Title
Hypersensitivity to non-steroidal anti-inflammatory drugs

Author
Zia-Amirhosseini, Parnian,

Publication Date
1994

Peer reviewed|Thesis/dissertation

HYPERSENSITIVITY TO NON-STEROIDAL ANTI-INFLAMMATORY DRUGS: EXPLORATION OF A THEORY

by

Parnian Zia-Amirhosseini

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

DEDICATION

To my parents: Iraj Zia-Amirhosseini and Paridokht Azadnejadi,
and in loving memory of my youngest brother Mamali

ACKNOWLEDGMENTS

I am grateful to my research advisor, Dr. Leslie Z. Benet, for helping me realize my potential. I thank him for being a wonderfully energetic, encouraging, and supportive mentor. This thesis would not have materialized without his friendship; his scientific, moral, and financial support.

I thank my parents for their courage to send away their young firstborn teenage daughter to experience an unknown world, and for their devotion toward ensuring my financial and emotional well-being throughout the years that I have lived away from home. Their efforts have not been in vain.

I thank my brother and sister, Alireza and Gisoo for their undying love, affection, and support throughout the long years of separation. I extend a special thanks to Gisoo for frequent letters and words of wisdom.

I acknowledge my late brother, Mohammad, for his love, his wonderful sense of humor, and his zest for life. My fond memories of him will live with me forever.

I thank my cousins, Firouzeh, Hadi, and Reza Nourkhalaj; my aunt Parvin Ghaemmaghami; and my friend Marta Munoz for giving me much needed love and support during my one year break from academia. They have been my family away from home. I extend a special thanks to Firouzeh for always having been there for me.

Many thanks to the following people for their friendship and support throughout the years: Roya Zommorodian, Farzaneh Turk, Roanna Ueda, Masoud Sadeh, Aria and AzitaTavana Roudsari.

I am grateful to my friend and colleague, Dr. Andreas Ding, for his collaborative efforts and his patience in teaching me about peptide mass spectrometry. I thank Dr. Hildegard Spahn-Langguth and Dr. Joseph Ojingwa for their friendship and for initiating me into the Benet laboratory.

I thank Dr. Frances M. Brodsky for scientific advice and encouragement. I appreciate Dr. Betty-Ann Hoener's and Dr. Anthony De Franco's efforts in serving on my thesis committee.

I thank the following members of the Benet laboratory: Harrison Wong for his friendship and excellent technical assistance; Dr. Claudia d'Uscio and Dr. Alfred Bischer for their friendship and good natured presence in the lab; Dr. Victoria Hale, Dr. David Lau, Beatrice Perotti, Saraswate Kenkare, and Graham Jang for their friendships.

Finally, I am grateful to my husband, Dr. Robert Z. Harris, for scientific discussions, proof reading this thesis, and wonderful moral support. His love and devotion made the last two years of my graduate work the most pleasant of my entire life.

TABLE OF CONTENT

		Page
Title Page		i
Dedication		ii
Acknowledgments		iii
Table of Contents		v
List of Tables		viii
List of Figures		ix
Glossary		xii
Abstract		xiii
1.0	**Introduction**	**1**
1.1	Background	1
	1.1.1 Non-steroidal anti-inflammatory drugs (NSAIDs)	1
	1.1.2 Toxicities of non-steroidal anti-inflammatory drugs	1
1.2	Reactivity of Acyl Glucuronides	3
	1.2.1 Acyl migration and hydrolysis	4
	1.2.2 Acylation of small nucleophiles by acyl glucuronides	5
	1.2.3 Covalent binding of acyl glucuronides to proteins	5
1.3	Mechanisms of Binding of Acyl Glucuronides to Proteins	9
1.4	Proposed Mechanisms for the Toxicities of Certain Carboxylic Acid Containing NSAIDs	9
1.5	Objectives and Overview of Thesis	13
2.0	**Formation and Stability of Plasma Protein Adducts of Tolmetin in Humans**	**15**
2.1	Introduction	15
2.2	Experimental Section	18
	2.2.1 Study design	18
	2.2.2 Sample handling	19
	2.2.3 Quantitation of covalently bound tolmetin	19
2.3	Results	20
2.4	Discussion	24
3.0	**Mechanism of Covalent Binding of Tolmetin Glucuronide to Human Serum Albumin**	**28**
3.1	Introduction	28
3.2	Experimental Section	31
	3.2.1 Materials	31

3.2.2 Isolation of tolmetin glucuronide from urine 31
3.2.3 Preparation of HSA adducts of tolmetin glucuronide 32
3.2.4 Quantitation of bound tolmetin by alkaline hydrolysis of the modified protein 33
3.2.5 Tryptic digestion of the protein 33
3.2.6 Isolation of the modified peptides by HPLC 34
3.2.7 Mass spectrometric analysis of the modified peptides 34
3.2.8 Edman sequencing 36
3.3 Results 36
3.4 Discussion 53

4.0 Synthesis and Characterization of Human Serum Albumins Modified by NSAIDs via the Nucleophilic Displacement Mechanism 58

4.1 Introduction 58
4.2 Experimental Section 59
4.2.1 Materials 59
4.2.2 Synthesis of modified HSA 59
4.2.3 Tryptic digestion of the protein 61
4.2.4 Isolation of the modified peptides by HPLC 61
4.2.5 Mass spectrometric analysis of the modified peptides 61
4.3 Results 62
4.4 Discussion 71

5.0 Production of Antibodies Against Protein Conjugates of Tolmetin Glucuronide and Reactivity of These Antibodies Against Several Drugs and Their Glucuronides 73

5.1 Introduction 73
5.2 Experimental Section 77
5.2.1 Materials 77
5.2.2 Isolation of glucuronides from urine 78
5.2.3 Synthesis of glucuronides 79
5.2.4 Preparation of antigens (protein conjugates) 79
5.2.5 Immunizations 80
5.2.6 Enzyme linked immunosorbent assay (ELISA) 80
5.2.7 Inhibition studies 81
5.3 Results 82
5.4 Discussion 87

6.0 Summary of Results and Future Directions **93**

6.1 Formation and Stability of Plasma Protein Adducts of Tolmetin in Humans 93
6.2 Mechanisms of Covalent Binding of Tolmetin Glucuronide to Human Serum Albumin 93
6.3 Human Serum Albumins Modified by NSAIDs via the Nucleophilic Displacement Mechanism 94
6.4 Immunogenicity of Protein Conjugates of Tolmetin Glucuronide and Reactivity of the Generated Antibodies Against Several Drugs and Their Glucuronides 95
6.5 Future Directions 95
6.5.1 Investigating the mechanism of covalent binding of tolmetin glucuronide to HSA *in vivo* 95
6.5.2 Determining the immunogenicity of a series of drug glucuronide-protein conjugates 96
6.5.3 Finding a measure of immunologic reactivity of certain NSAIDs in humans 96
6.5.4 Exploring the possible role of Human Leukocyte Antigen in causing the immunologic toxicity 97

7.0 References **98**

LIST OF TABLES

Table		Page
1.1	*In vivo* covalently bound drug, area under the plasma drug glucuronide concentration time curve and *in vitro* acyl glucuronide degradation rates.	8
2.1	Maximum observed concentrations of covalently bound tolmetin after single, and multiple dosing of Tolectin® and half-lives of the protein adduct following multiple dosing.	22
3.1	The extents of covalent binding (binding yield) for the five different incubations.	37
3.2	Summary of binding studies for incubations 1-3.	40
3.3	Summary of results of the binding study for incubation 4.	41
4.1	Drug containing fractions (monitored at 313 nm) with assigned binding sites.	67
5.1	Immunopathological classification of hypersensitivity reactions.	74

LIST OF FIGURES

Figures		Page
1.1	Pathways of arachidonic acid metabolism and eicosanoid generation.	2
1.2	Migration of the acyl group of the β-1-O-acyl glucuronide from C_1 to C_2.	4
1.3	Proposed mechanisms for covalent binding of acyl glucuronides to proteins.	10
2.1	Structures of tolmetin, zomepirac, and their acyl glucuronides.	16
2.2	Concentration time profile of covalently bound tolmetin after a 400 mg dose of Tolectin®.	21
2.3	Concentration time profile of covalently bound tolmetin after a ten day multiple dosing regimen of Tolectin®.	23
2.4	Concentration time profiles of tolmetin, 1-β-tolmetin glucuronide, and covalently bound tolmetin after a ten day multiple dosing regimen of Tolectin® in one volunteer.	25
3.1	Proposed mechanisms for covalent binding of acyl glucuronides in the absence and presence of sodium cyanoborohydride.	29
3.2	The nomenclature for fragmentation of peptides.	36
3.3	The HPLC chromatograms (λ=313) for incubations 1-3 and 5 (the control).	39
3.4	The HPLC chromatograms from incubation 3.	42
3.5	The CID mass spectrum for the molecular ion MH^+ 1363.6 corresponding to HPLC peak 3.	44
3.6	Characteristic fragment ions of tolmetin.	45
3.7	The MALDI-TOF spectrum of HPLC fraction 2.	47

Figures		Page
3.8	The CID mass spectrum for molecular ion MH^+ 1187.6 corresponding to HPLC peak 6.	49
3.9	The CID mass spectrum for molecular ion MH^+ 1119.4 corresponding to HPLC peak 9.	50
3.10	The CID mass spectrum for molecular ion MH^+ 1114.4 corresponding to HPLC peak 7.	52
4.1	The scheme for the synthesis of a drug-protein conjugate via the nucleophilic displacement mechanism.	60
4.2	HPLC chromatograms of the digested T-HSA adduct.	63
4.3	The CID mass spectrum of the ^{12}C isotope of molecular ion 986.4 corresponding to HPLC peak T-1.	64
4.4	Fragment ions consistent with tolmetin bound to lysine.	66
4.5	HPLC chromatograms of the digested Z-HSA adduct.	68
4.6	The CID mass spectrum of the $^{12}C^{35}Cl$ isotope (m/z 1221.6) of the molecular ion stable isotope cluster from HPLC fraction Z-2.	69
4.7	Fragment ions consistent with zomepirac bound to lysine.	70
5.1	Events leading to an immediate hypersensitivity (allergic) reaction.	76
5.2	Reactivity of serum obtained from a mouse that was immunized four times with TG-MSA conjugate.	83
5.3	Reactivity of antisera against TG-HSA and T+HSA.	84
5.4	Inhibition of antibody-antigen interaction by TG-MSA conjugate.	85
5.5	Inhibition of antibody-antigen interaction by tolmetin and tolmetin glucuronide.	86
5.6	Inhibition of antibody-antigen interaction by zomepirac and zomepirac glucuronide.	88

Figures **Page**

5.7 Inhibition of antibody-antigen interaction by fenoprofen, fenoprofen glucuronide, naproxen, and naproxen glucuronide. 89

5.8 Some of the inhibitors that were used in the inhibition experiments. 91

GLOSSARY

Absorb.	Absorbance
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CHDM	Comprehensive Hospital Drug Monitoring
CIDMS	Collision Induced Dissociation Mass Spectrometry
CFA	Complete Freund's Adjuvant
ELISA	Enzyme Linked Immunosorbent Assay
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin
IFA	Incomplete Freund's Adjuvant
LT	Leukotriene
LSIMS	Liquid Secondary Ion Mass Spectrometry
MS	Mass Spectrometry
MSA	Mouse Serum Albumin
MALDI-MS	Matrix Assisted Laser Desorption Mass Spectrometry
MALDI-TOF-MS	Matrix Assisted Laser Desorption Time of Flight Mass Spectrometry
SG	Suprofen Glucuronide
T	Tolmetin
TG	Tolmetin Glucuronide
TX	Thromboxane
Z	Zomepirac
ZG	Zomepirac Glucuronide

ABSTRACT

HYPERSENSITIVITY TO NON-STEROIDAL ANTI-INFLAMMATORY DRUGS: EXPLORATION OF A THEORY

Parnian Zia-Amirhosseini

We believe that the allergic like reactions to certain carboxylic acid containing drugs have an immune based mechanism. Thus we have theorized that a drug glucuronide-protein conjugate forms *in vivo* and this protein conjugate stimulates an immune response (i.e. antibody secretion). This theory makes at least three predictions: 1) a drug glucuronide is capable of chemically modifying a protein, 2) the drug glucuronide-protein conjugate is formed *in vivo* and persists in the body long enough to exert a toxic effect, 3) the drug glucuronide-protein conjugate is immunogenic (can trigger an antibody response). These predictions have been tested in this thesis:

1) Tolmetin glucuronide was reacted with human serum albumin (HSA) *in vitro*. The peptide from tryptic digestion of the reaction product revealed that HSA had been modified at several amino acids by the addition of either a tolmetin unit or a tolmetin glucuronide unit. These two modifications occur via a nucleophilic displacement or a Schiff base formation mechanism respectively. The formation of antibodies recognizing in part a common chemical moiety (i.e. glucuronic acid) on the tolmetin glucuronide modified sites of the protein could contribute to the observed cross-reactions among carboxylic acid containing non-steroidal anti-inflammatory drugs. HSA was also reacted with activated esters of both tolmetin and zomepirac to give protein that was modified by addition of a tolmetin or zomepirac group. The most reactive site on albumin for all the

above reactions, as measured by the highest percent of total binding, was lysine 199.

2) Tolmetin glucuronide binds to plasma proteins in humans. Covalently bound tolmetin was detected after a single dose of drug and was found to accumulate in the body upon multiple dosing. The plasma protein conjugates are long lived in humans.

3) The mouse serum albumin conjugate of tolmetin glucuronide stimulated an antibody response in mice. Both tolmetin and tolmetin glucuronide specific antibodies were generated in the responder mice. These antibodies cross-reacted with other drugs and their glucuronides to varying degrees.

1.0 INTRODUCTION

1.1 BACKGROUND

1.1.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs are a widely used class of compounds that are prescribed as analgesic, antipyretic, and anti-inflammatory agents. They exert their anti-inflammatory action by inhibiting the cyclooxygenase activity of prostaglandin synthase, thus decreasing the biosynthesis of prostaglandins and related eicosanoids (Fig. 1.1). NSAIDs constitute a chemically heterogeneous group of compounds. There are at least nine different subclasses of NSAIDs: 1) salicylates (e.g. aspirin, methyl salicylate, diflunisal, and salsalate), 2) pyrazolone derivatives (e.g. phenylbutazone, oxyphenbutazone, antipyrine, and aminopyrine), 3) para-aminophenol derivatives (e.g. acetaminophen and phenacetin), 4) indole acetic acid derivatives (e.g. indomethacin and etodolac), 5) fenamates (e.g. mefenamic, meclofenamic, flufenamic, tolfenamic, and etofenamic acids), 6) pyrrole acetic acid derivatives (e.g. tolmetin), 7) phenylacetic acid derivatives (e.g. diclofenac), 8) aryl propionic acid derivatives (e.g. ibuprofen, naproxen, flurbiprofen, fenoprofen, ketoprofen), and 9) oxicams (e.g. piroxicam) (Insel, 1990). The drugs listed as examples are all presently in use in the United States or Europe.

