo*. Studies in our laboratory have found that ZG is also quickly hydrolyzed *in vivo* when it is given intravenously to rabbits or guinea pigs (Chapter V). Nonetheless, the net effect is that probenecid dramatically reduces the apparent clearance of Z through conjugation with glucuronic acid. Metabolic clearance by other routes may also be affected by probenecid, since urinary recoveries of Z and ZG did not account for the total dose administered. The term $CL_{z \rightarrow other}$ does not necessarily represent the clearance of Z to hydroxyzomepirac, which was not measured with our assay. If instead $CL_{z \rightarrow other}$ represents metabolism to ZG which does not appear in the urine, the maximum value for $CL_{z \rightarrow zg}$ would be 119 ml/min (i.e. $CL_{z \rightarrow other} + CL_{z \rightarrow zg}$) in probenecid treated subjects (Table VI-1). This hypothetical maximum value for $CL_{z \rightarrow zg}$ in probenecid treated subjects is still much less than the minimum value in control subjects, 252 ml/min, which is based upon ZG recovered in the urine. Although the estimate for $CL_{z \rightarrow other}$ was not changed by probenecid administration, the AUC_z increased 4.3 fold and the apparent $CL_{z \rightarrow zg}$ was depressed which may result in an increased fraction of the total dose undergoing Phase I metabolism as was observed for naproxen when probenecid was given concurrently (166). This may explain the decrease in the amount of ZG excreted into the urine when probenecid was coadministered.

Probenecid does depress renal clearance of Z which is an expected result which occurred in previous studies of the probenecid/NSAID interaction. However, this contributes little to the mechanism of the interaction since renal clearance of Z is only a small fraction of its total clearance.

Other investigators have previously encountered difficulties when attempting to measure acyl glucuronides in plasma of humans. There has been disagreement between laboratories regarding the presence of clofibric acid glucuronides in the plasma of patients taking clofibrate. Veenendaal et al. (168, 180) found no conjugate of clofibric acid in plasma of control or probenecid treated subjects when either an acid hydrolysis treatment of plasma prior to measuring the parent drug was used or a direct HPLC assay of the conjugate was employed. In contrast, Faed and McQueen (22, 29), using an assay where the conjugate was cleaved by base prior to measuring the parent drug, found that in renal disease up to 30% of the total clofibric acid in plasma was the conjugate while in normal subjects the value was only 7%. Gugler (141) reported similar plasma levels of the conjugate in patients with renal failure when using β-glucuronidase to cleave the glucuronide, but found no conjugate detectable in healthy subjects. Meffin et al. (169) found that in rabbits plasma concentrations of clofibric acid glucuronide were only about 1% those of the parent drug, but that with concurrent probenecid the conjugate levels increased by a larger percentage than did clofibric acid levels. The discrepencies observed in human are probably due to methodological differences between laboratories which allowed variable extents of acyl migration and hydrolysis of clofibric acid glucuronide to occur in the plasma samples.

Two previous studies with the NSAID's indomethacin (164) and ketoprofen (24), which did attempt to measure glucuronide conjugates in human plasma during concurrent probenecid dosing, reported much less conjugate relative to parent drug. For indomethacin, probenecid increased the levels of its glucuronide conjugate (measured by β-glucuronidase hydrolysis) insignificantly, from 32 to 44 μg/ml (164). Ketoprofen had higher levels of the conjugate in plasma than indomethacin, and probenecid increased the average ratio of conjugate/ketoprofen plasma concentrations from 0.11 to 0.27 (24). However, in neither of these studies was sample handling or HPLC analysis controlled to prevent the possible degradation of the labile glucuronide. The measurement of low concentrations of the conjugate by the difference in the parent drug found after either hydrolysis with β-glucuronidase or addition of base is often inaccurate. Also, the isomeric conjugates formed by acyl migration are not susceptible to cleavage by β-glucuronidase (28, 61, 68, 72). Zomepirac may be unusual in that ZG levels *in vivo* in primates approximate those of Z. However, other animals have no measurable ZG levels after administration of Z to healthy animals (Chapter V). Until proper procedures are developed for the analysis of labile acyl glucuronides in biological fluids, the interpretation of pharmacokinetic studies with drugs metabolized to acyl glucuronides, especially the NSAID's, may be questioned.

E. Appendix VI-1. Derivation and assumptions for determining metabolic clearance of zomepirac to form zomepirac glucuronide after oral dosing.

The determination of $CL_{z \rightarrow zg}$, equation 7 in the text, requires that $CL_{R,z}$ is negligable relative to the total clearance of Z, CL_z, and that absorption is complete. Assumptions required for the pharmacokinetic model are that absorption is complete and that metabolism only occurs in the liver (25, 158). In the model presented here, F is the first pass availability through the liver, with complete absorption and no loss of drug prior to entering the liver. In Figure VI-3, Z' and ZG' represent the dose of Z which escapes first pass elimination by the liver and the glucuronide metabolite derived from that part of the dose, respectively. Based upon the following model, Figure VI-3, a mass balance approach was used to estimate $CL_{z \rightarrow zg}$.

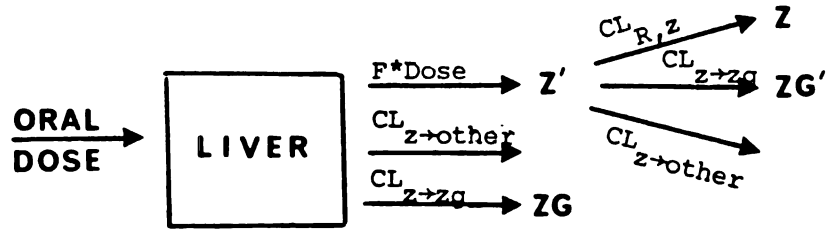


Figure VI-3. Pharmacokinetic model for Z absorption, first pass by the liver and subsequent elimination.

(a) $CL_z = CL_{z \to zg} + CL_{R,z} + CL_{z \to other}$

(b) $CL_z = Z'/AUC_z = F*Dose_z/AUC_z$

(c) $f_m = CL_{z \to zg}/CL_z = CL_{z \to zg}/(CL_{z \to zg} + CL_{R,z} + CL_{z \to other})$

(d) $f_L = CL_{z \to zg}/(CL_{z \to zg} + CL_{z \to other})$

(e) $ZG = (1 - F)*Dose_z*f_L$

(f) $ZG' = F*Dose_z*f_m$

(g) $ZG_{total} = ZG + ZG'$

Substituting equations (e) and (f) into (g),

(h) $ZG_{total} = (1 - F)*Dose_z*f_L + Dose_z*F*f_m$

which is rearranged to,

(i) $ZG_{total} = Dose*[f_m*F + f_L*(1 - F)]$

Because f_m approaches f_L as renal clearance approaches zero,

(j) $\lim_{CL_{R,z} \to 0} ZG_{total} = Dose_z*f_L = Dose_z*fm$

$= Dose_z*CL_{z \to zg}/CL_z$

Rearranging equation (j),

(k) $CL_{z \to zg} = ZG_{total}*CL_z/Dose_z$

Therefore, if renal clearance of Z is small relative to the total clearance, CL_z, then equation (k) can be used to estimate the metabolic clearance for the formation of ZG from Z.

CHAPTER VII

COVALENT BINDING OF ZOMEPIRAC TO ALBUMIN IN VITRO AND IN VIVO THROUGH ITS ACYL GLUCURONIDE METABOLITE

A. INTRODUCTION

The model compound used in our studies, zomepirac (Z), was previously marketed in the U.S. and Britain as an analgesic under the tradename of Zomax® (McNeil Pharmaceuticals, Springhouse, PA) (90). After several years on the market Zomax® was voluntarily withdrawn in March, 1983, because of a high incidence of immunological adverse effects some of which were anaphylactic reactions resulting in death (33, 34). Z is representative of a large and growing group of nonsteroidal antiinflammatory drugs (NSAIDs) of the aryl alkyl acid class (35) which includes tolmetin (Tolectin®), indomethacin (Indocin®), diflunisal (Dolobid®), ibuprofen (Motrin®) and benoxaprofen (Oraflex®). Ibuprofen has recently been approved for nonprescription sales under the tradenames fo Nuprin® and Advil®. Because of the serious toxicity of Z, as well as that of benoxaprofen (36, 37), this group of drugs have come under increasing scrutiny by both the public and government (38). Moreover, a recent review of prescription drugs withdrawn from the U.S. and British market because of serious adverse effects included three additional NSAIDs of this class, ibufenac, alclofenac and indoprofen, among the 25 drugs listed (181). With an estimated six million arthritics and 30 million persons with other chronic joint diseases in the U.S. (182), the market for antiiflammatory drugs is large and sales of these medications amount

to a billion dollars per year (183). The growth of this market and exposure of the public to NSAIDs is likely to continue with many new drug entities being developed or introduced.

A characteristic feature of aryl alkanoic acids is their ubiquitous carboxylic acid side chain which results in a common route of metabolism for these compounds. Drugs of this class often conjugate with glucuronic acid forming acyl glucuronides as the major metabolite. This metabolic pathway accounts for up to 90% of an orally administered ^{14}C-Z dose excreted in human urine (50). Another well known compound, that is metabolized to similar unstable acyl glucuronides which undergo acyl migration at physiological pH is bilirubin. Bilirubin was reported to irreversibly bind to albumin in vitro and in vivo through its acyl glucuronide (10, 11). Because of the common metabolic pathway for these antiinflammatory drugs and bilirubin, and the history of unexplained toxicies of NSAIDs, the covalent binding of bilirubin to albumin stimulated our investigations of potential irreversible binding of NSAIDs to plasma proteins. Bilirubin acyl glucuronide and zomepriac glucuronide (ZG) have similar stability problems. Both compounds undergo acyl migration (7, 28, 44) and react rapidly with methanol to form methyl esters (28, 47) at physiological pH. The electrophilic nature of the carbonyl group of the aglycone-glucuronic acid linkage is believed to be responsible for the irreversible binding of bilirubin to protein (11). Stogniew and Fenselau (120) have shown that clofibric acid glucuronide reacts in vitro with thiols, ethanethiol and glutathione, and suggested that the electrophilic nature of the acyl glucuronide may be involved in the toxicity of clofibrate. While the studies in our laboratory of irreversible binding of Z through ZG were

being conducted, VanBreeman and Fenselau reported in abstract form (184) that the ^{14}C-labelled acyl glucuronides of indomethacin, clofibric acid and flufenamic acid lead to irreversible incorporation of the ^{14}C with albumin and possibly DNA.

The possibility of irreversible binding of a drug to macromolecules in vivo through its electrophilic acyl glucuronide has stimulated interest in this phenomenon by several laboratories. A toxicological event has yet to be correlated with irreversible binding of NSAIDs to macromolecules. However, the strong relationships previously documented, correlating carcinogenicity, mutagenicity (14) and immunogenicity (13) with reactivity and the extent of covalent binding of organic compounds to macromolecules suggest the hypothesis that such binding might be intimately related to the toxicity of drugs metabolized to acyl glucuronides. The ubiquitous nature of carboxylic acids in xenobiotics (1) and the importance of glucuronidation as a route of metabolism for these acidic compounds (51, 54) demand that the possible irreversible binding of the acidic drugs to biological macromolecules through their acyl glucuronides be examined. The studies presented here are not designed with the intent of providing a cause-effect relationship between irreversible binding of Z to proteins in vivo and its immunological toxicity. Instead, the objectives of the experiments in this chapter are to prove that irreversible binding of Z to protein does occur via its acyl glucuronide, ZG; to examine the conditions which affect the binding; and finally to establish whether this irreversible binding occurs in vivo. For convenience, "binding" in this chapter will refer to irreversible binding of Z to protein. The exact nature of the binding was not addressed by the experiments here, however, some results

obtained did provide information which may contribute to an explanation of the mechanism of the binding.

B. MATERIALS AND METHODS

Chemicals

Zomepirac·Na·$2H_2O$, Zomax® 100 mg tablets, ^{14}C-zomepirac (11.7 μCi/mg, 322 μCi/mmole) and 5-(4-methoxybenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid (internal standard) were obtained from McNeil Pharmaceutical (Springhouse, PA). ZG was purified from human urine (prior to withdrawal of the drug) as previously described (28) (Chapter II). Isomeric conjugates of ZG, with Z linked to the 2,3 or 4 positions of glucuronic acid, were isolated by preparative HPLC (28) (Chapter II). Human plasma was collected from volunteers into heparinized (10 U/ml) tubes. Human serum albumin (Fraction V) (HSA), phenylmethylsulfonyl fluoride (PMSF) and β-glucuronidase, (Bovine Liver, Type B-10) were purchased from Sigma (St. Louis, MO). Other chemicals were reagent grade and other solvents for analysis were HPLC grade.

^{14}C-Zomepirac glucuronide from monkey urine

Initial studies of irreversible binding of Z to protein were done using ^{14}C-labelled ZG which was isolated and purified from urine of a rhesus monkey given a dose of radiolabelled Z. A male rhesus monkey (ca. 8 kg) was restrained in a metabolic chair, then administered an intraveneous dose of 23 μCi ^{14}C-Z which had been diluted with 23 mg Z in methanol such that the specific activity was 0.923 μCi/mg. The methanolic solution of Z was evaporated to dryness under nitrogen, then dissolved in 2 ml of 40% propylene glycol prior to administration.

Spontaneously voided urine was collected at intervals through 120 hours into a container with 1 ml 86% phosphoric acid which decreased the pH of the urine and prevented the loss of ZG (28) (Chapter IV). Urine was adjusted to pH 2 to 4 with phosphoric acid prior to storage at -20 °C.

The 0 to 11 hour urine sample (360 ml), which contained about 80% of the radioactivity excreted in the urine over 120 hours, was adjusted to pH 2, washed twice with 200 ml hexane, then extracted 4 times with 150 ml ethyl acetate. The extraction with ethyl acetate removed 80% of the radioactivity from the urine sample as determined by scintillation counting. The ethyl acetate extract was dried with magnesium sulfate, filtered, then taken to dryness with a rotoevaporator. The residue was dissolved in 4 ml of 25% acetonitrile/0.05 M sodium acetate, pH 4.5, and centrifuged to remove undissolved material; aliquots were then injected onto analytical and preparative HPLC. Analytical HPLC is as described in Chapter II using 45% methanol as the organic component of the mobile phase. Preparative HPLC was modified from that employed in Chapter II, with 50% methanol/sodium acetate, pH 5.1, as the mobile phase at a flow rate of 5 ml/min. The ZG peak eluted from successive preparative HPLC injections were combined, adjusted to pH 2 with phosphoric acid, then rotoevaporated to remove the methanol. Since these solutions of ZG were much more dilute than those obtained when preparative HPLC was done to isolate the isomeric conjugates of ZG (Chapter II), the ZG did not precipitate upon removal of the methanol. Instead, the aqueous solutions of ZG from the preparative HPLC were extracted with ethyl acetate, dried with magnesium sulfate, taken to dryness with a rotoevaporator and the residue was dissolved in dry methanol. The concentrated methanolic solution of purified ZG (ca. 189 μg/ml) was kept at -20 °C until used

for binding studies. Purity of ZG after preparative HPLC and subsequent workup was determined by analytical HPLC to be 85% of the radioactivity present. Recovery of ^{14}C-ZG after extraction and preparative HPLC was calculated to represent 2.4% of the total administered ^{14}C-Z dose.

In vitro binding studies with ^{14}C-labelled compounds

Duplicate incubations of radiolabelled Z and ZG were conducted at a concentration of 30 µg/ml (Z equivalents, 63,000 CPM/ml) with 0.5 mM (3 gm%) HSA in 0.15 M phosphoric acid, pH 7.5, at 37 °C. One ml aliquots taken after 2 minutes, 1, 6 and 20 hours of incubation were added to 3 ml acetonitrile to precipitate protein, centrifuged to separate the protein and supernatant, then the protein was washed 10 times with 3 ml methanol:ether (3:1) to remove reversibly bound Z and conjugates. The washed protein pellet was digested with 2 ml of 1 M NaOH for one hour at 50°C, neutralized with 0.5 ml glacial acetic acid, then suspended in 15 ml scintillation fluid (Aquasol®, New England Nuclear, Boston, MA) for counting of ^{14}C. Aliquots of the basic digest were taken and analyzed for protein concentration using the Bradford method (123) (Bio-Rad, Richmond, CA). Irreversible binding was expressed as CPM/mg protein and the fraction of the total radioactivity added to one ml of the incubation.

In vitro binding studies using unlabelled compounds

In vitro incubations of Z, ZG or ZG isomers with 0.5 mM (3 gm%) HSA or human plasma were carried out at 37°C. HSA solutions were prepared in 0.15 M potassium phosphate buffer adjusted to the desired pH with dilute NaOH or HCl. Z, ZG and isomers of ZG were dissolved in ethanol

prior to use, and aliquots of the solutions were added to HSA solutions to provide concentrations of 20 to 50 μg/ml (0.068 - 0.17 mM). Mixtures of isomeric conjugates of ZG were prepared by preincubation of ZG in phosphate buffer, pH 7.4, at 37°C for 45, 90 and 240 minutes (28). At the end of the incubation, 2000 U/ml β-glucuronidase was added followed by 15 minutes further incubation to assure complete hydrolysis of ZG. Isomeric conjugates of ZG are resistant to hydrolysis by β-glucuronidase (28) (Chapter IV) and HPLC analysis of the incubations showed that no ZG was present when the isomeric conjugates of ZG were studied for binding to albumin in vitro. Aliquots (0.5 ml) from the incubation reactions were stopped by precipitating the proteins with one part cold ethanol, 2 parts acetonitrile and 0.01 parts concentrated phosphoric acid to obtain pH 2 to 4. After centrifugation (2250 x g) for 10 minutes the supernatant was analyzed by HPLC using a method developed in our laboratory which prevents degradation of the labile glucuronides (98) (Chapters II and III). Quantification of the extent of covalent binding of Z to HSA was done using established methods as follows (185): the protein pellet obtained after precipitation above was washed exhaustively (10 times) with 6 volumes methanol:ether (3:1) to remove reversibly bound Z and conjugates. Residual protein was treated with 1 ml 1 M KOH (80°C x 1 hr) and the Z liberated was quantitated by HPLC (107) (Chapter III) after acidification (95 μl conc. H_3PO_4) and extraction of the protein digest with 5 ml dichloromethane containing 2 μg internal standard. A standard curve was constructed by spiking blank precipitated plasma or HSA with Z to concentrations of 0 to 2 μg/ml prior to base hydrolysis. Z is stable under the basic conditions employed and studies with both ^{14}C-labelled and unlabelled Z were conducted to ensure that the

exhaustive washing procedure completely removed adsorbed and reversibly bound Z from plasma or HSA protein precipitates. Drug not removed from plasma protein by the washing procedure is defined in this paper to be "irreversibly bound" to protein (185). Binding of Z *in vitro* was normalized to the molar amount of conjugate or Z present at the start of the incubation.

Conditions necessary for the hydrolysis of Z-protein adduct were optimized as follows. Incubation of 55 μg/ml ZG (Z equivalents) with 3 gm% (0.5 mM) HSA, pH 7.4, at 37 °C, for 6 hours was done to achieve maximal binding of Z to protein. After exhaustive washing of the protein as described above, the precipitated albumin was divided, then hydrolyzed in duplicate with either 1 or 2.5 M NaOH at 80°C. The hydrolysis of the Z-protein adduct was measured after 1, 3, 7 and 13 hours of incubation by extraction and HPLC determination of the liberated Z (107) (Chapter III). Zomepirac released was normalized to protein content of the hydrolysate.

In vivo binding studies in bile ligated guinea pigs

As described in Chapter V, two ml blood samples were collected serially for binding studies from bile ligated guinea pigs which had received intraveneous doses of Z or ZG. Some animals had also recieved an intraperitoneal dose of 50 mg/kg phenylmethylsulfonyl fluoride (PMSF) which decreased the plasma clearance of ZG, by inhibiting the hydrolysis of ZG by esterases *in vivo*, resulting in elevated plasma concentrations of the conjugate (Chapter V). A total of 31 plasma samples were obtained at 10 minutes, 2, 4 and 6 hours after the respective doses of Z or ZG. Using one ml plasma samples irreversible binding of Z to plasma

proteins was determined by the method described above for *in vitro* studies with unlabelled compounds. For *in vivo* studies the binding is expressed as ng of Z bound/mg protein, where protein was determined with the Bradford method (Bio-Rad, Richmond, CA) using HSA as the standard. Correlations of Z bound irreversibly to protein with the exposure to Z or ZG in plasma (AUC) utilized the plasma concentration data in Chapter V.