1.1.2 Toxicities of Non-Steroidal Anti-Inflammatory Drugs

NSAIDs are generally well tolerated. One of the more common toxicities of these drugs is gastric irritation. Less common, but potentially

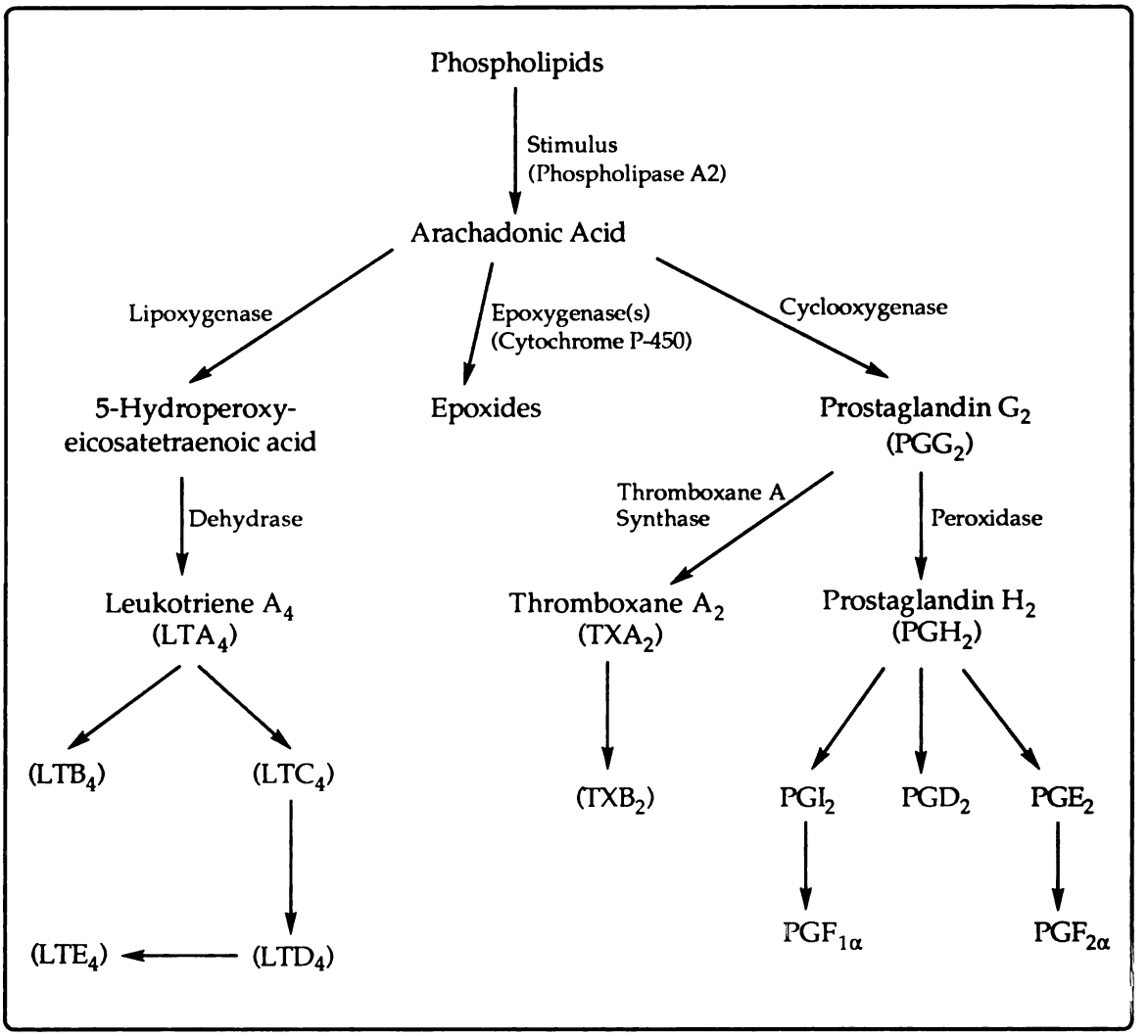

Fig. 1.1. Pathways of arachidonic acid metabolism and eicosanoid generation.

fatal, adverse effects caused by certain NSAIDs are hypersensitivity reactions. These include allergic reactions and organ toxicities.

Interestingly, six of at least 26 drugs which have been withdrawn from the British and U.S. markets during the past thirty years were NSAIDs (Bakke *et al.*, 1984; Hart *et al.*, 1987; Strom *et al.*, 1989). These are alclofenac, benoxaprofen, ibufenac, indoprofen, zomepirac, and suprofen; all of which are carboxylic acids. The drugs were withdrawn due to their causing either

severe organ toxicity (i.e., liver or kidney) or severe anaphylactic (allergic) reactions. Some other drugs, mostly acidic NSAIDs, which are still on the market, but for which hypersensitivity reactions have been observed, are diflunisal (Cook *et al.*, 1988), probenecid (Kickler *et al.*, 1986), aspirin (Amos *et al.*, 1971), and tolmetin (U.S. Congress *et al.*, 1982; Bretza, 1985). Patients who are allergic to one of these agent are not allowed to take any other NSAIDs because it is believed that there is some degree of cross-reactivity between these agents. Thus, if a patient allergic to aspirin takes a dose of ibuprofen, she may have an adverse reaction.

1.2 REACTIVITY OF ACYL GLUCURONIDES

Many NSAIDs, including all of those removed from the market, possess carboxylic acid groups. *In vivo*, the carboxyl group of these compounds is conjugated to glucuronic acid to form an ester (acyl) glucuronide. Acyl glucuronides of many drugs have been shown to be reactive compounds. The ester bond undergoes chemical hydrolysis, and enzymatic hydrolysis by β-glucuronidase and nonspecific esterases. Acyl glucuronides also undergo an isomerization reaction whereby the drug migrates from position 1 on the glucuronic acid ring to positions 2, 3, and 4 (Fig. 1.2) (Spahn-Langguth and Benet, 1992). Acyl glucuronides of a number of drugs have also been shown to react covalently with nucleophilic centers of small molecules (Stogniew and Fenselau, 1982) and proteins *in vitro* and *in vivo* (Spahn-Langguth and Benet, 1992).

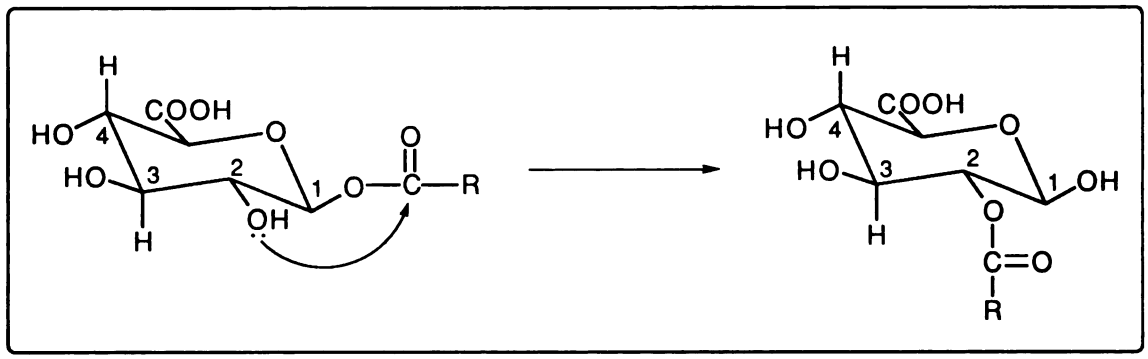

Fig. 1.2. Migration of the acyl group of the β-1-O-acyl glucuronide from C1 to C2. The acyl group can then migrate to C3 and subsequently to C4.

1.2.1 Acyl Migration and Hydrolysis

Carbohydrate chemists have thoroughly described acyl migration on sugars (Haines, 1976). The stability of acyl glucuronides of different drug molecules is quite variable and influenced by many factors. As studied in detail for zomepirac glucuronide (Hasegawa *et al.*, 1982; Smith *et al.*, 1985) and tolmetin glucuronide (Munafo *et al.*, 1990), the rates of acyl migration and hydrolysis depend upon the pH, temperature, and composition of the medium. Acyl glucuronides are most stable at pH 2-4, low temperatures, and in presence of esterase inhibitors.

The stability of acyl glucuronides is also affected by the presence of protein. The hydrolysis rate of oxaprozin glucuronide was accelerated in the presence of plasma proteins and albumin (Ruelius *et al.*, 1986). However, human serum albumin (HSA) stabilized tolmetin glucuronide, while bovine serum albumin (BSA) caused an increase in the rate of hydrolysis (Munafo *et al.*, 1990). The degradation rate of S-carprofen glucuronide in the presence of fraction-V and fatty-acid-free HSA at 37°C and pH 7.4 was higher than in the absence of these proteins. Presence of fatty-acid-free HSA decreased the degradation rate of R-carprofen glucuronide, while fraction-V HSA had no

effect on the degradation rate (Iwakawa *et al.*, 1988). Thus, the effect of protein on glucuronide stability is extremely variable. Degradation half-lives for the β-1-O-acyl glucuronides of various compounds in the physiologic pH range have been summarized by Spahn-Langguth and Benet (1992).

1.2.2 Acylation of Small Nucleophiles by Acyl Glucuronides

Clofibrate glucuronide was reported to react with the nucleophile ethanethiol to form the clofibrate thioester, whereas no thioesters were formed in an incubation containing clofibrate or clofibric acid with the nucleophile. Also, a mercapturic acid conjugate of clofibrate was isolated from human urine after administration of clofibrate. Based on the ethanethiol results and the fact that initial conjugation of xenobiotics with glutathione usually leads to their excretion as mercapturic acid (N-acetylcysteine) conjugates in human urine, the *in vivo* finding was interpreted as evidence for a glutathione conjugate precursor (Stogniew and Fenselau, 1982).

1.2.3 Covalent Binding of Acyl Glucuronides to Proteins

Covalent binding to proteins has been observed both *in vitro* and *in vivo* for acyl glucuronides of a large number of xenobiotics (Spahn-Langguth and Benet, 1992). For example, many non-steroidal anti-inflammatory drugs (benoxaprofen, indomethacin, flufenamic acid, oxaprozin, zomepirac, tolmetin, carprofen, fenoprofen, diflunisal, and suprofen), the uricosuric drug probenecid, the antihyperlipoproteinemic compounds clofibric acid (van Breemen and Fenselau, 1985) and beclobrate, the diuretic agent furosemide, and the anti-epileptic drug valproic acid form irreversible protein adducts.

The *in vitro* irreversible binding of acyl glucuronides was shown to be dependent on glucuronide concentration (Munafo *et al.*, 1990), pH (Smith *et*

al., 1986; Munafo *et al.*, 1990; Smith and Liu, 1993), and time (van Breemen and Fenselau, 1985; Wells *et al.*, 1987). For oxaprozin glucuronide, the highest yield of protein adduct was obtained after incubating the glucuronide and HSA at pH 7 for approximately one hour at 37°C. Similarly, for zomepirac glucuronide the highest yield of protein adduct was obtained after one hour of incubation at pH 9, although the level of protein adduct decreased very fast after this time due to its instability at this high pH. High concentrations of adduct were also observed after a 6 hour co-incubation of zomepirac glucuronide and HSA at pH 7 and 8 at 37°C (Smith *et al.*, 1986). Covalent binding of diflunisal glucuronide was greater with fatty acid-free human serum albumin than with human and rat plasma proteins (Watt and Dickinson, 1990). The covalent binding of suprofen glucuronide to HSA increased with increasing pH at 37°C and was time-dependent (Smith and Liu, 1993).

The isomeric conjugates of the β-1-O-acyl glucuronides also form protein adducts. Isomers of suprofen glucuronide exhibited time-dependent binding. This binding was 38% higher than that of the β-1-glucuronide (Smith and Liu, 1993). Covalent binding of valproic acid β-1-glucuronide to proteins was minor in buffered HSA solution and human plasma; however, approximately 7% of the isomers of valproic acid glucuronide formed covalent adducts with proteins (Williams *et al.*, 1992). Thus, certain isomeric conjugates seem to be more reactive toward protein than the β-1-glucuronide.

It may be possible to predict the extent of covalent binding of acyl glucuronides to proteins *in vitro* and *in vivo* by measuring their *in vitro* rate of degradation. Benet and coworkers (1993) have examined the degradation rates and covalent binding of acyl glucuronides of beclobrate, carprofen, fenoprofen, furosemide, tolmetin, and zomepirac with HSA *in vitro*. Three

of these drugs (beclobrate, carprofen, fenoprofen) have a chiral center, thus each produce a pair of enantiomeric glucuronides. The other three drugs do not possess any chiral carbons. Benet's group observed a linear correlation (r^2=0.995) between the rates of degradation of the 9 acyl glucuronides and the maximum extent of drug covalent binding (moles of drug irreversibly bound per mole of protein) to HSA *in vitro.*

The irreversible plasma protein binding for beclobrate, carprofen, fenoprofen, tolmetin, and zomepirac were also studied after oral administration of a single dose of each drug, at their usual therapeutic doses, to five different sets of healthy volunteers. A linear relationship was observed between the maximal amount of drug irreversibly bound and the extent of glucuronide present (measured as the area under the curve :AUC) for each drug. Furthermore, after normalizing the bound ratio (moles of drug per mole of protein) to the AUC of the corresponding glucuronide metabolite, a significant linear correlation (r^2 = 0.873) was obtained between the normalized *in vivo* bound amounts and the *in vitro* degradation rate constants of the glucuronides (Table 1.1) (Benet *et al.*, 1993). Thus, it may be possible to predict the *in vivo* covalent binding of acidic drugs to plasma proteins in man based on the degradation rate constant of the glucuronide metabolite after the amount of drug bound is normalized to the integrated levels of glucuronide present in plasma.

Moreover, Benet et al. (1993) observed a structure activity relationship for this group of drugs in relation to the reactivity of the drug acyl glucuronides and binding of the acyl glucuronides to proteins *in vitro* and *in vivo.* As the number of substituents on the carbon alpha to the carboxylic acid of the aglycone decreased, the degradation rate constant of its glucuronide (reactivity) and the number of moles bound per mole of HSA (protein

Table 1.1. *In vivo* irreversibly bound drug, area under the plasma drug glucuronide concentration time curve and in vitro acyl glucuronide degradation rates.* (from Benet *et al.*, 1993)

Parent Compound	Bound Drug (mole/mole protein) x 10^4	AUC Glucuronide (mole x hr/L) x 10^6	Bound/AUC 10^{-2}	k (hr^{-1})
Tolmetin	2.77 ± 1.54	3.72 ± 0.95	0.75	1.78
Zomepirac	2.33 ± 0.45	6.41 ± 2.14	0.36	1.54
R-Fenoprofen	1.02 ± 0.32	6.31 ± 5.65	0.16	0.71
S-Fenoprofen	3.23 ± 0.85	60.4 ± 24.7	0.054	0.36
Racemic Carprofen	1.92 ± 1.28	40.9 ± 7.3	0.047	0.32
(-) - Beclobric Acid	0.12 ± 0.03	8.16 ± 1.34	0.15	0.031
(+) - Beclobric Acid	0.20 ± 0.11	8.31 ± 1.63	0.24	0.027

*Measurement of drugs covalently bound to human serum albumin and area under the plasma concentration time curve for the glucuronide conjugates measured in five different groups of healthy volunteers following oral dosing of either 400 mg of tolmetin, 100 mg of zomepirac, 600 mg of racemic fenoprofen, 50 mg of racemic carprofen or 100 mg of racemic beclobric acid. When covalently bound drug is normalized to area under the curve for the respective glucuronide conjugates, an excellent correlation with the *in vitro* degradation rate constant (k) is obtained with an r^2 of 0.873.

binding) *in vitro* increased. The drugs exhibiting the greatest degree of binding, tolmetin and zomepirac, contain an unsubstituted α-carbon, whereas the compounds with an intermediate degree of binding, fenoprofen and carprofen, have a substituent on the α-carbon. The drugs with the least degree of binding, furosemide and beclobrate, are completely substituted at the α-carbon.

1.3 MECHANISMS OF BINDING OF ACYL GLUCURONIDES TO PROTEINS

Two mechanisms have been proposed to describe the irreversible binding of acyl glucuronides to proteins. The first is a nucleophilic displacement mechanism whereby a protein nucleophile (NH, O, S) attacks the carbonyl group of the aglycone (Fig. 1.3a)(Faed, 1984). In this mechanism, a bond (amide, ester, or thioester) is formed between protein and the drug, and glucuronic acid is liberated. The second mechanism involves the formation of a Schiff base (imine) intermediate (with an amino group of the protein) which can then undergo rearrangement to a more stable 1-amino-2-keto product (Fig. 1.3b). In this mechanism, the glucuronic acid moiety is retained as a component of the protein adduct (Smith *et al.*, 1990).