In vivo binding studies in humans

A clinical study of the disposition of Z and the effect of probenecid on Z disposition in healthy volunteers was conducted in our laboratory in September, 1982, and is presented in detail in Chapter VI. Briefly, after approval by the University Committee on Human Experimentation a single oral dose (100 mg) Zomax® tablet was given to six healthy male volunteers, ages 21-30. Probenecid (500 mg bid) was given three days prior to and concurrently with one of the Z doses in order to examine its effect on Z and ZG disposition. Plasma and urine samples taken from zero to 48 hours after each Z dose were assayed within two weeks for Z and ZG plasma concentrations using HPLC methods (107) (Chapter III). In order to minimize artifactual ZG loss prior to analysis, plasma and urine were adjusted to pH 2-4 with phosphoric acid, then frozen at -20°C until analyzed. Seven of the original twelve sets of plasma samples from the 1982 study were used for analysis of Z binding in the present study along with six control plasma samples that had been spiked with 2 µg/ml ZG. These frozen samples were examined retrospectively, 18 months after collection, for irreversible binding of Z using the procedure described above for the *in vitro* studies with unlabelled compounds.

C. RESULTS

Isolation of ZG from monkey urine after the dose of 23 μCi ^{14}C-Z provided a source of ^{14}C-ZG for use in the *in vitro* studies. The yield of ZG from the monkey urine after purification by extraction and preparative HPLC was only 2.4% of the total ^{14}C administered. In contrast to previous data on Z disposition in rhesus monkeys, where 85% of the labelled dose was excreted in the urine within 48 hours (26), we obtained only 43% of the dose in the urine after 45 hours. An additional difference was that only 35% of the ^{14}C present in the the 0-11 hour urine collection was ZG (Fig. VII-1), whereas the previous literature reported that 66% of the ^{14}C excreted in the urine from 0-48 hours after a dose of Z to the rhesus monkey was present as ZG (26). The poor overall yield of ^{14}C-ZG from monkey urine provided enough compound for only a limited number of *in vitro* studies to be performed. As will be mentioned below, the 85% radiochemical purity of the ^{14}C-ZG limited the interpretation of subsequent *in vitro* binding studies.

A preliminary study measuring the putative ^{14}C-Z which becomes irreversibly bound to albumin after 4 hours of incubation with ^{14}C-ZG found that incubations with ^{14}C-ZG produced 57 fold higher binding of ^{14}C-Z to albumin than the control incubations with ^{14}C-Z. Further *in vitro* studies examined the time dependence of binding when ^{14}C-ZG was incubated with albumin at pH 7.4, 37°C. The reversibly bound Z, ZG and isomers of ZG in the supernatant after the first precipitation of albumin was measured by HPLC. As shown in Figure VII-2, binding of ^{14}C-Z upon incubation of labelled ZG with albumin was time dependent with an apparent plateau or maximum achieved after 6 hours, while control incubations with ^{14}C-Z produced no time dependency in binding. The rate

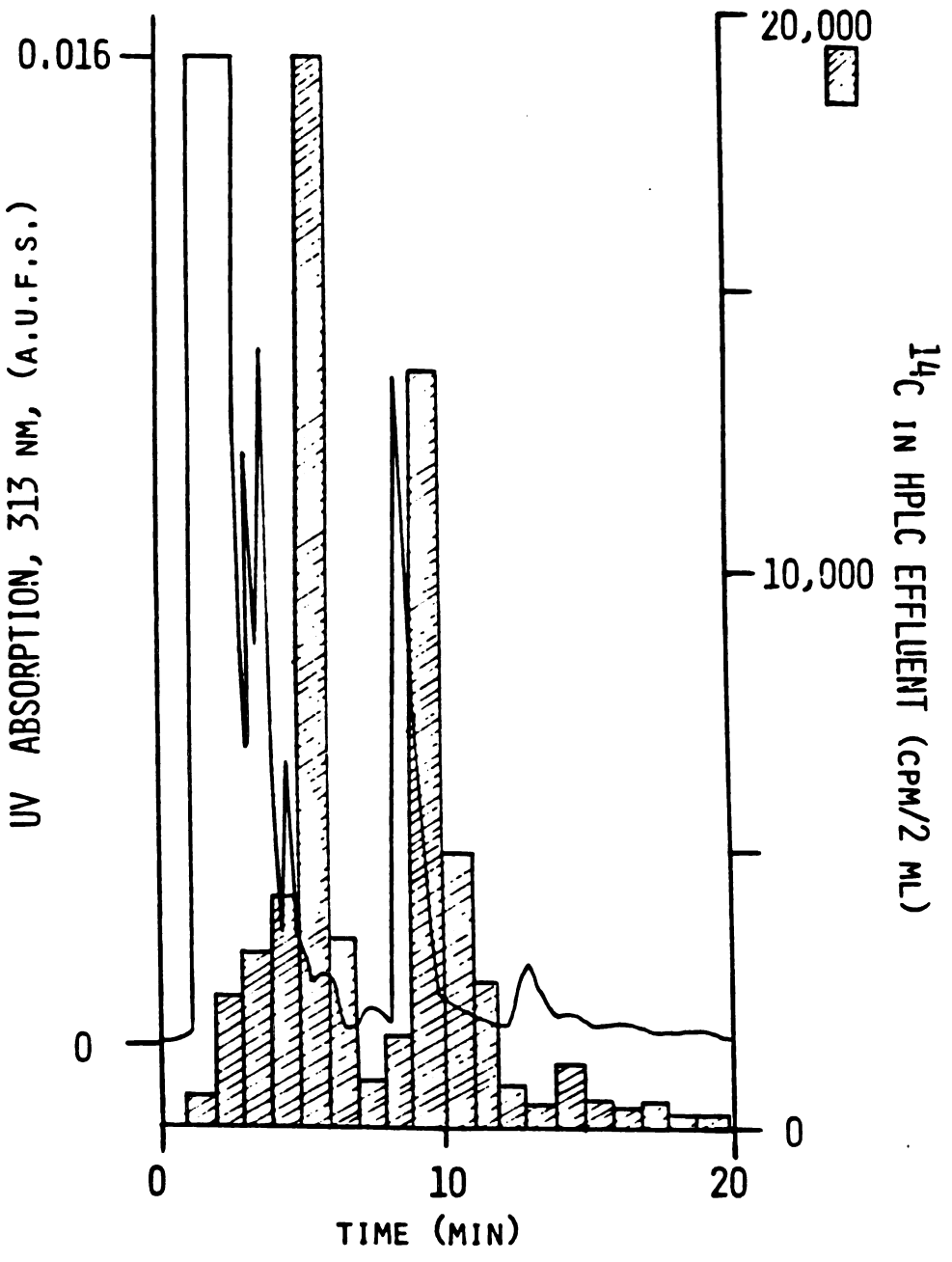


Figure VII-1. Profile of the ethyl acetate extract of urine collected from 0 to 11 hours after an intravenous dose of ^{14}C-labelled zomepirac to a rhesus monkey. Left ordinate is UV absorption at 313 nm. Superimposed (right ordinate) is the radiochemical analysis of the HPLC effluent which has been shifted two minutes to the right for clarity.

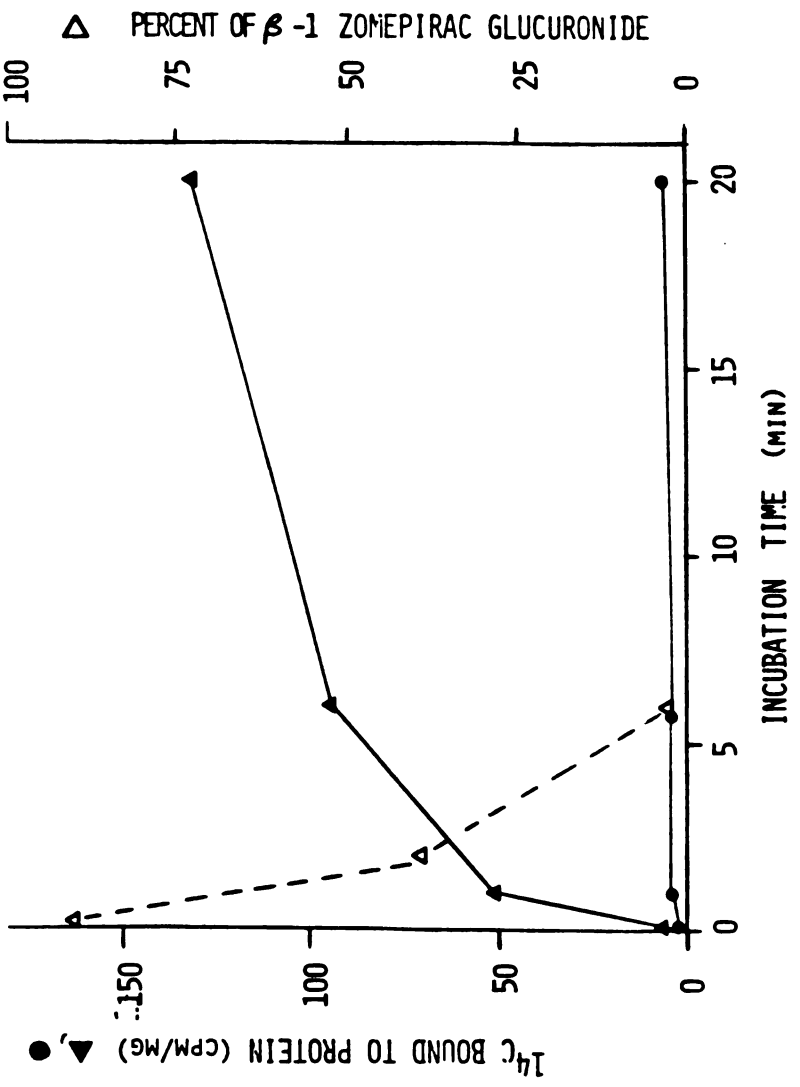


Figure VII-2. The irreversible binding of ^{14}C to albumin after incubation of zomepirac glucuronide, ▲, and zomepirac, ●, with 0.5 M human serum albumin at pH 7.4, 37 °C. The degradation of zomepirac glucuronide is superimposed, Δ, right ordinate.
Note: 50 cpm/mg = 2.38% of the total conjugate present at the start of the incubation.

of binding appeared to be temporally related to the concentration of β-1 ZG present, as determined by HPLC (Fig. VII-2). Unfortunately, the fraction of the ^{14}C in the incubation which became bound was at most 7% of the total ^{14}C, which is less than the impurities unaccounted for with the 85% radiochemically pure ^{14}C-ZG. Additional studies with the remaining ^{14}C-ZG produced similar time dependent binding of putative Z to albumin via ZG, which averaged about 5% of the ^{14}C becoming irreversibly bound after 6 hours of incubation. The impurities in the ^{14}C-ZG isolated from monkey urine precluded a definitive correlation between binding of Z to protein and exposure to ZG <u>in vitro</u>. Also, the entity which became irreversibly bound was not conclusively established to be Z when ^{14}C incorporation was used to determine the extent of binding. The results and their limitations prompted a search for an alternative procedure to assess Z binding to albumin.

If a Z-protein adduct does form with albumin, it is likely to be through formation of an amide, ester or sulfhydryl ester with a lysine, tyrosine or cysteine moiety, respectively, on albumin (186, 187). Since a protein adduct possibly formed through these functional groups should be labile to base, and Z was found to be stable in aqueous base, a method was developed where the Z putatively bound to albumin or plasma proteins was first cleaved from the protein by base hydrolysis prior to extraction and quantification by HPLC. Both 1 and 2.5 M NaOH liberated Z from the irreversible protein adduct. The amount of Z released from the protein upon hydrolysis did not vary with either base concentration, nor length of hydrolysis longer than one hour. Therefore, hydrolysis with 1 M NaOH at 80 °C for one hour was adequate and was used for all incubation studies performed using unlabelled compounds. Hydrolysis of

the Z-protein adduct and analysis of Z liberated from protein by HPLC also proves that the entity bound is Z and not an impurity from the monkey urine nor a product of spontaneous oxidation *in vitro*. An example of the chromatography obtained after extraction and HPLC analysis of the protein hydrolysate as described in Methods is shown in Figure VII-3.

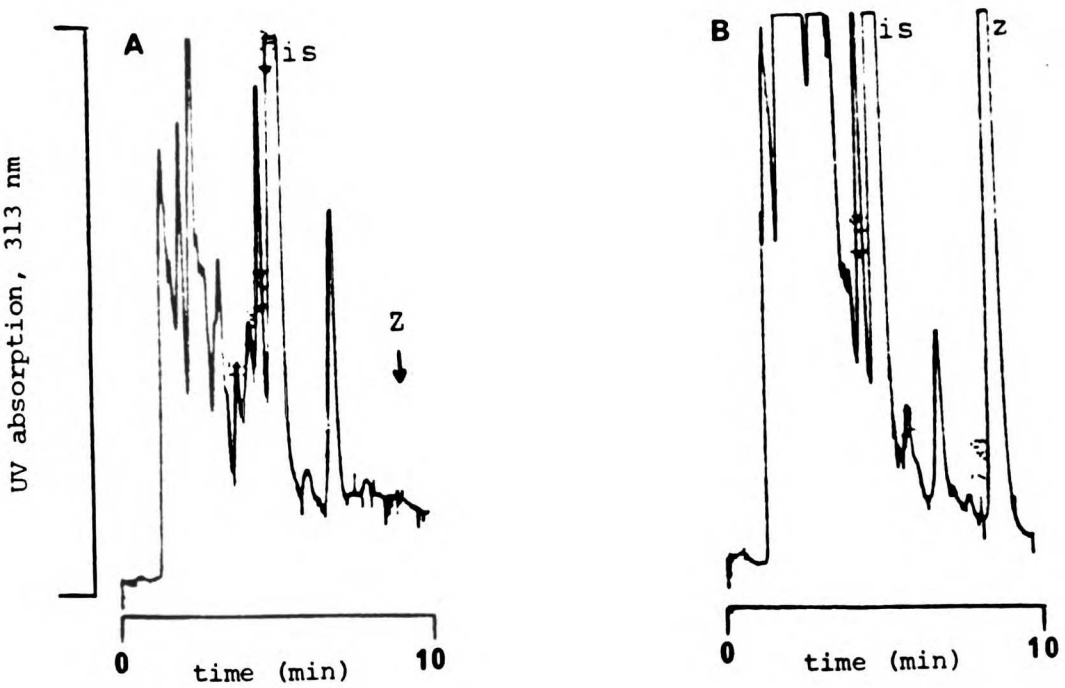


Figure VII-3. HPLC chromatograms of the extract of a protein sample after hydrolysis to liberate Z. A, blank sample with 2 µg internal standard; B, sample with 1 µg Z.

Using the hydrolysis and extraction procedure for the determination of Z binding to proteins allowed the use of purified unlabelled ZG for *in vitro* binding experiments. Mixtures of the isomeric conjugates of ZG as well as the purified isolated conjugates of ZG were also examined for

possible binding to albumin. Irreversible binding of Z to protein occurred when either ZG or a mixture of its isomeric conjugates was incubated with HSA or plasma at physiological pH. As shown in Figure VII-4, the binding was time dependent, and reached a plateau after 3 hours. The relative fraction of the total drug bound was 60% higher with ZG than with the mixture of ZG positional isomers. Control incubations with Z produced no binding. Not shown in Figure VII-4 are analyses carried out at 12 and 24 hours which showed a slight decrease in the concentration of bound drug, presumably because of slow hydrolysis of the Z-protein adduct. The rate and extent of binding was pH dependent, as shown in Figure VII-5. The formation rate of Z-protein adduct increased when the pH was raised from 5 to 9, but the conjugates eventually rearranged by acyl migration and hydrolyzed at the higher pH. The Z-protein adduct also hydrolyzed at the higher pH values. Further experiments conducted with Z-HSA adduct at pH 7.4 and 10.0 (data not presented), as well as the use of 1 N KOH to hydrolyze the Z-protein adduct, document the labile nature of the adduct under basic conditions. When the supernatant obtained from the in vitro incubations was analyzed by HPLC, the concentration of ZG, as expected (28) (Chapter IV), decreased with time producing isomeric conjugates of ZG with eventual slow hydrolysis to Z. Thus for ZG, hydrolysis and acyl migration compete with covalent Z-protein adduct formation.

Mixtures of the isomeric conjugates of ZG formed after 90 minutes of incubation of ZG in buffer at pH 7.4, 37°C, yielded Z-protein adduct after they were incubated with albumin (Fig. VII-4). The pattern of

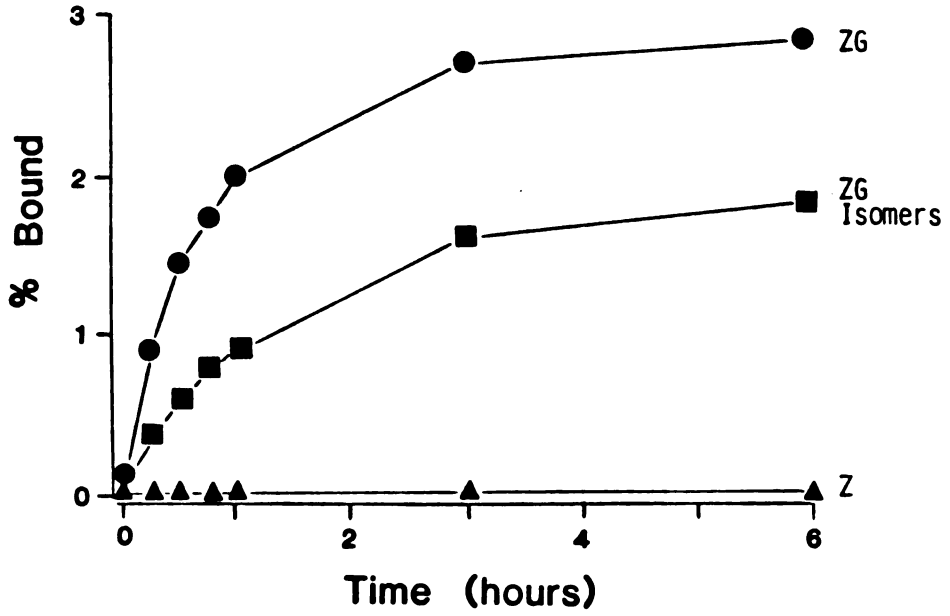


Figure VII-4. The irreversible binding of zomepirac (Z) to human plasma in vitro after incubation with zomepirac glucuronide, ZG, ●; isomers of ZG, ■; or Z, ▲; at pH 7.4, 37 °C. Initial concentrations are 20 - 50 µg/ml. Data points are the mean of replicate incubations.

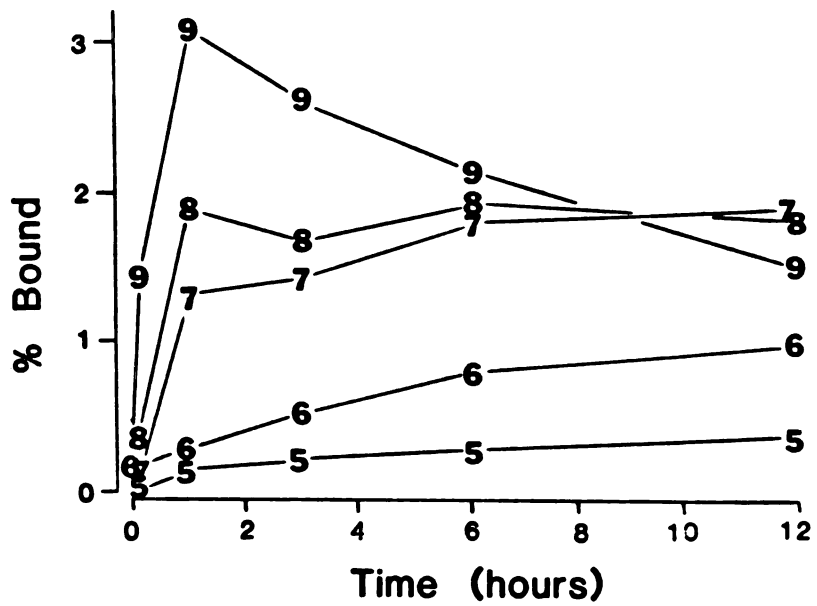


Figure VII-5. The effect of pH on the irreversible binding of zomepirac to albumin when albumin was incubated with 50µg/ml zomepirac glucuronide at 37 °C in 0.15 M phosphate buffer. Numbers on the curves indicate the pH for each data point. Data are the mean of duplicate incubations.

isomers present changes over the time course of incubation in buffer because of sequential acyl migration about the sugar (Fig. IV-1). For preliminary studies on the relative ability of the individual isomers of ZG to cause binding, mixtures of the isomers formed after 45, 90 and 240 minutes of incubation in buffer were compared with respect to binding to albumin *in vitro*. As shown in Table VII-1, all isomeric mixtures incubated with albumin produced less Z bound to albumin than β-1 ZG. There was a decline in the rate and extent of Z binding with increasing time allowed for acyl migration to occur. In all cases the binding of Z was less at 12 hours than at 6 hours of incubation. However, when the supernatant of the incubations with isomers were analyzed by HPLC, the isomer peak did not disappear due to the addition of HSA. From these preliminary experiments, no isomer of ZG could be exclusively associated with the binding of Z to protein.

To investigate the possible relationship between the individual isomeric conjugates of ZG and the binding of Z to protein, each individual purified isomeric conjugate was incubated at pH 7.4 with HSA (Fig. VII-6). Incubations were sampled frequently during the first hour before appreciable rearrangement to the other isomeric conjugates could occur. Because the available quantities of purified isomers were limited these incubations were only done in duplicate. Irreversible binding of Z was observed with each isomer incubated with albumin, though at different rates. Therefore, ZG and all of its isomers formed by acyl migration are capable of leading to formation of Z-protein adduct *in vitro*.