1.4 PROPOSED MECHANISMS FOR THE TOXICITIES OF CERTAIN CARBOXYLIC ACID CONTAINING NSAIDS

As mentioned above, a number of NSAIDs have been observed to cause hypersensitivity reactions in patients. Three hypotheses have been put forth regarding the mechanism of toxicity of certain NSAIDs.

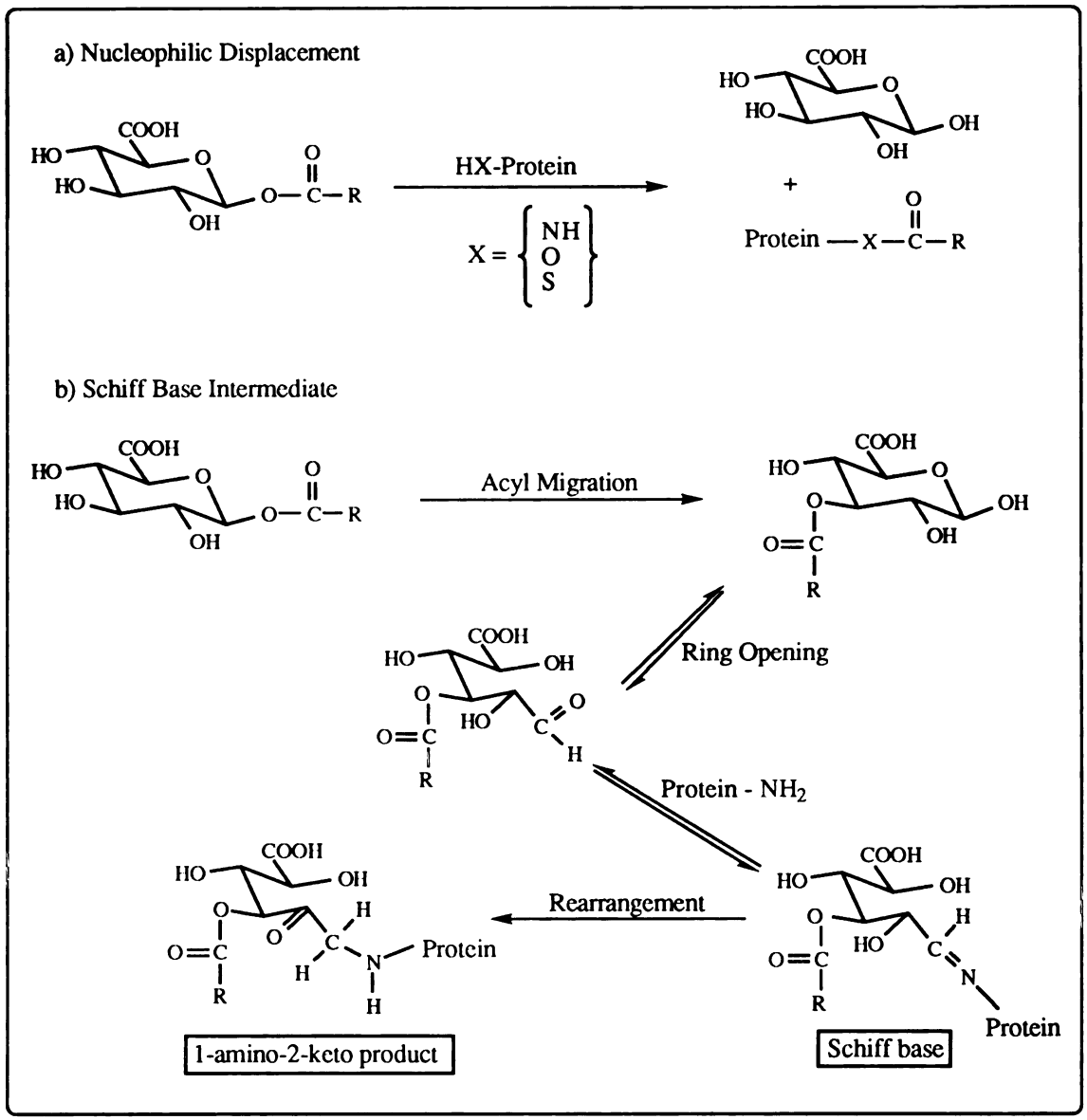

Fig. 1.3. Proposed mechanisms for covalent binding of acyl glucuronides to proteins: a) nucleophilic displacement mechanism, b) Schiff base intermediate mechanism.

One hypothesis states that the hypersensitivity reactions are caused by the pharmacologic action of the NSAIDs. The theory is that since the prostaglandin synthesis arm of arachidonic acid metabolism is inhibited by these drugs, the result is an increase in the production of leukotrienes (Fig. 1.1) (Rainsford, 1988). Leukotrienes (LT) were originally recognized as the slow-reacting substance(s) of anaphylaxis (SRS-A) in perfusates of lung after immunologic challenge. SRS-A is composed of the peptidoleukotrienes LTC_4, LTD_4, and LTE_4. These leukotrienes increase microvascular permeability, plasma exudation, and mucus secretion. They can cause cardiac depression by reducing myocardial contractility and coronary blood flow. They are also potent bronchoconstrictors (Hecker *et al.*, 1992). Thus it is reasonable to hypothesize that an increase in production of leukotrienes by NSAIDs can induce allergic reactions such as asthma and anaphylaxis. Supporting evidence that indicates exacerbation of asthma may occur via this mechanism exists in the literature (Szczeklik, 1986). However, there is no supporting evidence for the occurrence of anaphylactic reactions via this pathway.

In fact, when Szczeklik (1986) administered an oral challenge test with therapeutic doses of a number of NSAIDs (aspirin, indomethacin, fenoprofen, ibuprofen, mefenamic acid, paracetamol, benzydamine, phenylbuatzone, and sulfinpyrazone) to patients who had had an allergic reaction to dipyrone and/or aminopyrine, two NSAIDs, two to twelve months earlier, none of the patients had adverse reactions. However, the majority of them had a positive intradermal skin test to 0.01% aqueous solution of dipyrone. Seventy percent of them responded positively to 0.001% dipyrone, and 50% responded to 0.001% aminopyrine. The positive skin tests indicate the allergic status of the patients with respect to dipyrone

and aminopyrine. However, if their initial allergic response had been due to an over-production of leukotrienes, then oral administration of other NSAIDs should have caused an adverse reaction. Thus these studies suggest that a sensitization to the drugs had taken place in the allergic patients as opposed to an over-production of leukotrienes (Szczeklik, 1986).

The second hypothesis states that the reactivity of acyl glucuronides toward smaller nucleophiles such as glutathione may lead to the observed toxicity of some acidic drugs and may also enhance the toxicity of other xenobiotics by glutathione depletion (Stogniew and Fenselau, 1982; Faed, 1984).

The third hypothesis states that both the anaphylactic reactions and organ toxicities occur via an immune based mechanism. As described above, carboxylic acid containing NSAIDs are capable of forming protein adducts *in vivo*. The acyl glucuronide is hypothesized to act as a hapten which upon covalent binding to a protein becomes immunogenic and causes antibody production (Spahn-Langguth and Benet, 1992). When IgE antibodies are produced an anaphylactic reaction ensues. Furthermore, organ toxicity may result from covalent binding to tissue proteins and subsequent antigen-antibody complex formation (Abbas *et al.*, 1991). In support of this hypothesis, drug specific antibodies have been observed in aspirin hypersensitive patients (Amos *et al.*, 1971) and in patients receiving valproic acid therapy (Williams *et al.*, 1992). The low levels of valproic acid specific antibodies are consistent with the relatively low toxicity of this drug.

However, there have been no attempts to establish a model for systematically studying the mechanism of hypersensitivity reactions and the cross-reactivities caused by NSAIDs. My objective has been to study this

problem with an emphasis on the role of the protein conjugates of acyl glucuronides in causing the adverse reactions.

1.5 OBJECTIVES AND OVERVIEW OF THESIS

The overall aim of this thesis is to investigate the mechanism of hypersensitivity and cross-reactivity of certain non-steroidal anti-inflammatory drugs. The working hypothesis of this thesis is that the acyl glucuronide of these drugs covalently modifies a protein *in vivo*. The modified protein is then recognized as non-self by the immune system, which starts a cascade of events that leads to sensitization and antibody secretion. Thus the drug-protein conjugate generated via the reaction of drug acyl glucuronide with the protein may cause the observed toxicities. The studies presented in this thesis have been done primarily with tolmetin, a NSAID that has been reported to cause hypersensitivity reactions and is a close chemical congener of zomepirac.

Three different components of this hypothesis have been examined:

Firstly, for the hypothesis to be valid, it must be shown that the drug-protein conjugate is formed *in vivo* and is long lived. The formation and *in vivo* stability of the protein conjugates of tolmetin after a single dose and multiple doses of the drug are discussed in Chapter 2.

Secondly, to address the issue of cross-reactivity more systematically, it is necessary to establish the mechanism of binding of tolmetin glucuronide to protein. The cross-reactivities of various NSAIDs occur due to similarities in chemical structures of the compounds (i.e. tolmetin and zomepirac) or due to the presence of a common chemical moiety, namely the glucuronic acid, within the protein adduct. Thus it is important to determine if the product of

the covalent binding reaction of tolmetin glucuronide to protein contains tolmetin bound to protein (nucleophilic mechanism) or tolmetin glucuronide bound to protein (Schiff base mechanism). To this end, tryptic digests of the modified protein were analyzed by HPLC and mass spectrometry to identify binding sites on the protein and the type of modification. For these studies, human serum albumin was chosen as a model protein. Tryptic digests of modified HSA after its reaction with tolmetin glucuronide were examined. This work is presented in Chapter 3. Moreover, chemically synthesized HSA conjugates of tolmetin and its structural congener, zomepirac, were studied. This work is presented in Chapter 4.

Thirdly, for the hypothesis to be valid, the modified protein (the protein conjugate) must be recognized as non-self and cause an antibody response (i.e. be immunogenic). To address this issue and the problem of cross-reactivity of the potentially generated antibodies with other NSAIDs, immunogenicity of mouse serum albumin modified by its reaction with tolmetin glucuronide was investigated in mice. This work is presented in Chapter 5.

2.0 FORMATION AND STABILITY OF PLASMA PROTEIN ADDUCTS OF TOLMETIN IN HUMANS

2.1 INTRODUCTION

Tolmetin [1-methyl-5-(4-methylbenzoyl)-1H-pyrrole-2-acetic acid] (Fig. 2.1) is an anti-inflammatory drug used in the treatment of rheumatoid arthritis. The structurally related NSAID zomepirac, [5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid] (Fig. 2.1), was withdrawn from the market due to its causing hypersensitivity reactions, mainly of the anaphylactoid type (U.S. Congress *et al.*, 1982). Similar reactions have been observed with tolmetin (U.S. Congress *et al.*, 1982; Bretza, 1985), and the current package insert indicates that such anaphylactoid reactions occur in less than 1% of patients.

As described in Chapter 1, tolmetin, zomepirac, and other carboxylated NSAIDs are metabolized to acyl glucuronides (Fig. 2.1), which undergo both acyl migration and covalent binding to proteins. Glucuronide conjugates of tolmetin and zomepirac are necessary intermediates for the formation of covalent adducts with albumin (Smith *et al.*, 1986; Munafo *et al.*, 1990), since tolmetin and zomepirac themselves do not react directly.

A number of studies have examined the stability of NSAID adducts formed with plasma proteins in humans after administration of a single drug dose. Pharmacokinetic evaluation of these data indicate that the protein adducts exhibit significantly longer half-lives than the parent drugs and their primary metabolites. After a single oral dose of racemic fenoprofen,the concentration of plasma protein adducts for both R- and S-fenoprofen reached

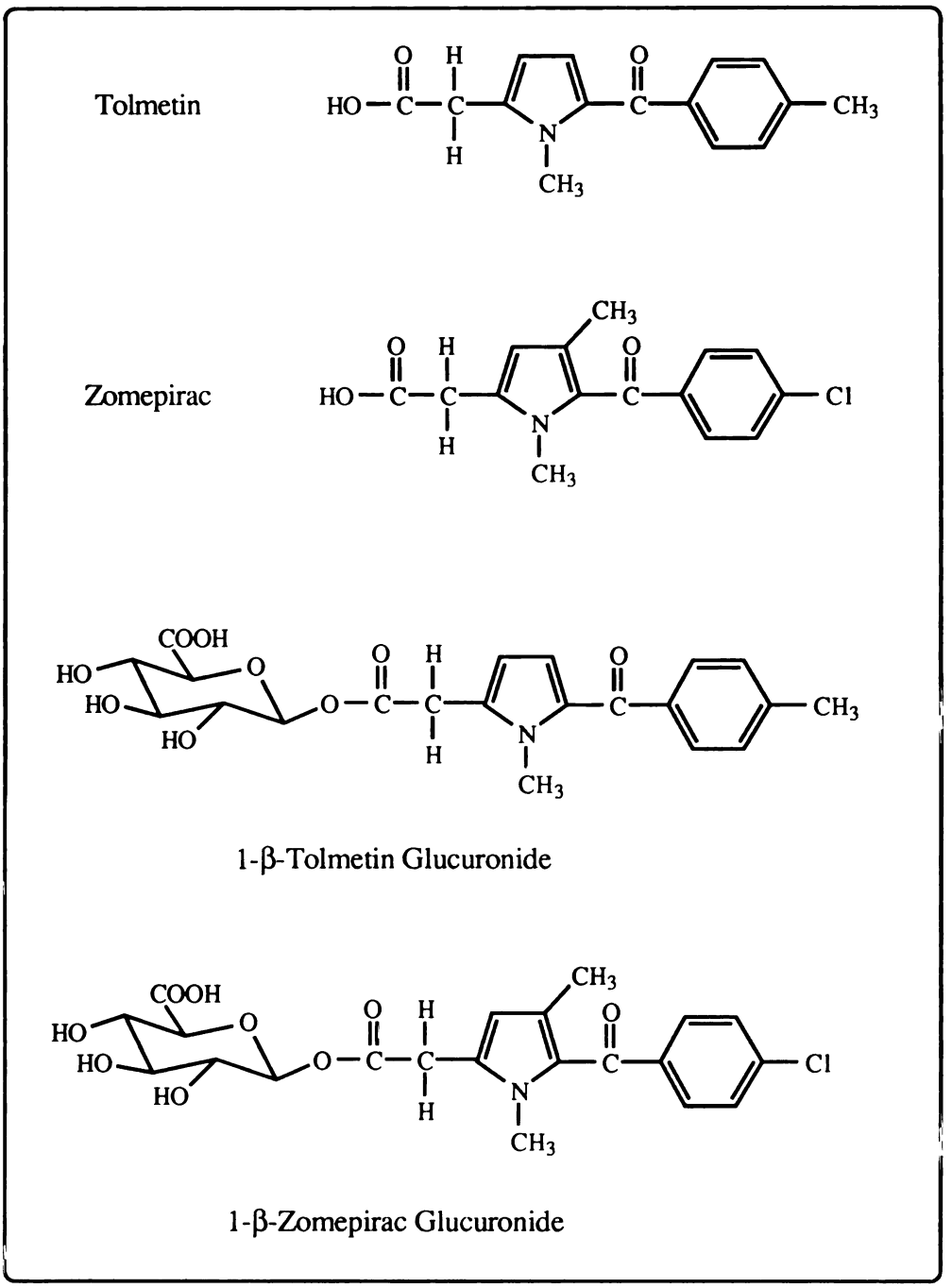

Figure 2.1. Structures of tolmetin, zomepirac, and their acyl glucuronides.

maximum values approximately two hours after the dose and remained constant for up to 14 hours after the dose (the last time point studied) (Volland *et al.*, 1991). Thus, the plasma protein adduct of fenoprofen is quite stable *in vivo*. Similarly after a single oral dose of tolmetin, the concentration of covalently bound tolmetin remained constant for 22 hours (the last time point examined) (Hyneck *et al.*, 1988).

Two studies have examined the *in vivo* stability of a protein adduct of an NSAID, diflunisal, after multiple dosing of the drug. Covalent binding of diflunisal to proteins has been studied in rats after multiple intravenous. doses of the drug. Covalent adducts of the drug were not only detected in plasma, but also in liver, kidney, small intestine, large intestine, and bladder (Dickinson and King, 1993; King and Dickinson, 1993). The half-lives of the protein adducts (range of 18-67 hours) in plasma and in the different tissues examined were several folds greater than the half-lives of diflunisal and diflunisal glucuronide. Also after multiple doses of diflunisal in humans, the average half-life for the plasma protein adduct of diflunisal was found to be 10 days (McKinnon and Dickinson, 1989).

As discussed in Chapter 1, small molecular entities (i.e., drugs or their metabolites) may become immunogenic upon covalent binding to a protein and cause direct immunologic reactions or immune-based organ toxicity (Park *et al.*, 1987). Therefore, it is toxicologically important to obtain detailed quantitative data on the *in vivo* formation and stability of covalent adducts of drugs in common clinical use. Here, the formation and stability of plasma protein adducts of tolmetin have been examined after oral administration of a single dose and after a multiple dosing regimen to six healthy human volunteers. This work is only the second study that has investigated the *in*

vivo stability of protein adducts of a NSAID after multiple doses of the drug in humans.

2.2 EXPERIMENTAL SECTION

2.2.1 Study Design

The study was approved by the Committee on Human Research at the University of California, San Francisco. Volunteers (3 females, 3 males) were between the ages of 24 and 40. A complete medical history was obtained and a physical examination was done on each participant before and after the study. Routine blood and urine chemistry values for all volunteers were within the normal range. Individuals with evidence of physical or laboratory abnormalities, history of cardiac, gastrointestinal, renal, pulmonary, or hematologic disorders, chemical dependency, or those who had ingested a medication (including over-the-counter drugs but excepting oral contraceptives) during the week prior to the study date were excluded. Subjects allergic to tolmetin or any other NSAID were also excluded. All volunteers gave their witnessed, informed, written consent. No adverse reactions were observed during or upon completion of the study. The laboratory test results, including total plasma protein and albumin concentrations, of the one subject who was on oral contraceptives were within the average range.

On the first day of the study, immediately after time zero sampling, each subject took a 400 mg capsule of tolmetin (Tolectin®, McNeil Pharmaceutical, Spring House, PA) with 200 ml of water. Blood samples (12 ml) were drawn 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, and 14 hours after the dose, and in the morning and evening of days two and three of the study. On the

morning of the fourth day, a ten-day multiple-dosing regimen of one 400 mg capsule every 12 hours was started. The last dose was administered on the morning of study day fourteen, and blood samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14 hours after the last dose. On days fifteen and sixteen, morning and evening blood samples were obtained. Additional blood samples were taken on the mornings of days seventeen, eighteen, nineteen, twenty-one and twenty-four, and then weekly for six subsequent weeks (days 28, 35, 42, 49, 56, and 63). The subjects were asked to fast for 10 hours during the nights prior to the first and the fourteenth days of dosing and were served breakfast two hours after dosage. They were ambulatory but stayed inside the study unit during the first fourteen hours of these two days.

2.2.2 Sample Handling

Blood samples were collected in precooled, heparinized Vacutainers® (Becton Dickinson, Rutherford, NJ) and centrifuged at 4° C. In order to reduce the degradation rate of tolmetin glucuronide, plasma was adjusted to pH 2 to 4 with concentrated phosphoric acid (10 µl/ml) immediately after centrifugation and stored at -20° C. Samples were analyzed within several weeks of collection. Concentrations of tolmetin, and its β-1-glucuronide were determined by HPLC (Hyneck *et al.*, 1987) using zomepirac as the internal standard.

2.2.3 Quantitation of Covalently Bound Tolmetin

Covalent binding of tolmetin to plasma proteins was determined by a previously used method (Hyneck *et al.*, 1988). Briefly, plasma proteins were precipitated by addition of cold isopropanol (0.5 ml), followed by cold acetonitrile (1.5 ml), to plasma samples (0.5 ml) and controls. The protein

pellet was washed 7 times with 3 ml aliquots of methanol:ether (3:1) to remove reversibly-bound tolmetin and tolmetin glucuronide. The number of washes necessary for removal of free or reversibly bound tolmetin was determined by performing the washing and assay procedures on blank plasma spiked with tolmetin (50 μg/ml, the highest maximal concentration observed in our study). The dried protein pellet, containing covalently bound tolmetin, was heated with 0.25 M KOH (1 ml) at 80° C for 1.5 hours to release tolmetin from the adduct. The mixture was acidified by addition of phosphoric acid (45 μl of a 43% solution in water) and, after addition of internal standard (150 μl of a 2 μg/ml solution of zomepirac), extracted with dichloromethane (5 ml). The organic phase was evaporated to dryness under nitrogen and reconstituted into 300 μl acetonitrile-0.5 M sodium acetate (pH 4.5) (25:75, vol/vol) for HPLC analysis. Standard curves were constructed from dried blank protein pellets to which tolmetin was added before base hydrolysis. Protein concentration in each of the hydrolysates was measured using bicinchoninic acid protein assay (BCA assay, Pierce, Rockford, IL). Covalently bound tolmetin concentrations are expressed as nanograms of tolmetin per milligram of protein.

2.3 RESULTS

The mean value ± SE of the concentrations of covalently bound tolmetin at different time points after a single dose are shown in Fig. 2.2. Measurable bound amounts were initially observed in three volunteers at half an hour, in two of them at one hour, and in the last at three hours after drug administration. Maximum bound concentrations in the volunteers were reached at different times. The mean ± SD of these maximum values

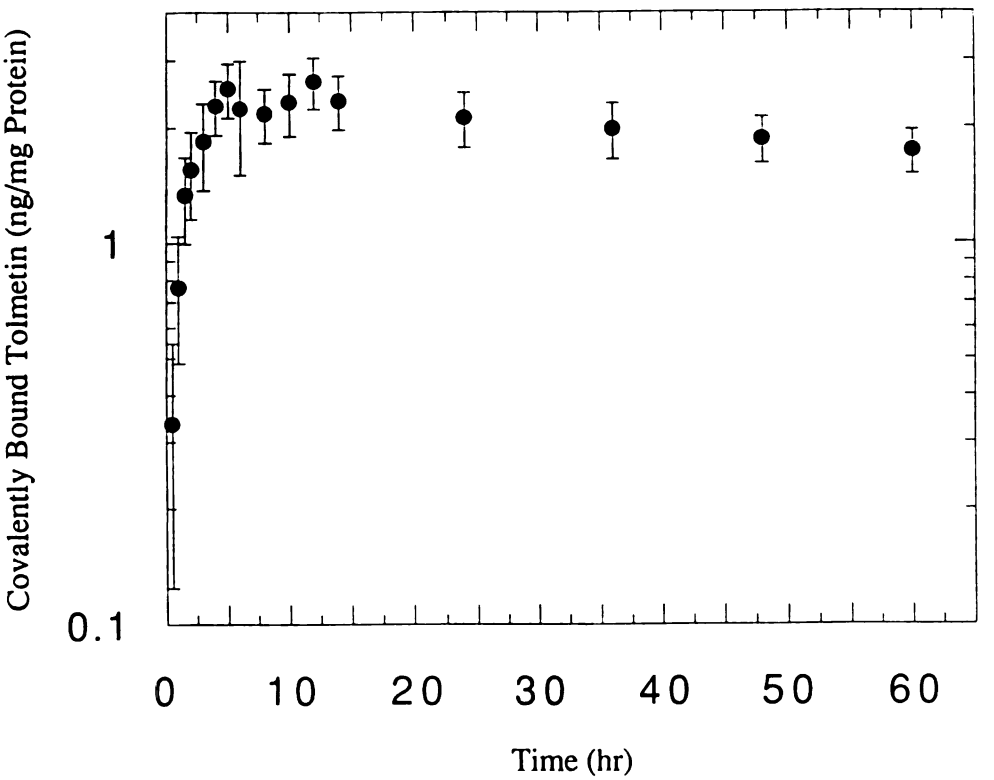

Figure 2.2. Concentration-time profile of covalently bound tolmetin after a 400 mg dose of Tolectin®. Average values of the concentrations in all six volunteers ± standard errors are shown.

after a single dose was 2.72 ± 0.98 ng drug/mg protein (Table 2.1). These values are within the range of the maximum adduct concentrations reported following single oral doses of tolmetin to healthy young (Hyneck *et al.*, 1988) and elderly (Munafo *et al.*, 1993) volunteers. The mean covalent binding

Table 2.1. Maximum observed concentrations (C_{max}) of covalently bound tolmetin per milligram protein (P) after single, and multiple dosing of Tolectin® and half-lives of the protein adduct following multiple dosing.

	Single Dose	Multiple Doses	
Volunteers	C_{max} (ng/mg P)	C_{max} (ng/mg P)	Half-lives (days)
1	1.14	26.9	4.8
2	2.66	20.3	3.7
3	3.00	25.5	6.1
4	3.67	37.1	5.5
5	3.76	24.6	4.4
6	2.16	18.8	4.3
Avg. ± SD	2.72 ± 0.98	25.5 ± 6.5	4.8 ± 0.9

profile of tolmetin after multiple dosing is depicted in Fig. 2.3. The maximum bound concentrations and half-lives observed in all subjects are given in Table 2.1. The mean ± SD maximal concentration reached was 25.5 ± 6.5 ng/mg protein. This value is approximately an order of magnitude higher than that observed after a single dose (2.72±0.98). Adduct levels were measurable in plasma for up to fourteen days in all subjects and detectable in

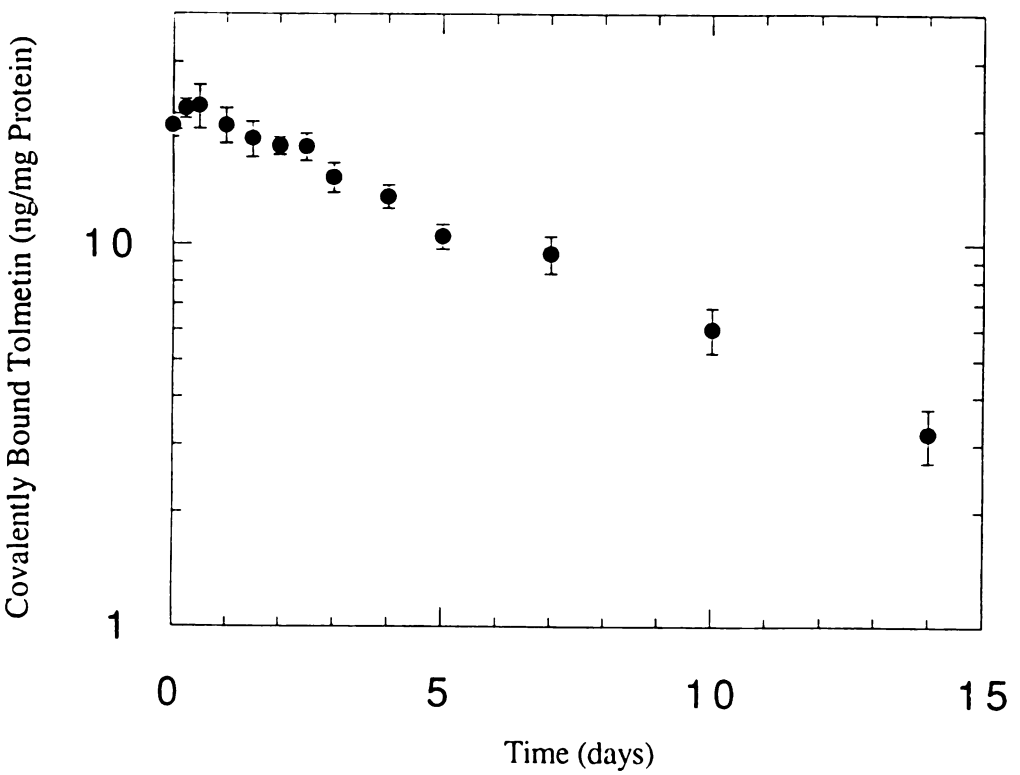

Figure 2.3. Concentration-time profile of covalently bound tolmetin after a ten day multiple dosing regimen of Tolectin®. Average values of the concentrations in all six volunteers ± the standard errors are shown. Time zero corresponds to the time when the last dose of the multiple dosing regimen was administered.

two of the subjects twenty-one days after the cessation of the multiple dosing regimen. The protein adduct is long-lived relative to the drug and its glucuronide metabolite, as depicted in Fig. 2.4 for one volunteer, with an average half-life, determined following multiple dosing, of 4.8 ± 0.9 days (Table 2.1). The mean value of maximal concentration of covalently bound tolmetin after multiples doses were 129% (± 56) of the concentration of tolmetin glucuronide and 2.5% (± 0.8) of that of tolmetin in all six volunteers. The concentration values of bound tolmetin, tolmetin, and tolmetin glucuronide for a representative subject are shown in Fig. 2.4.

2.4 DISCUSSION

The results show that formation of protein adduct occurs even after a single dose of tolmetin, and that much higher concentrations of adduct are present in plasma after multiple doses of tolmetin. This accumulation is important since clinical use of tolmetin invariably involves multiple doses. The adduct has a markedly longer half-life (4.8 ± 0.9 days) than the drug and its metabolites (apparent half-life of five hours) (Hyneck *et al.*, 1988), but a shorter half-life than albumin (approximately 19 days). McKinnon and Dickinson (1989) have observed an average terminal half-life of 10 days for protein adducts of diflunisal (another NSAID) and 13.5 days for adducts of probenecid after a 6-day oral regimen of diflunisal with oral co-administration of probenecid during the last 2 days of the regimen. Probenecid, being an acidic drug, interferes with the secretion of diflunisal glucuronide, thus increasing body exposure to this reactive metabolite.