These *in vitro* studies suggested that formation of an aglycone-protein adduct might occur for Z *in vivo*, as with bilirubin glucuronides

Table VII-1. The irreversible binding of Z to albumin when mixtures of isomeric conjugates of zomepirac glucuronide are incubated with albumin at pH 7.4, 37 °C.

Incubation time (min)	Percent of total conjugate which becomes bound[a]			
	Time for acyl migration to occur[b] (min)			
	0	45	90	240
0	0.09[c]	0.095	0.13	0.12
15	0.93	0.42	0.37	0.34
30	1.46	0.66	0.62	0.60
45	1.75	0.95	0.81	0.79
60	1.98	0.99	0.89	0.80
180	2.67	1.64	1.60	1.38
360	2.78	1.95	1.82	1.58
720	2.28	1.39	1.31	1.02
Ratio of 720/360	0.82	0.71	0.73	0.65

[a]Z bound irreversibly to albumin was determined and normalized to the amount of total conjugates present at the start of the incubation just prior to the addition of albumin, 0.5 mM.
[b]Acyl migration of zomepirac glucuronide (50 μg/ml) was done in 0.15 M phosphate buffer, pH 7.4, 37 °C, for the designated time.
[c]Values are the mean of duplicate incubations.

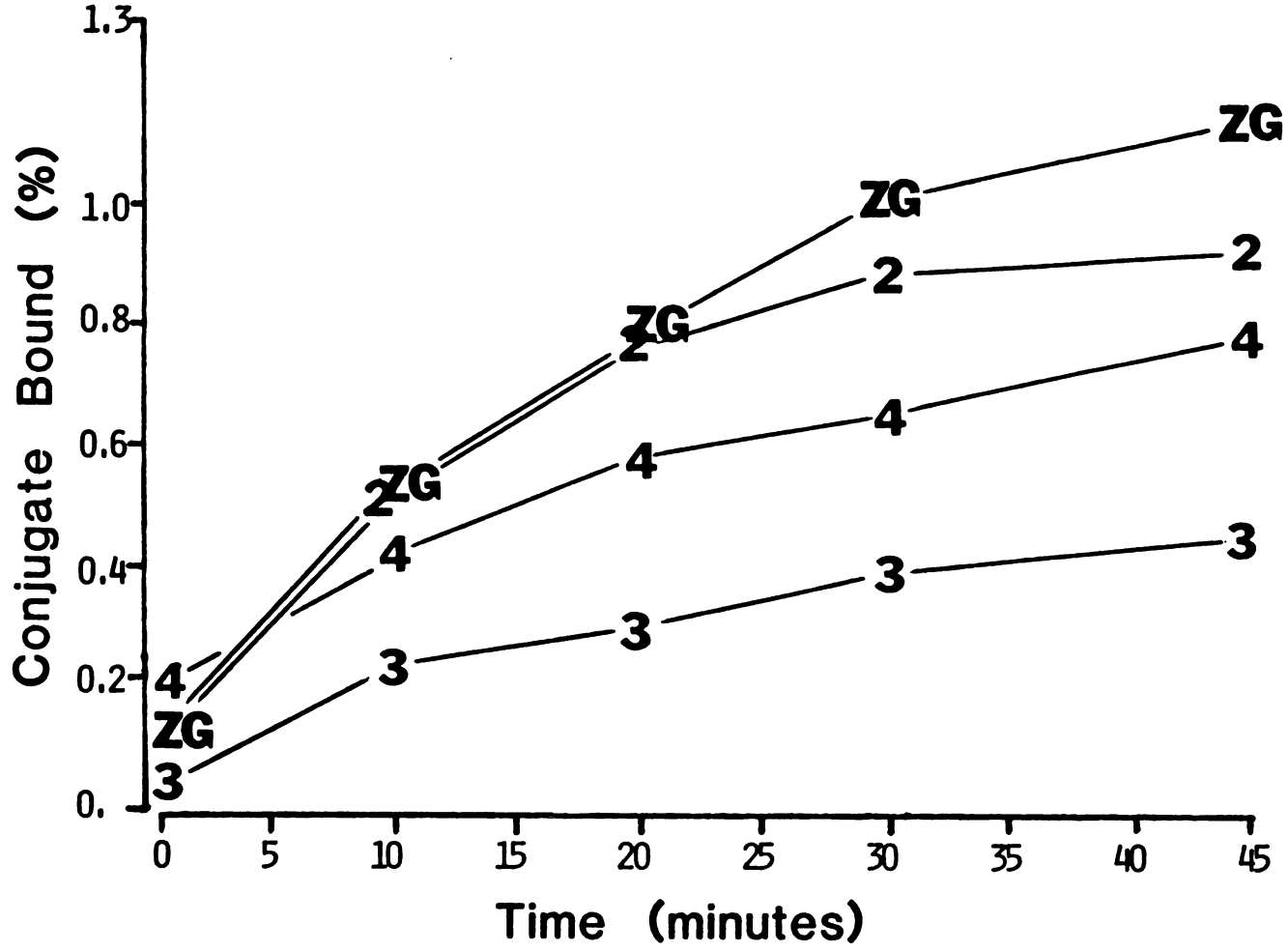


Figure VII-6. The irreversible binding of Z to albumin in vitro when albumin is incubated with each of the purified isomeric conjugates of zomepirac glucuronide at pH 7.4, 37 °C. Numbers indicate the position of the acyl group on the sugar ring of glucuronic acid. Data are the mean of duplicate experiments.

(10, 11), if ZG levels in plasma were to become appreciable. When possible binding of Z to plasma proteins *in vivo* was determined in plasma obtained from bile ligated guinea pigs, there was a significant amount of Z binding, especially in animals treated with PMSF. As shown in Figure VII-7, there was no correlation between Z bound to protein and exposure to Z *in vivo* for guinea pigs (A, r=0.14) when exposure was expressed as AUC_z, but a strong relationship was found between Z bound and AUC_{zg} (B, r=0.84). As discussed in Chapter V, levels of ZG were not appreciable in guinea pig plasma unless PMSF was given concurrently. Thus, binding of Z to protein *in vivo* was highest for animals treated with PMSF.

Z disposition in human was much different than that in the guinea pig as no inhibitor of esterases or cholestasis was needed for ZG to reach appreciable concentrations in human plasma. In the 6 volunteers given 100 mg Z orally, the molar concentrations of ZG detected in plasma were similar to Z levels (Chapter VI). The total area under the plasma concentration vs. time curve (AUC) of ZG and Z were 180 ± 57 and 164 ± 64 μg-min/ml (n=6), respectively, expressed in Z equivalents. The peak concentrations after the Z dose were 1.09 ± 0.42 and 1.60 ± 1.74 μg/ml for ZG and Z, respectively. When probenecid was administered concurrently the plasma clearance of both ZG and Z decreased dramatically, resulting in increased plasma concentrations of both which translated into an increased AUC to 499 ± 134 and 708 ± 308 μg-min/ml, for ZG and Z, respectively, which represent 2.8 and 4.3 fold increases over those seen without probenecid.

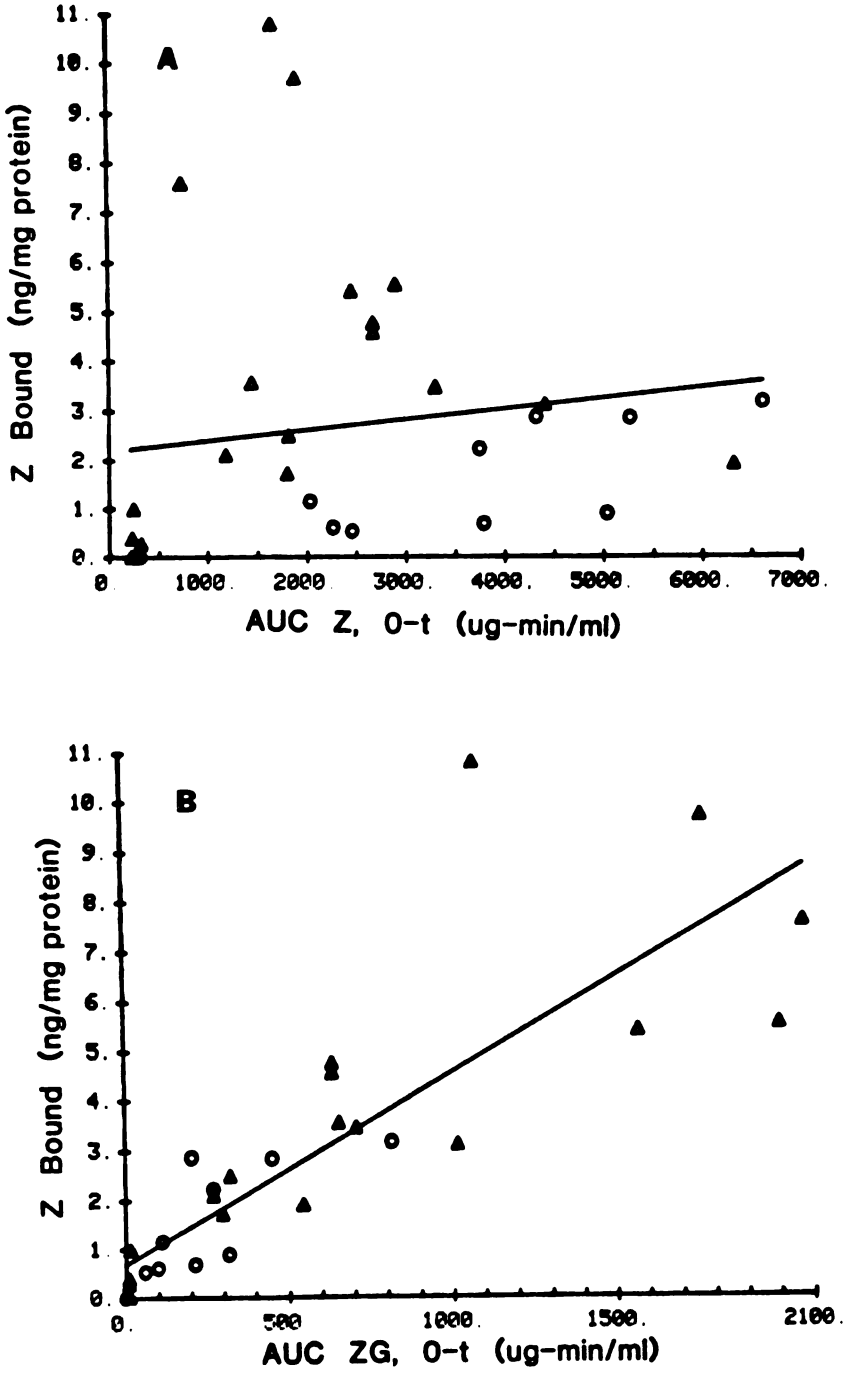


Figure VII-7. The correlations of the irreversible binding of zomepirac to plasma protein in the bile duct ligated guinea pigs relative to AUC_z, A, and AUC_{zg}, B. Triangles correspond to animals treated concurrently with phenylmethylsulfonyl fluoride; circles are control animals. Figure A, slope = $2.12 * 10^{-4}$, r = 0.142, p = 0.447; figure B, slope = $3.87 * 10^{-3}$, r = 0.843, $p < 0.001$, n = 31.

Z-protein adduct was detected in plasma from all subjects given a single oral dose of Z. For each subject the extent of binding was linearly related to the cumulative AUC (0-t) of ZG. Representative plots of this relationship for two subjects are shown in Figure VII-8. Superimposed on the plot (right ordinate) are concentrations of ZG measured at each time point (t) corresponding to a particular cumulative $AUC_{zg}(0-t)$. At zero time no binding of Z was observed. Subsequently irreversible binding increased with increasing $AUC_{zg}(0-t)$ until a maximum was reached when all of the reversibly bound Z and ZG in plasma had been cleared. The maximum amount of drug bound in all subjects was observed at 8-12 hours after the oral Z dose which corresponded to the period during which ZG concentrations in plasma approached zero. Thereafter, as observed in the in vitro experiments, the extent of Z bound decreased gradually. Control plasma samples spiked with 2μg/ml ZG, which is twice the average maximum ZG concentration observed in vivo, had low concentrations of Z-protein adduct (0.30 ± 0.07 ng Z/mg protein, n=6) as compared to the maximum binding measured in vivo.

In each volunteer the concentration of irreversibly bound drug increased linearly with AUC (0-t) of ZG and Z (Fig. VII-9). Interestingly, the linear relationship between Z-protein adduct formed and exposure to ZG had a slope of 4.77×10^{-3} (Fig. VII-9) which is similar to that obtained in the guinea pig, 3.87×10^{-3} (Fig. VII-7). Concurrent administration of probenecid led to an elevated total AUC of ZG and increased Z-protein adduct formation. The correlation between the average maximum adduct detected for each subject and total AUC of ZG is shown in Figure VII-10.

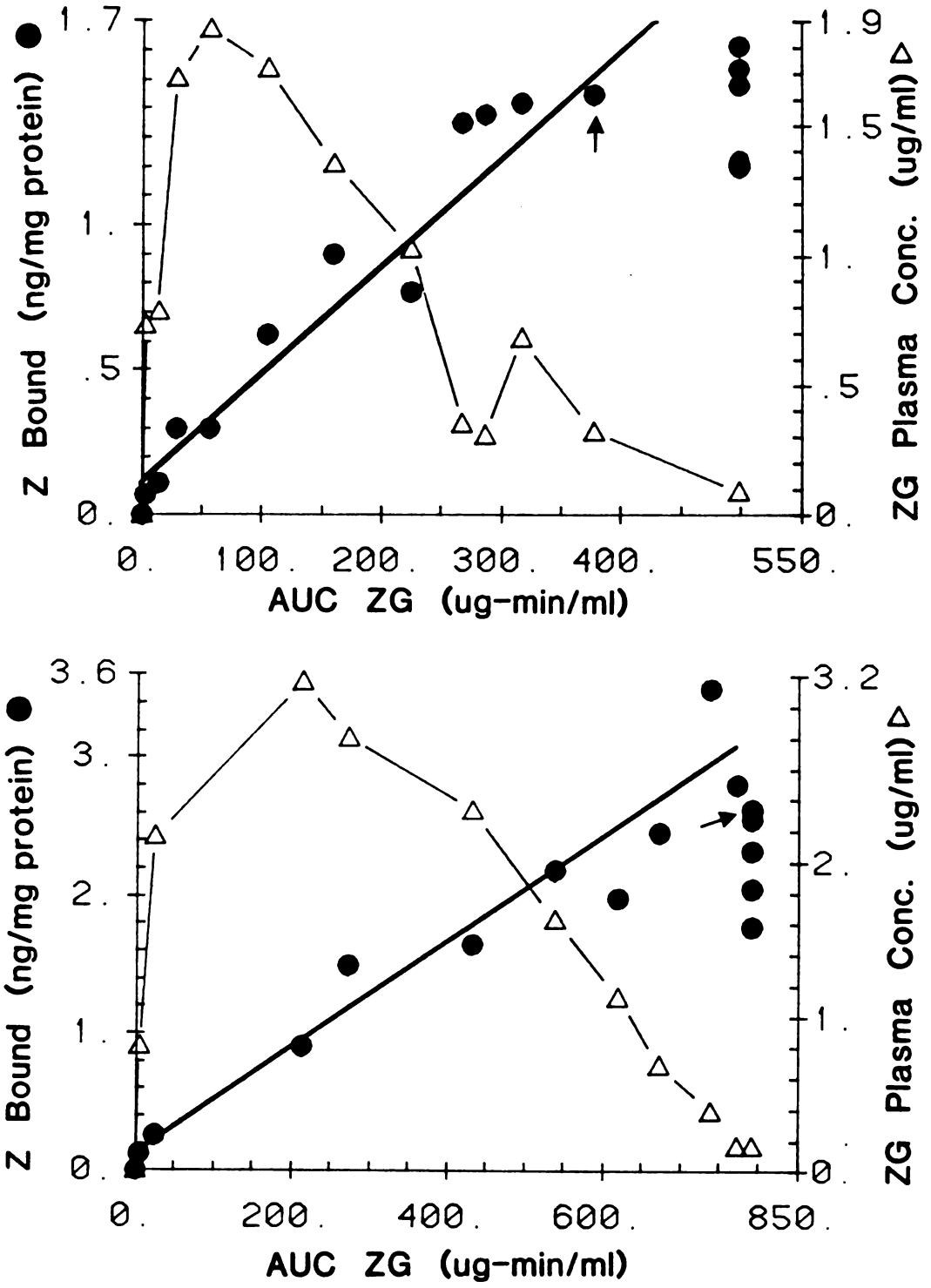


Figure VII-8. The irreversible binding of zomepirac to plasma protein in vivo in two representative human subjects, ● (left ordinate), after 100 mg oral zomepirac dose. Plasma concentrations of zomepirac glucuronide, △ (right ordinate) are superimposed. Fitted regressions from zero to 12 hours (indicated by the arrow) after the dose: subject PS-ZP (top), r = 0.961, p 0.001; subject BW-ZP (bottom), r = 0.967, p 0.001.

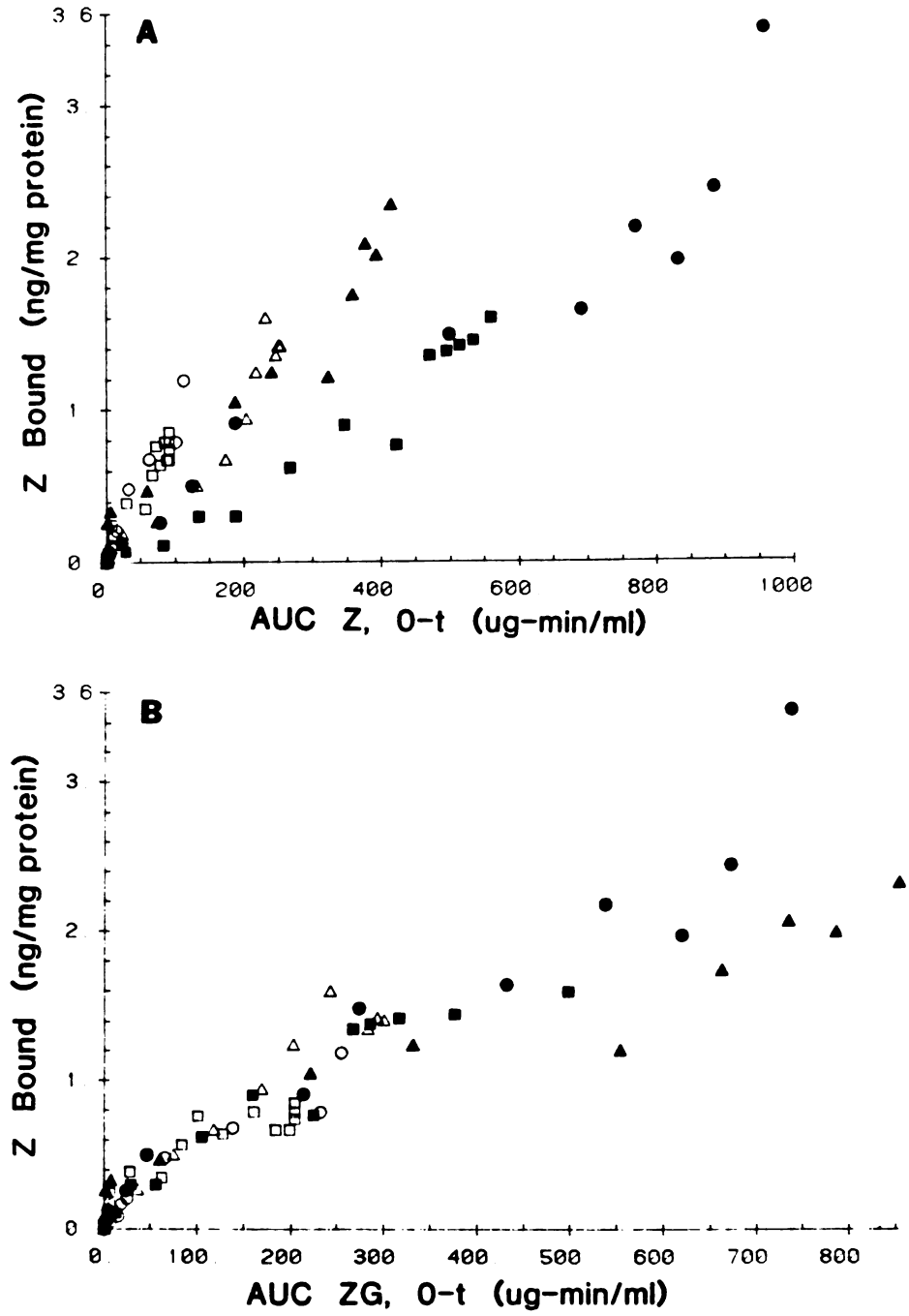


Figure VII-9. Combined data of zomepirac binding irreversibly to plasma proteins in human subjects after an oral dose of 100 mg zomepirac. Closed symbols are subjects given concurrent probenecid (500 mg b.i.d.); open symbols are control subjects. Each symbol shape represents an individual subject. Data are from initial dosing to the maximum binding at 8 to 12 hours after the dose. Figure A, slope = 0.00283, r = 0.900; B, slope = 0.00477, r = 0.934, n = 79.