As described in Chapter 1, two mechanisms have been proposed to describe the covalent binding of acyl glucuronides to proteins. The

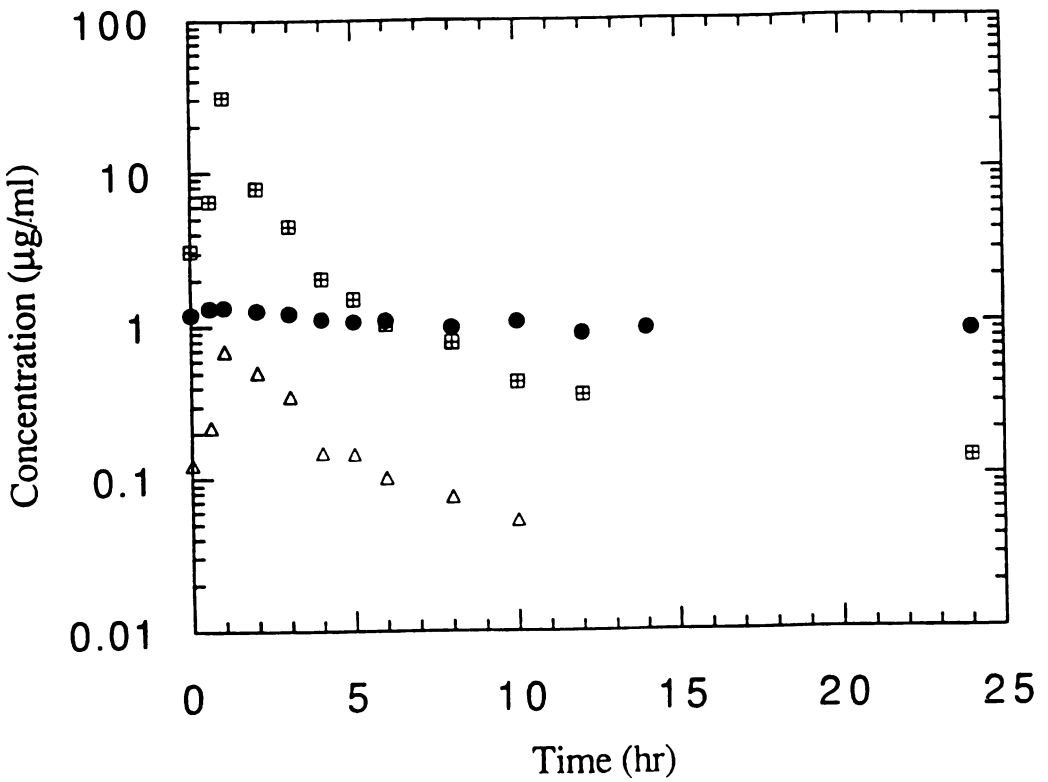

Figure 2.4. Concentration-time profiles of tolmetin (squares), 1-β-tolmetin glucuronide (triangles), and covalently bound tolmetin (circles) after a ten day multiple dosing regimen of Tolectin® in one volunteer. Time zero corresponds to the time when the last dose of the multiple dosing regimen was administered.

nucleophilic displacement mechanism leads to regeneration of glucuronic acid and formation of an amide, ester, or a thioester bond between the protein and the drug (Fig. 1.3a). The Schiff base mechanism involves a condensation reaction with an NH_2 group, generating an imine (-C=N-) which, potentially, could undergo rearrangement to a more stable 1-amino-2-keto product (Fig. 1.3b). In this case, the glucuronic acid would be retained in the adduct. Both mechanisms may occur *in vivo*. As will be described in Chapter 3, both mechanisms occur simultaneously *in vitro*, but the relative importance of each route depends on the acyl glucuronide-protein concentration ratio. It is not obvious which mechanism would yield the most stable adduct *in vivo*.

It is reasonable to assume that adduct formation in plasma involves primarily albumin because it is the major plasma protein. With this in mind, the short half-life of the adduct relative to albumin must reflect either cleavage of covalently bound drug in the circulation, or accelerated proteolytic degradation of the protein moiety of the adduct. However, it is also possible that adduct formation involves reaction of tolmetin glucuronide with plasma proteins that have shorter half-lives than albumin.

In vitro binding of tolmetin glucuronide to rat and sheep tissue homogenates have also been observed (Ojingwa *et al.*, 1994). Recently, binding of other NSAIDs, diclofenac (Pumford *et al.*, 1993) and diflunisal (King and Dickinson, 1993), to tissue proteins has been detected in experimental animals after drug administration. A number of instances of hepatic toxicity have been reported for diclofenac (Zimmerman, 1990) and a few cases of severe generalized hypersensitivity reactions have been reported for diflunisal (Cook *et al.*, 1988).

Many of the cases of adverse reactions to tolmetin, zomepirac and a number of other NSAIDs have been observed following intermittent use of

the drugs (U.S. Congress *et al.*, 1982; Australian Adverse Drug Reactions, 1993), suggesting the occurrence of a sensitization process in these individuals. The long half-life of the adduct may aid this process. If adduct formation is responsible for the hypersensitivity reactions of tolmetin (and other NSAIDs), then sensitized individuals should exhibit anti-adduct antibodies. Low titers of anti-adduct antibodies have been detected in patients taking the acidic drug valproic acid, which does not cause hypersensitivity reactions (Williams *et al.*, 1992). However, tolmetin glucuronide is apparently much more reactive (*in vitro* $t_{1/2}$ of 23 min) than valproic glucuronide (*in vitro* $t_{1/2}$ of 4.5 days) (Williams *et al.*, 1992; Benet *et al.*, 1993). Thus, adduct formation with tolmetin is likely to be more extensive than with valproic acid, and anti-adduct antibody titers in patients taking tolmetin are likely to be higher.

3.0 MECHANISM OF COVALENT BINDING OF TOLMETIN GLUCURONIDE TO HUMAN SERUM ALBUMIN

3.1 INTRODUCTION

Two mechanisms have been proposed to describe the covalent binding of acyl glucuronides to proteins. The first is a nucleophilic displacement mechanism whereby a nucleophile (NH_2, OH, SH) of the protein attacks the carbonyl group of the aglycone (Fig. 3.1a). This reaction leads to the regeneration of the glucuronic acid and formation of a bond (amide, ester, or thioester) between the protein and the drug. The second is a mechanism whereby an amino group of the protein reacts with the aldehyde group of the glucuronic acid to form a Schiff base intermediate (imine). This intermediate then undergoes Amadori rearrangement to form a more stable 1-amino-2-keto product (Fig. 3.1b). In this mechanism, the glucuronic acid moiety is retained as a component of the protein adduct. In the presence of an imine trapping reagent (e.g. sodium cyanoborohydride-$NaCNBH_3$) the Schiff base is reduced and a more stable amine product is generated (Fig. 3.1b)

Three different approaches have been utilized to characterize the covalent modification of albumin by acyl glucuronides : 1) competitive inhibition of adduct formation by molecules that are known to bind specific sites on albumin reversibly (Wells *et al.*, 1987), 2) inhibition of adduct formation by chemical modification of various nucleophiles (Fehske *et al.*, 1980; Han *et al.*, 1987; Ojingwa, 1991), 3) mass spectrometric analysis of tryptic digests of the albumin adduct of acyl glucuronides (Ding *et al.*, 1993).

Different amino acid residues have been implicated in the covalent binding of acyl glucuronides of a number of drugs to albumin. For example,

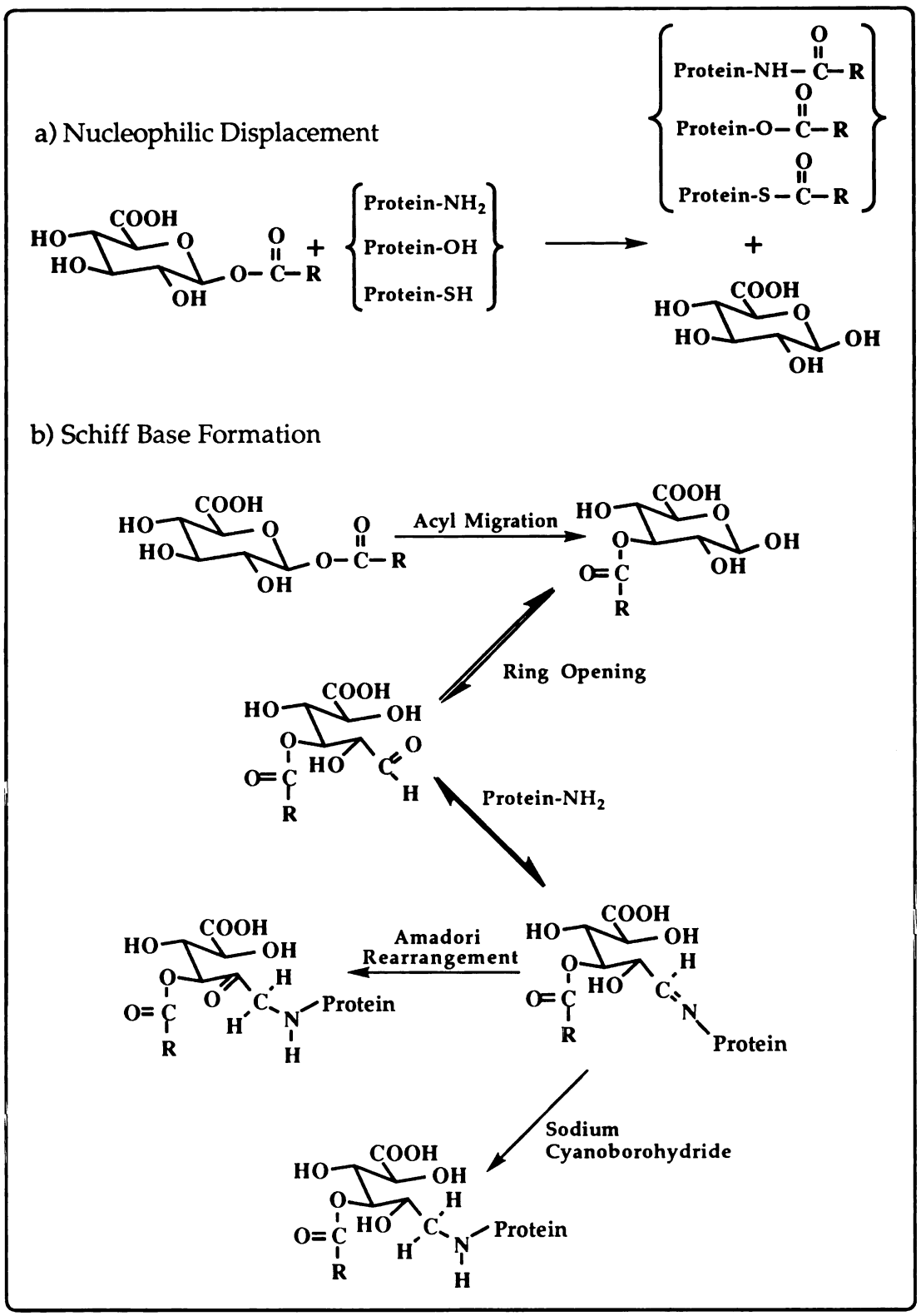

Figure 3.1. Proposed mechanisms of covalent binding of acyl glucuronides with protein: a) nucleophilic displacement mechanism, b) Schiff base formation mechanism with Amadori rearrangement and in the presence of a reducing agent (e.g. $NaCNBH_3$).

molecules (including oxaprozin) that bind specifically to the benzodiazepine binding site on albumin were found to inhibit the covalent binding of oxaprozin glucuronide (Ruelius *et al.*, 1986). A significant reduction in irreversible binding and degradation of oxaprozin glucuronide, but not reversible binding, resulted from reacting the tyrosine at the benzodiazepine-binding site (tyrosine-411) with tetranitromethane, diisopropyl fluorophosphate, or *p*-nitrophenylacetate (Wells *et al.*, 1987). Ruelius et al. (1986) have also implicated tyrosine as the binding site for oxaprozin glucuronide. In contrast, another group (van Breemen and Fenselau, 1985) hypothesized that sulfhydryls act as the primary nucleophiles; however, these scientists found that blocking the sulfhydryls on bovine serum albumin (BSA) could not prevent all of the binding of flufenamic glucuronide. Moreover, blockage of thiols on HSA with *p*-hydroxymercuribenzoate did not have any effect on the extent of binding of zomepirac glucuronide (Smith *et al.*, 1990). Furthermore, irreversible blockage of sulfhydryl groups of albumin had no effect on irreversible binding of oxaprozin glucuronide, while chemical modification of lysine residues led to a minor decrease in the extent of binding (Ruelius *et al.*, 1986). These studies suggest that the reactive residues on albumin are lysines and tyrosines.

Recently, the binding of tolmetin glucuronide (TG) to HSA in the presence of sodium cyanoborohydride has been investigated (Ding *et al.*, 1993). At least six of the peptides generated by tryptic digestion of the adduct contained tolmetin (T). After subjecting the isolated peptides to liquid secondary-ion mass spectrometry (LSIMS) and collision-induced dissociation mass spectrometry (CIDMS), all six peptide fragments were found to contain tolmetin linked covalently via glucuronic acid to a lysine residue. The modified residues were lysines 199, 195, 525, 351, 541, and 137. In the absence

of the imine trapping reagent, it was unclear what the major binding sites would be and whether the Schiff base formation mechanism would be observed. It is possible that both the Schiff base formation and the nucleophilic reaction occur concurrently.

As described in Chapter 1, if the Schiff base formation mechanism occurs, the glucuronic acid group is retained within the protein adduct. The presence of this common chemical moiety could play a role in the observed immunological cross-reactivities. Identification of the modified residues is important, because in addition to giving a first clue to the mechanism of binding, the modified residues may be a part of the antigenic determinants (parts recognized by antibodies) of the protein adduct. Thus, the mechanism of binding of TG to HSA has been examined here in the absence and presence of $NaCNBH_3$. The effect of increasing concentrations of TG at a constant concentration of HSA on the overall binding yield and the mechanism of binding have also been investigated.

3.2 EXPERIMENTAL SECTION

3.2.1 Materials

All chemicals were purchased from Sigma Chemical Company (St. Louis, Miss.) unless otherwise specified.

3.2.2 Isolation of Tolmetin Glucuronide from Urine

Tolmetin glucuronide was isolated and purified as previously described (Munafo *et al.*, 1990). Briefly, urine of a volunteer was collected during the period 0-8 hours after ingestion of a 400 mg tolmetin capsule (Tolectin®, McNeil Pharmaceutical, Springhouse, PA). The pH of the urine was adjusted

to 3 by addition of concentrated phosphoric acid (5 µl/ml urine). After gravity filtration, the urine was extracted twice with equal volumes of chloroform, and then the aqueous phase was extracted twice with ethyl acetate. After drying the ethyl acetate phase with sodium sulfate, the filtered solvent was evaporated using a rotoevaporator and the residue was reconstituted in 45% methanol in 0.05 M ammonium acetate aqueous solution (pH 4.5) and further purified by HPLC. A semi-preparative HPLC column (Ultrasphere ODS 5m, 250 mm x 10 mm) was used. The mobile phase was 45% methanol in 0.05 M ammonium acetate aqueous solution (pH 4.5) and the flow rate was 2.5 ml/min. The effluent was monitored at 313 nm. The main collected fraction, representing β-1-tolmetin glucuronide was lyophilized and again purified by HPLC to approximately 90-96% purity (assessed by HPLC analysis).

3.2.3 Preparation of HSA Adducts of Tolmetin Glucuronide

Three different concentrations of tolmetin glucuronide (0.3, 1.5, and 7.5 mM) were reacted with fatty acid free HSA (0.45 mM). In a fourth reaction mixture, TG (1.5 mM) was reacted with HSA (0.45 mM) in the presence of sodium cyanoborohydride. The control mixture contained tolmetin (1.5 mM), glucuronic acid (1.5 mM), and HSA (0.45 mM).

The reaction was carried out at 37°C in phosphate buffer (150 µl, 0.1M, pH 7.4) for six hours. One third of the mixture (50 µl) was used to quantitate bound tolmetin after hydrolysis of the modified protein. In order to separate the modified protein from the smaller molecules, the rest of the sample was subjected to six cycles of centrifugal filtration using Amicon centricons (30KD molecular weight cutoff). The mixture was diluted with water before each filtration step and lyophilized after the last step.

3.2.4 Quantitation of Bound Tolmetin by Alkaline Hydrolysis of the Modified Protein

The protein was precipitated by addition of cold isopropanol (50 µl) and cold acetonitrile (150 µl). The precipitated protein was then pelleted by centrifugation. The pellet was washed six times with a mixture of methanol:ether (3:1, 300 µl) and then dried overnight. The dried protein pellet was hydrolyzed in potassium hydroxide (0.25 M, 100 µl) at 80°C for one hour to release bound tolmetin. A portion (80 µl) of the hydrolysate was neutralized with 7.2 µl of 43% phosphoric acid and then 24 µl of the internal standard solution (zomepirac, 2 µg/ml) was added. This mixture was extracted with dichloromethane (0.8 ml). After evaporation of the organic phase, the residue was reconstituted in 40 µl of acetonitrile 0.5 M sodium acetate (pH 4.5, 25:75, vol/vol) and analyzed by high performance liquid chromatography (HPLC) (Hyneck *et al.*, 1988). Protein concentration was measured by the bicinchoninic acid protein assay (BCA assay, Pierce, Rockford, IL) in the remaining portion of the hydrolysate (20 µl) after dilution with water (780 µl).

3.2.5 Tryptic Digestion of the Protein

The protein residue (containing HSA and modified HSA, ~ 44 nmol) was dissolved in 300 µl of a buffer solution containing 6 M guanidine·HCl, 100 mM Tris ,and 1 mM EDTA (pH 8.3). This solution was heated at 55°C for 40 min with 27 µmoles of dithiothreitol (11 µl of 2.5 mmol/ml) to reduce the disulfide bonds in the protein. The sulfhydryl groups were then alkylated with sodium iodoacetate (45 µmoles, 40°C, 35 min). The mixture was then dialyzed overnight against ammonium bicarbonate buffer (50 mM, pH 7.75) using a Bethesda Research Labs microdialysis apparatus equipped with an

8000 Da molecular weight cutoff membrane (Pierce, Rockford, IL). The dialyzed protein was treated with 2% (wt/wt) trypsin (L-tosylamido-2-phenylethyl chloromethyl ketone-treated,type XIII).

3.2.6 Isolation of the Modified Peptides by HPLC

The tryptic peptides were separated by HPLC using a Vydac protein and peptide C18 column (250 x 4.6 mm, 300 pore size) and a Carlo Erba dual-wavelength UV detector. For this analysis, a linear gradient starting with 99% solvent A (0.1% (vol/vol) trifluoroacetic acid in water) to 45% solvent B (0.08% trifluoroacetic acid in acetonitrile) within 90 min at a flow rate of 1 ml/min. The eluent was monitored at 215 nm (peptide trace) and 313 nm (modified peptide trace) since tolmetin has a strong absorbance at this latter wavelength. The modified peptide fractions were collected and subjected to another HPLC purification step using a Vydac phenyl column (250 x 4.6 mm) and a linear gradient starting from 5% to 30% solvent B within 50 min. These fractions were then lyophilized and redissolved in 5 µl of the HPLC mobile phase before mass spectrometric analysis.

3.2.7 Mass Spectrometric Analysis of the Modified Peptides

To determine the molecular masses of the peptides in each of the collected HPLC fractions, an aliquot (10-20%) of the redissolved sample was added to 1 µl of the liquid matrix (1:1 thioglycerol-glycerol with 1% TFA) and examined by LSIMS. The mass spectra were recorded with a Kratos MS-50S double-focusing mass spectrometer equipped with a cesium ion source (Aberth *et al.*, 1982; Falick *et al.*, 1986). The runs were performed using an acceleration voltage of 6 keV and resolution of 2000. Thus the m/z values for the MH^+ ions reflect the ^{12}C monoisotopic components. In some cases,

determination of molecular masses was accomplished by matrix-assisted laser desorption mass spectrometry (MALDI-MS) using a Kratos Kompact MALDI 3 time-of-flight mass spectrometer (Kratos Inc., Manchester, U.K.). The instrument was set to linear mode and high voltage. The laser power was set to 43% and the matrix was α-cyano-4-hydroxy cinnamic acid. Approximately 50 shots were averaged. The m/z values obtained with this technique reflect average masses.

The masses of the modified peptides were compared to the known list of masses of the peptides generated from tryptic digestion of carboxy methylated HSA. Knowing that a covalent modification by tolmetin (nucleophilic displacement mechanism) would add 239 mass units to the mass of the peptide and a covalent modification by tolmetin glucuronide (the Schiff base mechanism) would add 415 mass units to the mass, the probable modified peptides were identified. To confirm the amino acid sequence of the modified peptides and to localize the site of modification, tandem mass spectrometry (MS/MS) was carried out using a Kratos Concept IIHH four-sector-tandem mass spectrometer with $E_1B_1E_2B_2$ configuration (Walls *et al.*, 1990) equipped with a continuous flow-FAB probe, a cesium ion source and a charge coupled device detector. The solvent contained 5% acetonitrile, 2% thioglycerol and 0.1% TFA in water. The flow rate was set to 3 µl/min using a syringe pump (Applied Biosystems, Foster City, CA). Collision induced dissociation (CID) of the ^{12}C component of the protonated peptide molecules was performed in a collision cell in the field-free region after B_1. The CID nomenclature for peptide dissociation processes follows that published in the literature (Roepstorff and Fohlman, 1984; Biemann, 1988) (Fig. 3.2).

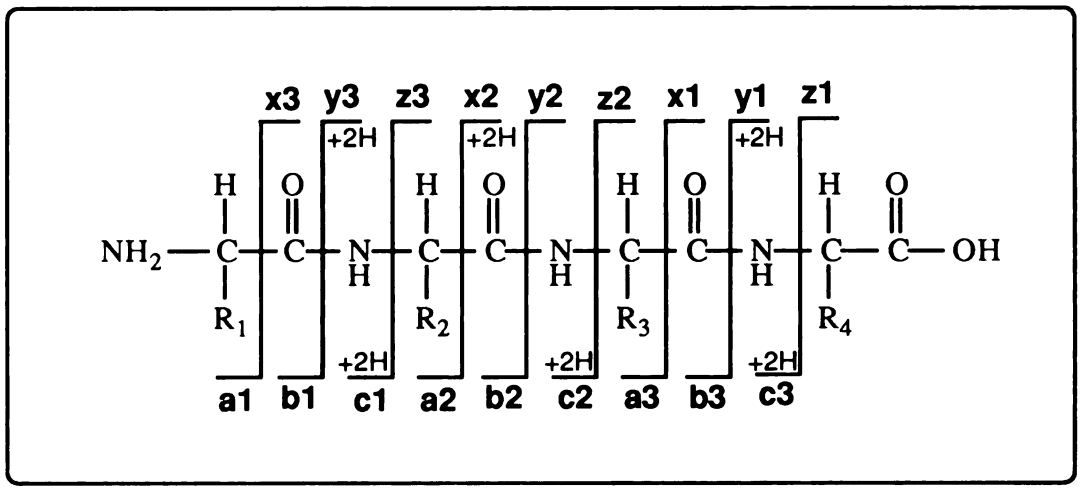

Figure 3.2. The nomenclature for fragmentation of peptides (adapted from Roepstorff and Fohlman, 1984).

3.2.8 Edman Sequencing

A few of the HPLC fractions were analyzed by Edman sequencing due to low amounts of peptides. The sequencing was performed using an ABI 470A gas phase sequencer equipped with a 130A HPLC system (Foster City, CA). The procedures were performed according to the manufacturer's manual (Hunkapiller, 1985).

3.3 RESULTS

The extents of covalent binding (binding yield) for the five different incubations are shown in Table 3.1. Percent molar ratio of bound tolmetin to HSA was determined after HPLC analysis of the hydrolysate of the protein pellet and after HPLC analysis of the tryptic digest of the protein. When the calculations for percent bound tolmetin were done after tryptic digestion, the absorbance of unbound tolmetin was subtracted from the total observed

Table 3.1. The extents of covalent binding (binding yield) for the five different incubations.

		Percent Molar Binding Ratios (T equivalents bound per mole HSA x 100)		
Incubations	Amounts of the Reactants (μmoles)	Determined after Hydrolysis	Determined after Tryptic Digestion	Average of Values Presented in Columns 3 & 4
1	0.045 TG : 0.068 HSA	1.2	1.2	1.2
2	0.225 TG : 0.068 HSA	5.2	6.6	5.9
3	1.125 TG : 0.068 HSA	10.9	13.1	12.0
4	0.225 TG : 0.068 HSA ($NaCNBH_3$)	25.3	29	27.2
5	0.225 T : 0.068 HSA	0.0	0.0	0.0

absorbance. As expected, the binding yield increases as the concentration of TG increases; however, the increase is not linear. Due to the presence of the imine trapping agent ($NaCNBH_3$), the average binding yield for incubation 4 (27.2%) is approximately five times higher than that of incubation 2 (5.9%) (Table 3.1).

The HPLC chromatograms (λ = 313 nm) for incubations 1-3 and 5 (the control) are given in Fig. 3.3 These modified peptide traces look similar for the first three incubations. The identified binding sites, the type of modification, and the percent of total binding attributed to each site are summarized for incubations 1-3 in Table 3.2. Similar TG binding sites were observed for incubation 4 (1.5 mM TG with $NaCNBH_3$) as those obtained by Ding et al. (1993); however, two minor T binding sites were also identified in this mixture (Table 3.3). The results from incubation 3 (7.5 mM TG) are discussed below.

Both the peptide HPLC trace and the modified peptide HPLC trace for incubation 3 are depicted in Fig. 3.4. The peak with the highest area in the chromatogram (69.5 min) represents absorbance from unbound tolmetin. The peaks with retention times less than 40 min were also observed in the control incubation (Fig. 3.3, incubation 5) and in digests of untreated HSA. The fractions represented by the numbered peaks (45-67 min) were collected and subjected to mass spectrometric analysis (Fig. 3.4).

The following fractions were identified as peptides that were modified by a tolmetin glucuronide group (the product of the Schiff base formation mechanism):

The fraction eluting at 56.5 min (17% of total absorbance) gave a strong molecular ion (MH^+) at m/z of 1363.6 by LSIMS (Fig. 3.4, peak 3, Table 3.2). This m/z value corresponds to the mass of residues 198-205 of carboxy

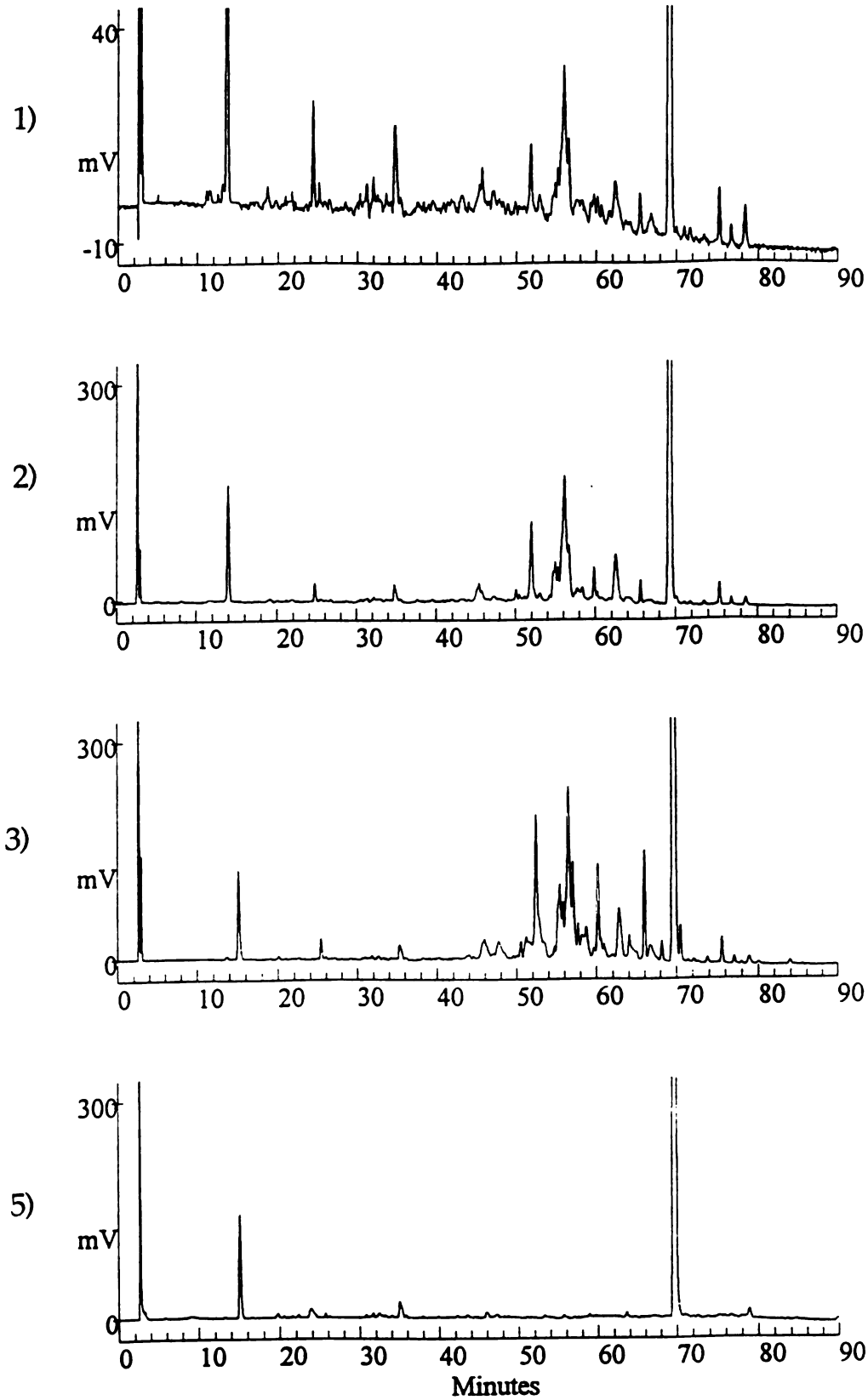

Figure 3.3 The HPLC chromatograms (λ=313) for incubations 1-3 and 5 (the control).

Table 3.2. Summary of binding studies for incubations 1-3. Peak assignments were mainly based on the CIDMS examinations of the fractions collected from the tryptic digest of incubation 3 (7.5 mM TG). Peptide identification for incubation 2 (1.5 mM TG) was done by molecular ion measurements only and for incubation 1 (0.3 mM TG) peptide identification was done by comparing the retention times of the HPLC peaks.

Modified Peptide	HSA Residues	Modified Amino Acids	MH^+ (m/z)	HPLC Peak No.	HPLC Retention Time (min)	1) 0.3 mM TG	2) 1.5 mM TG	3) 7.5 mM TG
						Percent of Total Binding§		
ASSAK*QR	191-197	TG-Lys 195	1162.4	1	46	9	6	3
ATK*EQLK	539-545	TG-Lys 541	1232.5	2	52.5	11	13	16
LK*C(Cm)ASLQK	198-205	TG-Lys 199	1363.6	3	56.5	29	31	17
K*QTALVELVK	525-534	TG-Lys 525	1543.8	10	66.5	nd	nd	3
ATK*EQLK	539-545	T-Lys 541	1056.5	4	57	9	8	6
ER*QIK	520-524	T-Arg 521	912.4	5	57.5	nd	nd	2
LK*C(Cm)ASLQK	198-205	T-Lys 199	1187.6	6	60	nd	5	7
LS*QR*FPK	219-225	T-Ser 220 T-Arg 222	1114.4	7	63	10	12	6
C(Cm)C(Cm)TES*LVNR	476-484	T-Ser 480	1379.6	8	64	nd	nd	2
AEFAEVS*K	226-233	T-Ser 232	1119.4	9	66	6	3	6
		Sum of % Binding for TG-Binding Sites				49	50	39
		Sum of % Binding for T-Binding Sites				25	28	29
		% of Total Binding Identified				74	78	68

§ The relative importance of each binding site was determined from the ratio of the respective peak area to the total absorbance after the absorbance of unbound tolmetin had been subtracted.

nd: not detected

Cm: carboxy methylation of cysteine (C)

Table 3.3. Summary of results of the binding study for incubation 4 (1.5 mM TG with $NaCNBH_3$). Peak assignments were based on the CIDMS examination of some of the fractions collected from tryptic digests and a comparison of the retention times of the HPLC peaks.