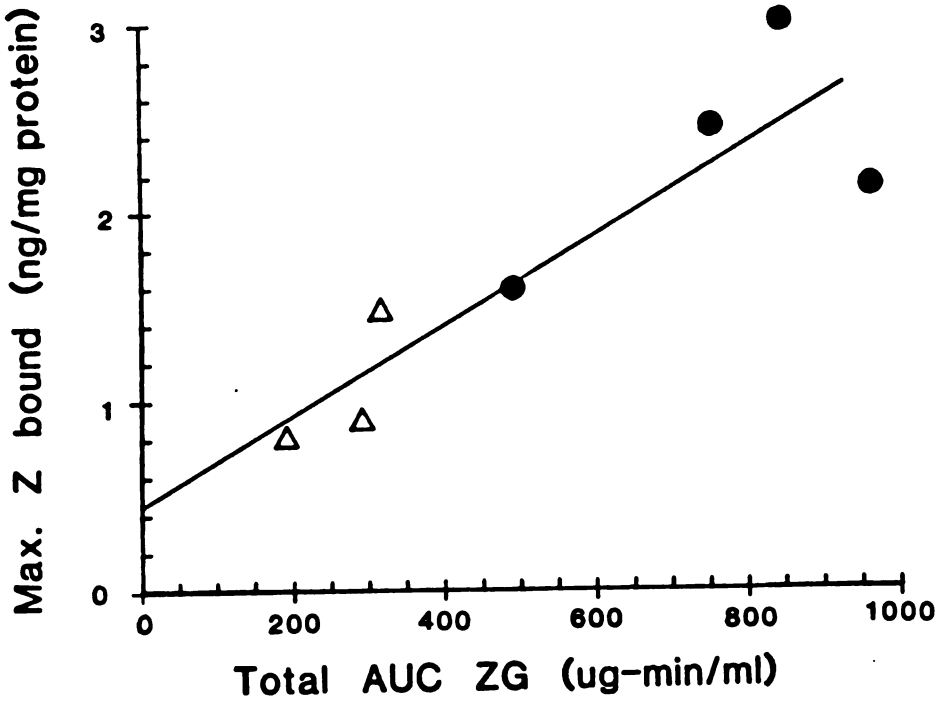


Figure VII-10. The maximum irreversible binding of zomepirac to plasma proteins after a 100 mg oral dose of zomepirac was given to control human subjects, △; and subjects given probenecid concurrently, ● (500 mg bid). Data are the average of the 3 highest values obtained for each subject.

D. DISCUSSION

The results document that irreversible binding of Z to albumin and plasma proteins occurs *in vitro* and *in vivo*. The binding is probably covalent, although the exact nature of the binding to albumin has not been delineated. This is the first report of irreversible binding of a drug to proteins *in vivo* through an acyl glucuronide metabolite. Although previous data have been published for bilirubin binding *in vivo* through its acyl glucuronide (10, 11), the elevated plasma concentrations of bilirubin glucuronide and subsequent binding of bilirubin only occur in human or animals with cholestasis due to liver disease or experimentally induced cholestasis (10, 11, 12). In contrast, ZG accumulates in plasma of healthy humans with concommitant binding of Z to plasma proteins.

The use of radiolabelled ZG or other acyl glucuronides provides a method to examine binding of the drug to proteins which obviates the need for complex assays of the ligand which is bound. However, as experienced here, radiochemical purity is often not satisfactory when only a small fraction of the radioactivity becomes incorporated irreversibly with the protein or macromolecule. The nature of the ligand which becomes bound can be questionable even if radiochemical purity is complete, since oxidation may occur *in vitro* for some compounds, especially when incubations are done with tissues containing enzymes (185). It was shown that Z was indeed bound by the ability to hydrolyze the Z-protein adduct and then identify the liberated Z by HPLC (Fig. VII-3). This procedure also allowed use of unlabelled ZG which was more pure than the ^{14}C-ZG isolated; in addition, a also larger quantity of unlabelled glucuronide was available. The ability to obtain

Z after hydrolysis of the Z-protein adduct, however, does not eliminate the possibility that glucuronic acid may be attached to the protein as well.

The *in vitro* studies of Z binding to albumin or plasma protein conclusively document that the conjugate, ZG, was necessary for the formation of the covalent adduct of Z and protein. The binding was time-dependent, eventually reaching a plateau after 3 to 6 hours of incubation with albumin or plasma protein (Fig. VII-4), even though the isomers of ZG which can also lead to binding (Table VII-1) were still present in the incubation medium. The decrease in Z-protein adduct after 6 hours of incubation (Table VII-1), the loss of the adduct observed after the maximum binding at pH 9 (Fig. VII-5), and preliminary stability studies of the Z-protein adduct at pH 7.4 and 10 showed that the adduct formed between Z and albumin was slightly labile under mild basic conditions. From the effect of pH on the rate of binding *in vitro*, it is apparent that the binding is also catalyzed by basic conditions (Fig. VII-5). The observed time-dependent binding can be rationalized as follows. Since β-1 ZG is more reactive than the isomeric conjugates, there is a rapid formation of Z-protein adduct at early times. Subsequently, when all ZG has either rearranged to form isomers or hydrolyzed, the rate of binding via the isomers is less and reaches a point when the rate of formation equals the rather slow rate of Z-protein adduct hydrolysis. Finally, after much of the conjugates have hydrolyzed, the rate of adduct cleavage is greater than its formation and the concentration of Z-protein adduct decreases.

The unexpected finding that the isomers of ZG also lead to binding of Z to albumin does not fit well with the hypothesis that only the more

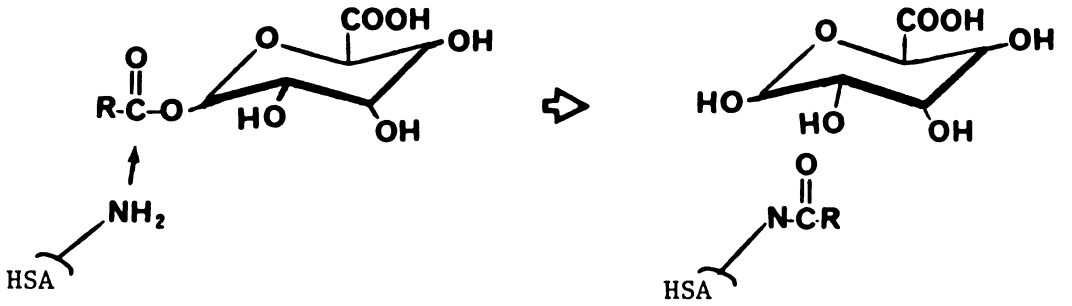
unstable and potentially more reactive, β-1 ZG, results in binding through an electrophilic mechanism as suggested by Stogniew and Fenselau (48) for clofibric acid glucuronide and by McDonagh *et al.* (11) for the binding of bilirubin to albumin through its acyl glucuronides. The isomeric conjugates of ZG would be expected to behave as simple esters and thus not to be very reactive. The isomeric conjugates of ZG are much more stable than ZG (28) (Chapter VI), but will rearrange by acyl migration faster than they hydrolyze. It is possible that the isomeric conjugates could rearrange to form β-1 ZG, or perhaps α-1 ZG (never identified), which might then react with albumin. The very labile isomeric conjugate of ZG, fraction 2 on HPLC (Chapter II), which was never identified, could also be suspected as a possible reactive intermediate. The HPLC data from the supernatant of the incubations of ZG with protein do not support the idea that a particular degradation product of ZG is reactive, as the profile of isomers in the supernatant was unchanged and fraction 2 was still present. Moreover, when the pure isomeric conjugates of ZG were incubated with albumin, and binding was assessed during the first 45 minutes when rearrangement from the 3- or 4- position to the 1-O-acyl position is unlikely, all of the isomers led to the formation of Z-protein adduct at similar rates (Fig. VII-6). Taken together, there is substantial evidence showing that the isomeric conjugates of ZG can lead to Z binding to albumin.

An alternative mechanism which accomodates the results of the *in vitro* binding experiments better than the above mentioned electrophilic mechanism, is shown in Figure VII-11. Formation of a Schiff's base (imine) between the free aldehyde of glucuronic acid and a lysine residue of albumin would require acyl migration prior to binding. The

pH dependence of the formation and the hydrolysis of the Z-protein adduct would agree with the formation of an imine linkage between Z and protein. There is a large amount of literature precedent supporting this alternative mechanism of binding by forming an imine. Glycosylation of hemoglobin forming an imine, which then undergoes an Amadori rearrangement (188, 189,) to form the more stable 1-amino-2-keto isomer, has been thoroughly studied (190, 191, 192). Similar reactions have occurred with other proteins and more recently the glycosylation of albumin has been documented (193, 194). In normal, nondiabetic humans 3% of the hemoglobin (191) and 10% of the albumin (193, 193) is glycosylated. The rate of glycosylation of albumin is estimated to be nine times greater than that of hemoglobin (913). Similar binding might be expected to occur with glucuronic acid when the 1-position of the uronic acid is free such that anomerization to the open chain form can occur. There is also carbohydrate literature which reports that glucuronic acid and its salts react more rapidly than glucuose with amines, sometimes followed by instantaneous Amadori rearrangement (195, 196, 197, 198). Besides the requirement that the 1 position of glucuronic acid be free for the formation of an imine, the formation of the more stable Amadori product with retainment of the acyl group requires that the 2 position not be acylated. At present, the favored explanation for the binding of acyl glucuronides to albumin is through formation of an imine with possible Amadori rearrangement.

Based upon the results of our in vitro data and previous results documenting irreversible binding of bilirubin to proteins, it was expected that similar binding of Z would occur with albumin and other proteins in vivo, if plasma concentrations of ZG became appreciable. To

A) Nucleophilic attack at the carbonyl, with elimination of glucuronic acid.



B) Acyl migration from the 1 position of glucuronic acid, ring opening to yield the free aldehyde, then formation of a imine (Schiff's base) with a lysine residue of albumin (HSA). Amadori rearrangement may occur after formation of the imine.