Modified Peptide	HSA Residues	Modified Amino Acids	MH^+ (m/z)	Percent of Total Binding§
ASSAK*QR	191-197	TG-Lys 195	1164.4	13
ATK*EQLK	539-545	TG-Lys 541	1236.6	6
LK*C(Cm)ASLQK	198-205	TG-Lys 199	1365.6	30
K*QTALVELVK	525-534	TG-Lys 525	1545.8	20
LAK*TYETTLEK	349-359	TG-Lys 351	1713.8	4
K*YLYEIAR	137-144	TG-Lys 137	1472.6	2
LS*QR*FPK	219-225	T-Ser 220 T-Arg 222	1114.4	1
AEFAEVS*K	225-233	T-Ser 232	1119.4	3
Sum of % Binding for TG-Binding Sites				75
Sum of % Binding for T-Binding Sites				4
% of Total Binding Identified				79

§ The relative importance of each binding site was determined from the ratio of the respective peak area to the total absorbance after the absorbance of unbound tolmetin had been subtracted.

* Designates modified amino acids.

Cm: carboxy methylated

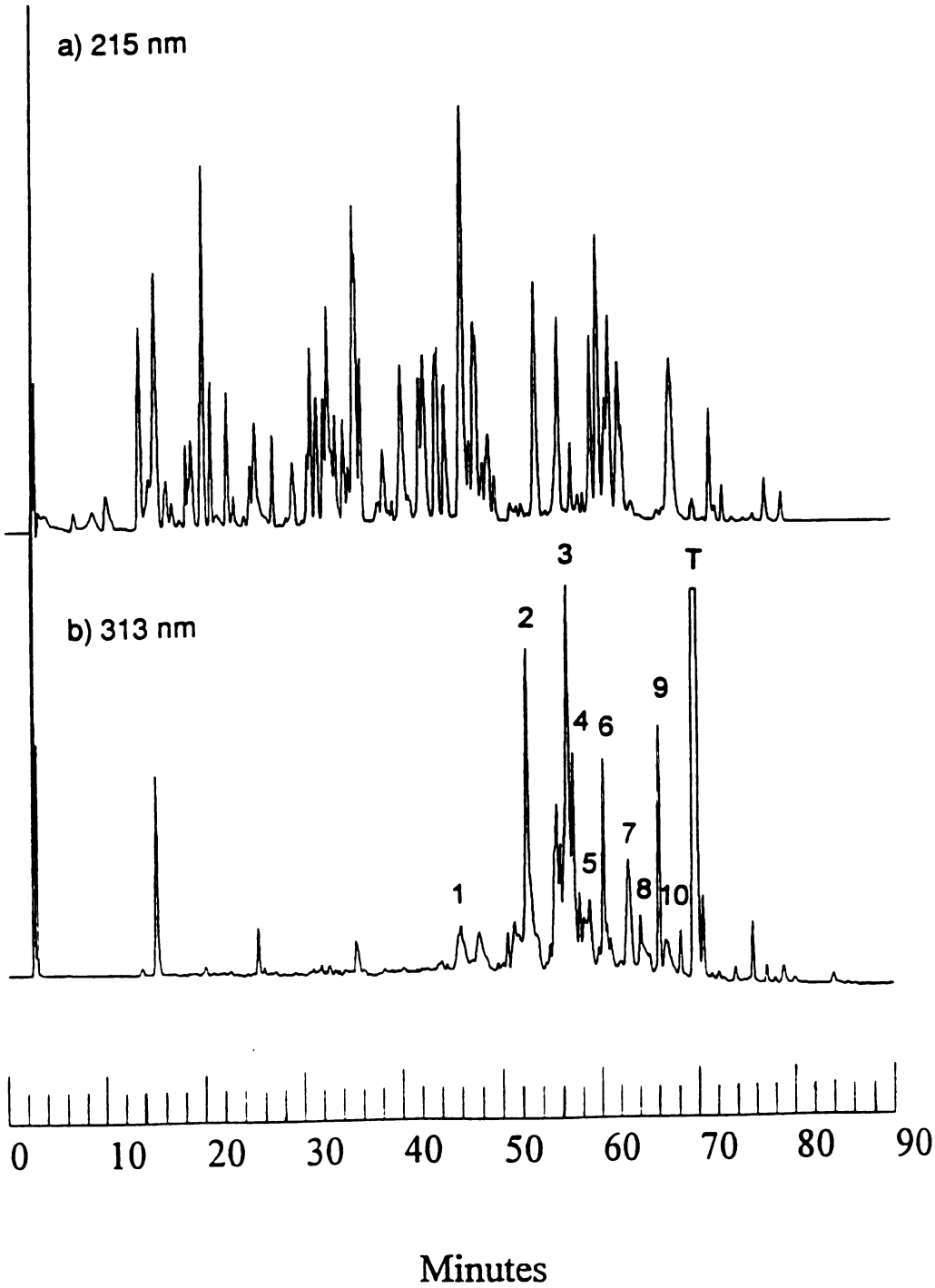

Figure 3.4 The HPLC chromatograms from incubation 3 (7.5 mM TG: 0.45 mM HSA): a) the peptide trace (λ=215), b) the modified peptide trace (λ=313).

methylated HSA (Leu-Lys*-Cys(CH_2COOH)-Ala-Ser-Leu-Gln-Lys; MH^+ with m/z of 948.6) plus the mass of a covalently bound tolmetin glucuronide group (415 mass units). The presence of both tolmetin and glucuronic acid was confirmed by the CID spectrum of this molecular ion (Fig. 3.5). The most abundant ions in the CID spectrum (m/z 94, 119, 122, 212, and 240) reflect fragmentation of tolmetin (Fig 3.6). The presence of tolmetin linked to a lysine via glucuronic acid was demonstrated by the ion peak (K*) at m/z 516 which corresponds to a lysine immonium ion (101 mu) plus tolmetin glucuronide (415 mu); and the ion peak at m/z 260 which corresponds to a lysine immonium ion plus glucuronic acid (159 mu). Other peptide fragment ions (a2, b2, b3, a4, b4, b5, a6, y7) were also shifted by 415 mass units. These shifts indicate the presence of tolmetin glucuronide. By analyzing these fragment series and comparing the CID spectra of the modified peptide with the known amino acid sequence of HSA, modification of lysine 199 by tolmetin glucuronide was unequivocally identified. As discussed earlier, this type of modification results from the Schiff base formation mechanism (Fig. 3.1b).

Similarly, two other fractions contained peptides that were bound to tolmetin glucuronide. The tryptic digest peak eluting at 66.5 min (peak 10, 3% of total absorbance) contained a fraction with MH^+ 1543.8. This mass corresponds to amino acids 525-534 of HSA (Lys*-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys) modified by a tolmetin glucuronide group at the N-terminal lysine. The small peak eluting at 46 min (peak 1, 3% of total absorbance) contained a fraction that gave a MH+ ion with m/z 1162.4 corresponding to the mass of the protonated peptide containing residues 191-197 of HSA (Ala-Ser-Ser-Ala-Lys*-Gln-Arg, 747.4) plus the mass of tolmetin glucuronide (415). The only possible binding site for the glucuronide in this peptide is lysine 195.

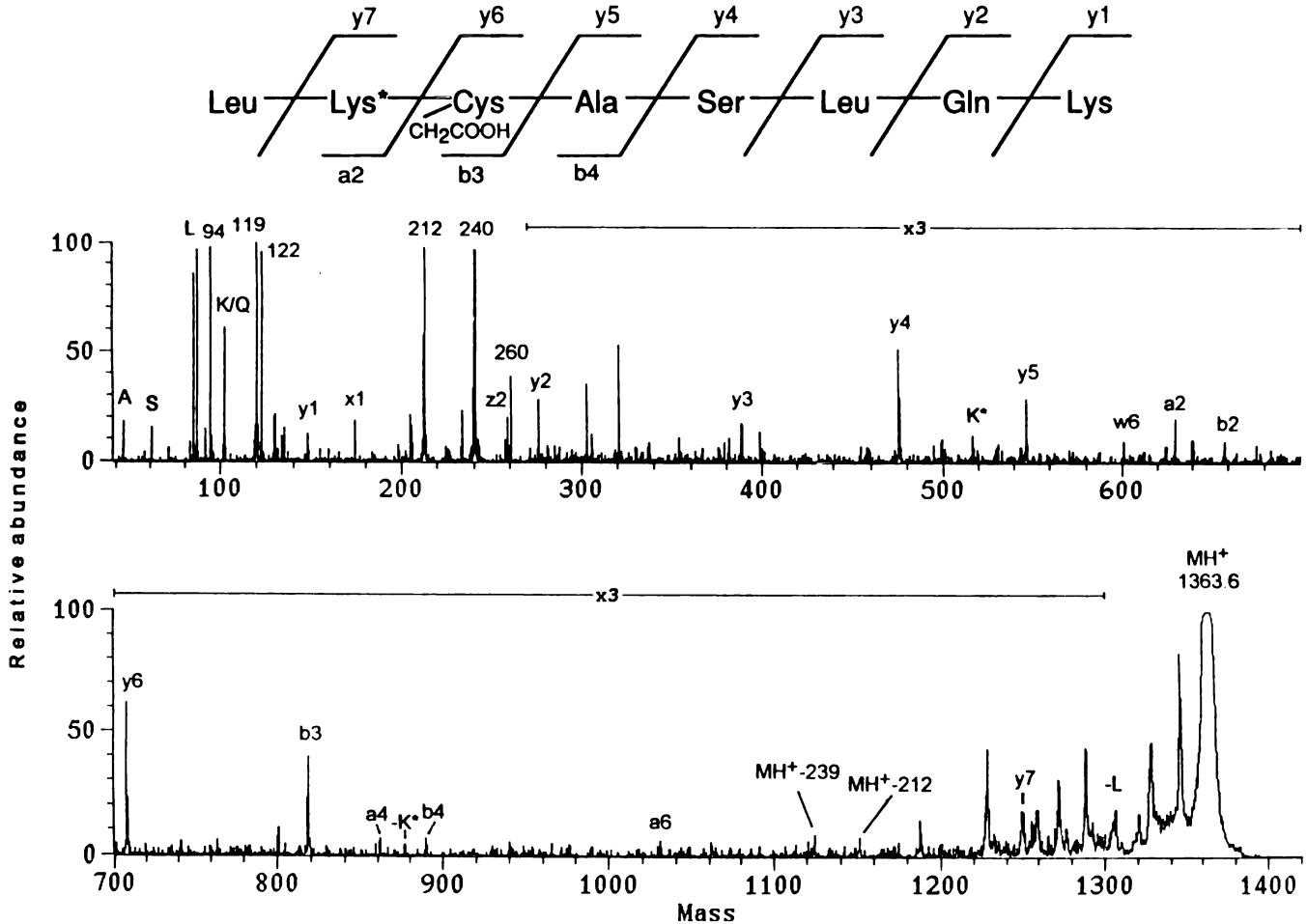

Figure 3.5. The CID mass spectrum for the molecular ion MH^+ 1363.6 corresponding to HPLC peak 3. Significant ions indicating the sequence of the modified peptide are shown above the spectrum. Fragments containing lysine 199 (Lys*) show a shift of 415 mu which corresponds to the mass of tolmetin glucuronide.

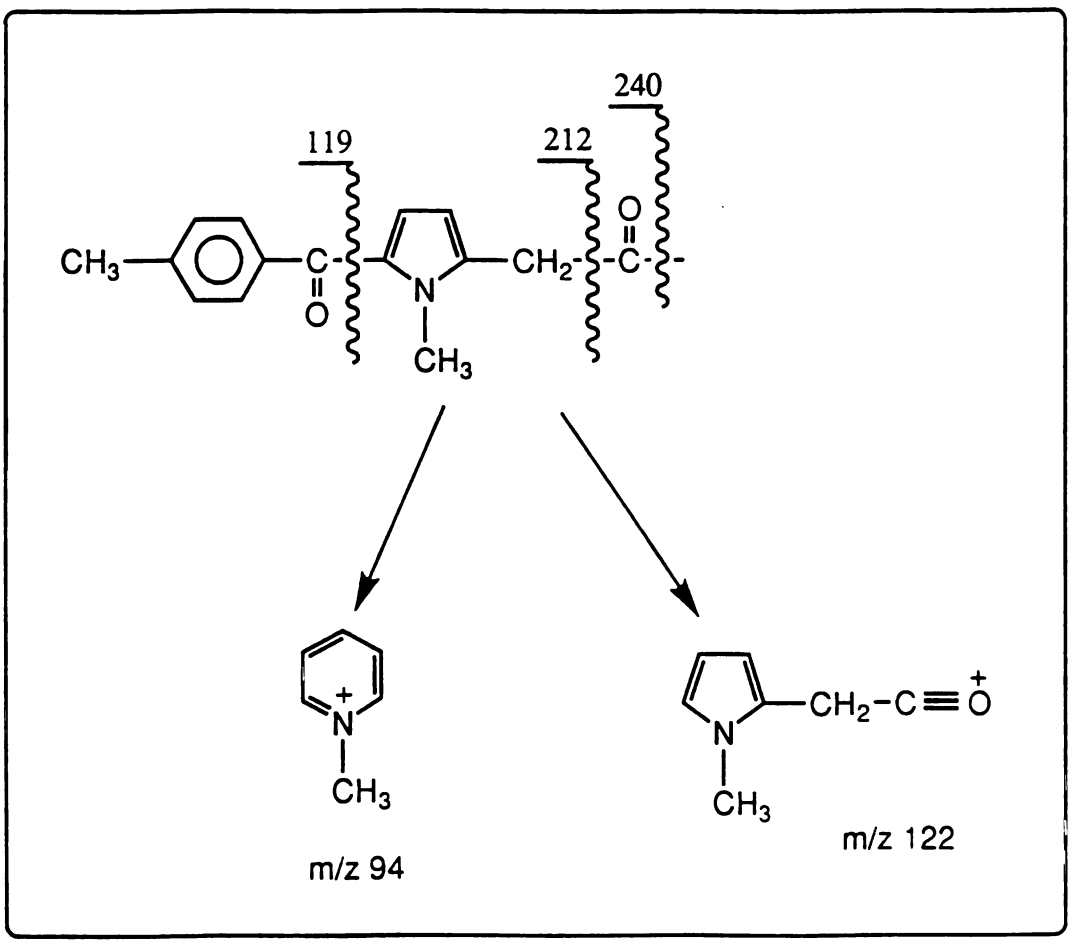

Figure 3.6 Characteristic fragment ions of tolmetin.

If the serines in the peptide had reacted with TG via a nucleophilic attack, modification of the peptide by one tolmetin group (239 mu) or two tolmetin groups (478 mu) would have been observed. The mass values obtained by addition of 239 to 747.4 or 478 to 747.4 do not agree with the observed m/z of 1162.4 for this modified peptide.

Furthermore, LSIMS analysis of the HPLC fraction eluting at 52.5 min (peak 2, 16% of total absorbance) (Fig. 3.4, Table 3.2) did not yield a molecular ion which would match a tryptic peptide of HSA modified by tolmetin or its glucuronide. Edman sequencing of this fraction yielded a complete sequence of an unmodified peptide representing the major component (MH^+ 1419.6) which was derived from tryptic digestion of human haptoglobin. MALD-TOF-MS detected several molecular ions not seen in LSIMS before (Fig. 3.7). Considering that the largest signal (1419.6) was due to an impurity of the HSA supply, none of the smaller signals could be definitely assigned to an expected HSA peptide. However, the peak at m/z 1233 (as seen in Fig. 3.7) may represent the peptide Ala-Thr-Lys*-Glu-Gln-Leu-Lys (amino acid residues 539-545 of HSA, MH^+ 817.4) plus a tolmetin glucuronide group (415 mu). The tolmetin glucuronide is most likely bound to lysine 541. Lysine 541 has been previously identified as a TG binding site (Ding *et al.*, 1993) and it was also identified as a TG binding site for incubation 4 of this study.