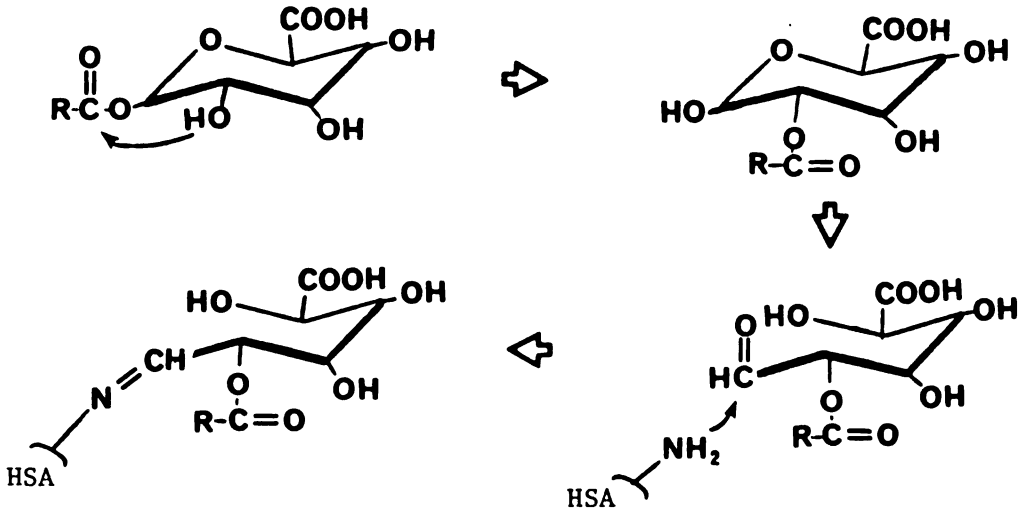


Figure VII-11. Two possible mechanisms for the irreversible binding of an acyl glucuronide to human serum albumin (HSA).

establish an correlation between binding of Z to plasma proteins and ZG, a measure of ZG exposure *in vivo* was needed. Since the Z-protein adduct is probably cleared at the rate of albumin turn over by the body, which is very slow relative to the elimination of Z and ZG, the adduct should accumulate once it is formed. Binding should be dependent upon the concentration of ZG present and the time of exposure, thus AUC was selected as the measure of exposure. AUC has been proposed as the most appropriate value to quantitate exposure to genotoxic chemicals (199). The converse relationship has also been proposed to estimate exposure to glucose (i.e. diabetic control) (191) or toxic substances (200) by determining the extent of irreversibly bound ligand found *in vivo*.

When binding of Z to plasma proteins in samples obtained from the human clinical study were examined, a linear relationship was observed between exposure to ZG as measured by AUC_{zg} and the extent of Z binding *in vivo* (Figs. VII-8 and VII-9). When probenecid was given concurrently it decreased the renal clearance of ZG, and thus increased exposure to ZG with a resultant increase in the formation of irreversibly bound Z (Figs. VII-9 and VII-10). Therefore, the extent of covalent binding observed was clearly dependent on both the concentration and residence time of the glucuronide in plasma. Without the previous *in vitro* data it would be difficult to delineate whether the binding of Z to plasma proteins *in vivo* was due to Z or ZG, as the plasma concentration profiles of the parent drug and metabolite are similar in humans (Chapter VI). This is reflected in the high correlations for both AUC_z and AUC_{zg} with Z binding to plasma proteins *in vivo* (Fig. VII-9).

The plasma concentration of irreversibly bound Z was maximum at 8-12 hours, then decreased slowly until it was on the average 65% of the

maximum at 24 or 48 hours after the dose. This slow disappearance of Z-protein adduct which was also seen during the incubations of ZG with albumin *in vitro* was due presumably to slow hydrolytic release of Z from the adduct. The irreversible binding of Z through its acyl glucuronide metabolite may act as a reservoir for Z in the body which is then very slowly eliminated, since albumin is normally catabolized at a much slower rate than the clearance of most drugs. Although covalently bound drug may represent only a small fraction of a single dose as presented here for Z, it is likely that upon repeated, multiple doses bound drug may accumulate and eventually represent a larger fraction of the drug in the body or in a particular organ. Furthermore, it might be expected that disease states or advanced age which compromise the excretion of ZG could lead to increased ZG levels and increased adduct formation, as was seen with bilirubin (12). Irreversible binding of bilirubin, however, does not occur in healthy individuals (12), because bilirubin conjugates do not normally accumulate, whereas Z binding reported here was significant in healthy young male volunteers. Whether this is unique for Z, because of the high levels of the acyl glucuronide, ZG, seen *in vivo*, is not yet known.

A more definitive correlation between formation of Z-protein adduct and exposure to ZG *in vivo* was achieved by the animal studies. Guinea pigs do not normally have much accumulation of ZG, however, levels of ZG could be manipulated by administration of the esterase inhibitor, PMSF. This animal model did not exhibit the strong correlation in plasma concentrations between ZG and Z which was observed in humans. Binding of Z to protein in the guinea pig was highly correlated with AUC_{zg} ($r=0.84$); while no relationship was seen with AUC_z ($r=0.14$) (Fig. VII-

7). The similar slope of Z binding vs. AUC_{zg} for the human data (Fig. VII-9, B) and the guinea pig data (Fig. VII-7, A) support the hypothesis that the binding of Z to plasma proteins in humans is due to the exposure to ZG, not Z.

Zomepirac (Zomax®) was withdrawn from the market because of a high incidence of immunologic reactions which included anaphylaxis (33, 34). As yet, no relationship can be drawn between the irreversible binding of Z to protein as found in these studies and the incidence of immunological reactions observed clinically for Z in man. However, it is generally accepted that immunogenic sensitizing ability and the ability to form adducts with proteins are correlated (13), although such a relationship is often difficult to prove in humans. The studies presented here show that Z becomes bound to albumin and plasma proteins. Because ZG is not extremely reactive, with the ability to distribute from the organ (presumably the liver) where it is synthesized into the blood, it is unlikely that its reactivity is limited to albumin. Thus, formation of adducts with other tissue proteins, leading to potential haptens, would also be expected in vivo. The ability of ZG to bind irreversibly with many tissues would also make it difficult to identify the specific adduct which may be responsible for the observed toxicity (201).

These studies were initiated because of the similarities between ZG and bilirubin acyl glucuronides and the previous observation that bilirubin binds irreversibly to albumin through its acyl glucuronide. This phenomenon is probably a general one which occurs with other acyl glucuronides of drugs or endogenous compounds. Conjugation of acidic compounds with glucuronic acid is a major route of metabolism for many xenobiotics and represents the primary route of elimination for NSAIDs.

As a class of drugs, the NSAIDs do require concern for serious toxicity. A recent review of drugs withdrawn from the market place because of toxicity included, zomepirac, benoxaprofen, ibufenac, alclofenac and indoprofen among the 25 drugs listed (181). All are NSAID's of the aryl-alkyl acid class which are eliminated as acyl glucuronides. The studies presented here document what is likely to be a general phenomenon for a common metabolic pathway of xenobiotics in humans, and raises the question of whether such binding may be related to potential toxicity.

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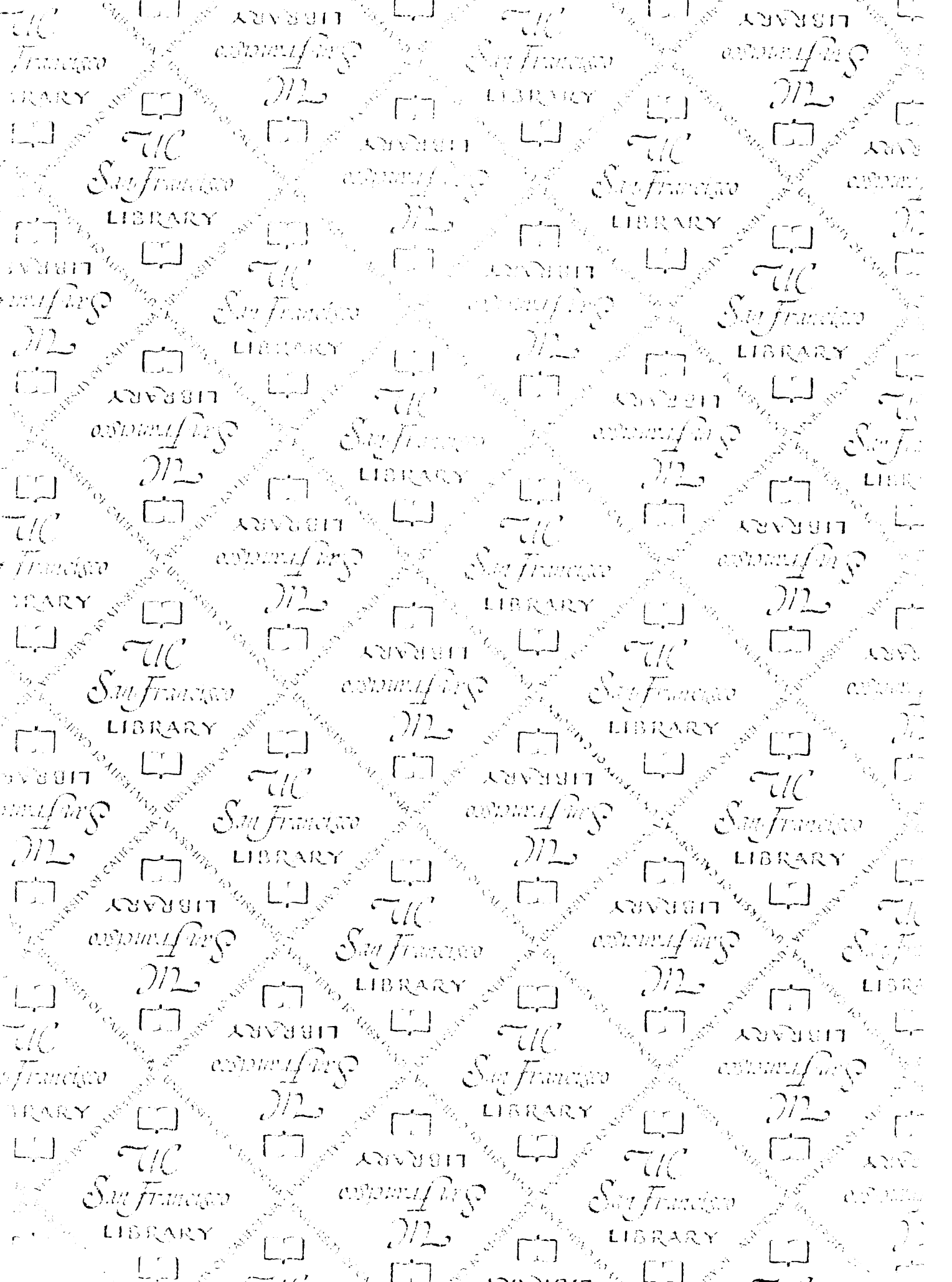
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