The following fractions were identified as peptides that were modified by a tolmetin group (product of the nucleophilic displacement mechanism):

The fraction eluting at 60 min (peak 6, 7% of total absorbance) gave a molecular ion with MH^+ 1187.6 (Fig. 3.4, Table 3.2). This corresponds to the mass of the protonated peptide composed of residues 198-205 of HSA (948.6) plus the mass of tolmetin (239). The presence of many fragment ions, diagnostic for the tolmetin group (94, 119, 122, 212, 240, MH^+-239), in the CID

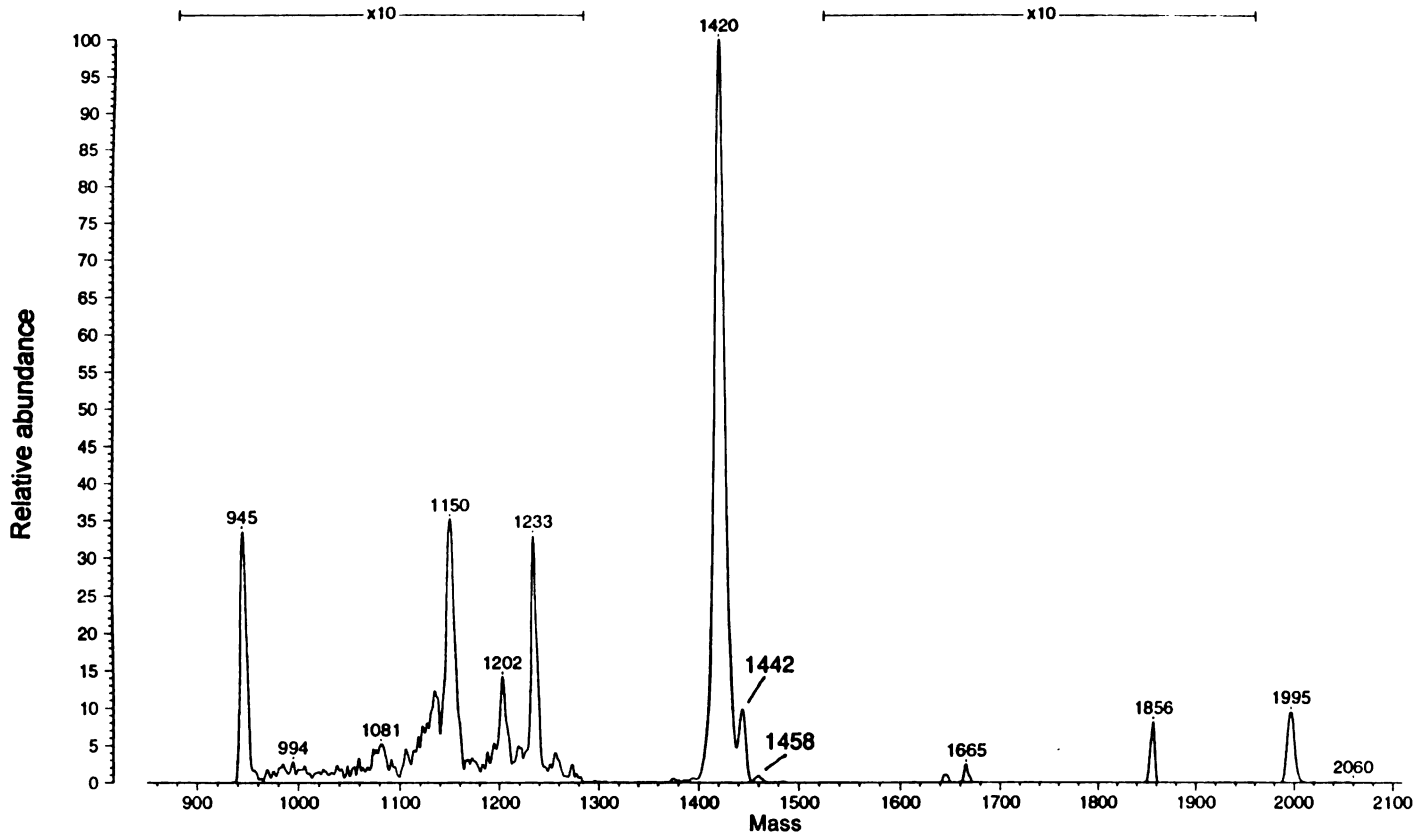

Figure 3.7 The MALDI-TOF spectrum of HPLC fraction 2. The ion with m/z 1233 (average mass) may represent HSA amino acid residues 539-545 plus a tolmetin glucuronide group. Here lysine 541 would be the most likely target for covalent binding of the glucuronide.

spectrum revealed modification by tolmetin (Fig. 3.6, Fig. 3.8). The C-terminal sequence ions (e.g. y1 to y7, several z ions, and w ions) and the N-terminal ions (b2, b3, d3, a5, b5, c5, a6, b6, c6, a7, b7, c7) confirmed the identification of the peptide as that corresponding to amino acids 198-205 of HSA. The shift of the y7 ion and all of the observed N-terminal ions by 239 mass units confirmed modification of lysine 199 by a tolmetin group (not the glucuronide). Other useful peaks were K* (m/z 340), -K* (m/z 876.6), MH^+-T-NH3 (MH^+-256, m/z 931.6), MH^+-T-$(CH_2)_3NH_3$ (MH^+-298, m/z 889.6) (Fig. 3.8).

Similarly, the fraction eluting at 57 min (peak 4, 6% of total absorbance) contained the peptide corresponding to residues 539-545 of HSA that was modified with a tolmetin unit at lysine 541 (Fig. 3.4, Table 3.2).

Two of the fractions contained peptides that were modified at serine residues only. The peak eluting at 66 min (peak 9, 6% of total absorbance) gave MH^+ ion of m/z 1119.4. The information obtained from the CID spectrum (Fig. 3.9) confirmed the peptide to be that containing amino acids 226-233 of HSA that was modified by addition of a tolmetin group. The almost complete N- and C-terminal fragment series showed clearly (e.g. b7, and y2-y6 shifted by 239 mu) that the serine 232 was the binding site. Only a negligible peak representing the immonium ion for the modified serine (mass 299) was observed. However, strong ions were observed for MH^+-T-H_2O (MH^+-257, m/z 862.4) and MH^+-T (MH^+-239, m/z 880.4) fragments (Fig. 3.9). Similarly, the fraction eluting at 64 min (peak 8, 2% of total absorbance) was identified as the peptide Cys(CH_2COOH)-Cys(CH_2COOH)-Thr-Glu-Ser*-Leu-Val-Asn-Arg of HSA modified by the binding of tolmetin at serine 480 (Fig. 3.4, Table 3.2).

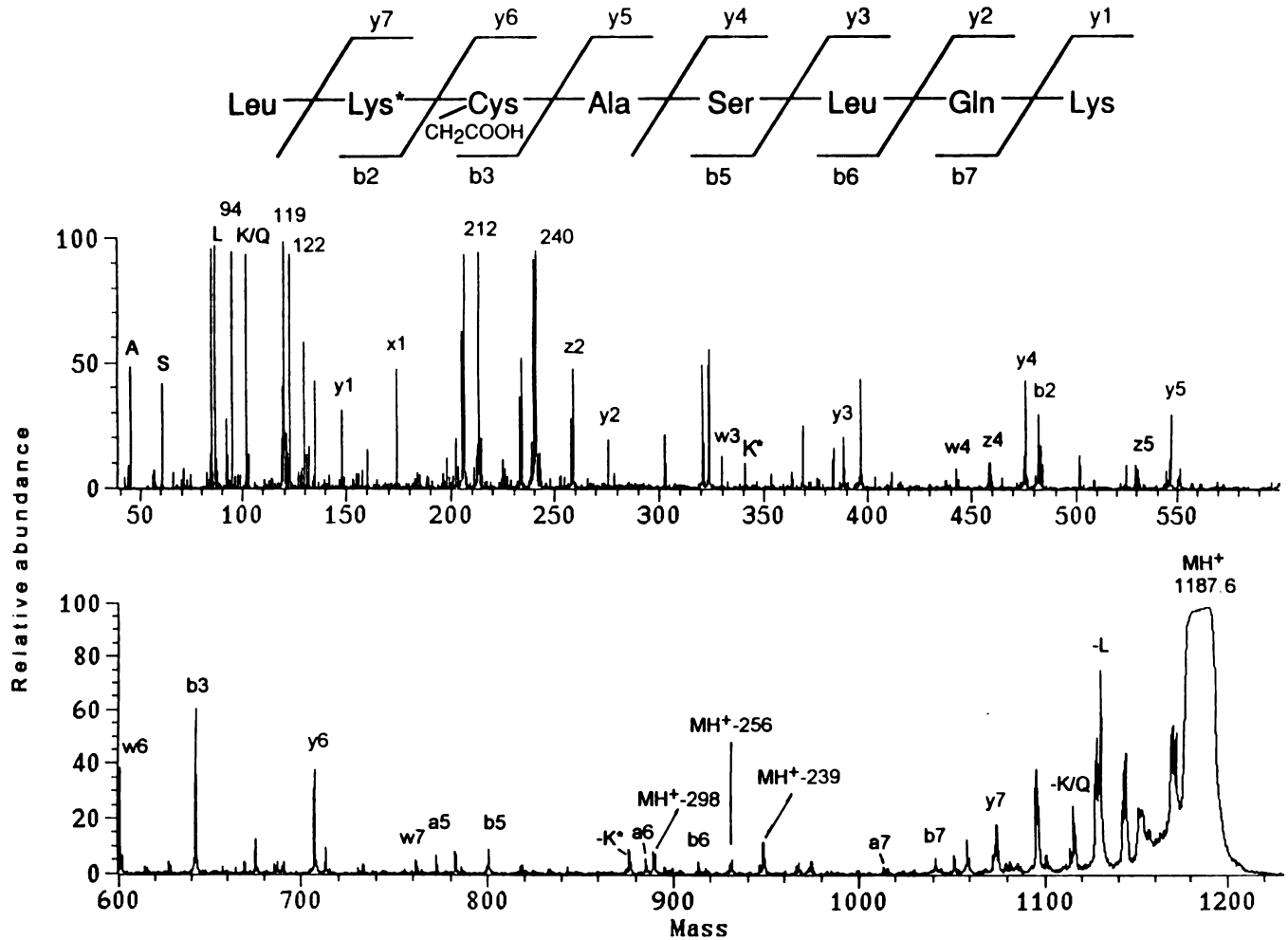

Figure 3.8. The CID mass spectrum for molecular ion MH^+ 1187.6 corresponding to HPLC peak 6. Significant framents indicating the sequence of the modified peptide are depicted above the spectrum. The fragments containing lysine 199 (Lys*) show a shift of 239 mass units which corresponds to the mass of tolmetin.

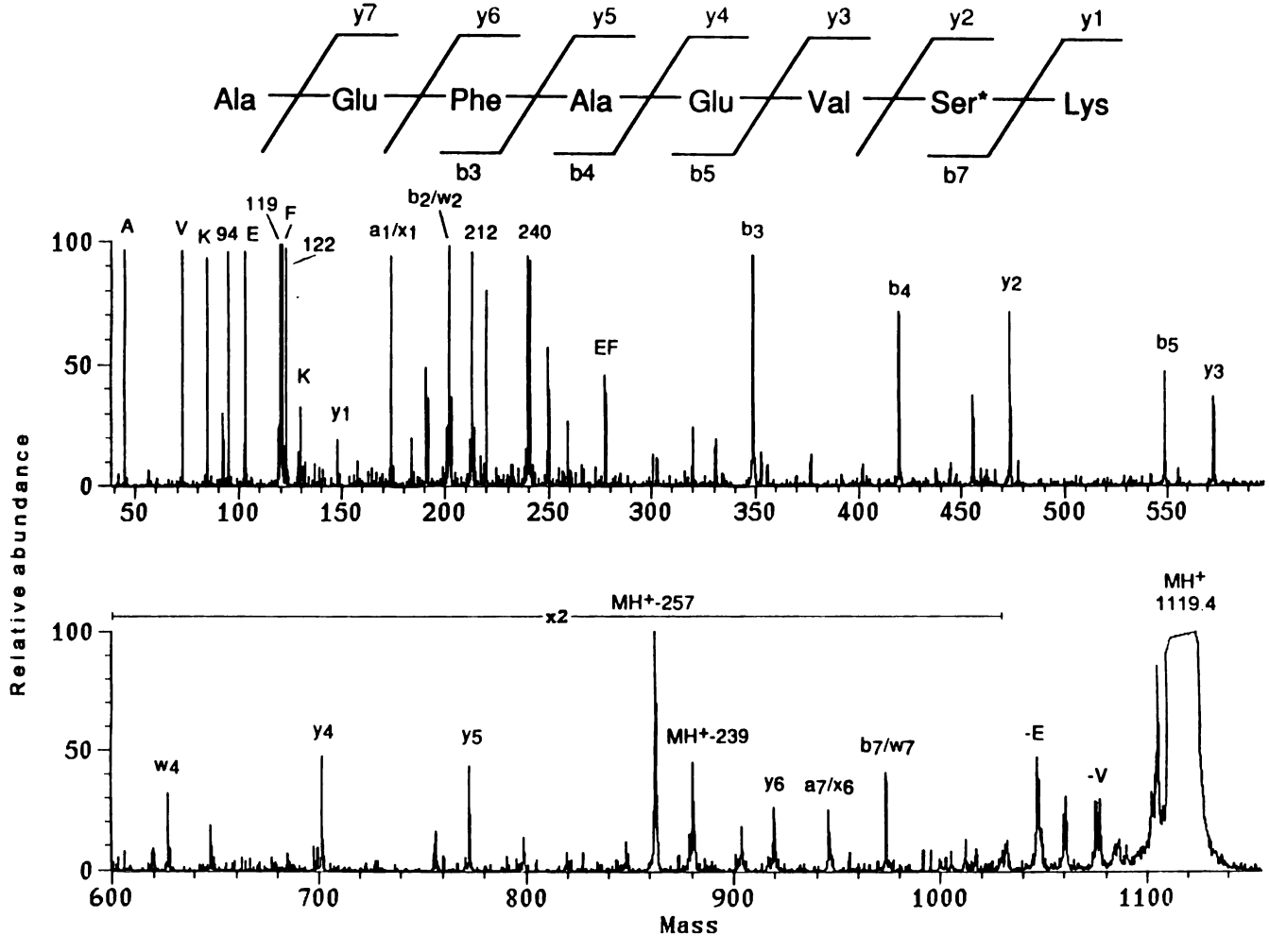

Figure 3.9. The CID mass spectrum for molecular ion MH^+ 1119.4 corresponding to HPLC peak 9. Significant fragments indicating the sequence of the modified peptide are depicted above the spectrum. The fragments containing serine 232 (Ser*) show a shift of 239 mass units which corresponds to the mass of tolmetin.

The fraction eluting at 63 min (peak 7, 6% of total absorbance) gave a few molecular ions in LSIMS. The CID spectrum of the most abundant molecular ion (MH^+ 1114.4) is shown in Fig. 3.10. The presence of a tolmetin group is again confirmed by several low molecular mass ions (94, 119, 122, 212, and 240) and some ions derived from the loss of the tolmetin group (MH^+ - 213 and MH^+ -239). A complete y-series and an almost complete a/b series confirmed the sequence to be Leu-Ser-Gln-Arg-Phe-Pro-Lys (amino acid residues 219-225 of HSA). Interestingly, the spectrum showed evidence for covalent binding of tolmetin (239 mu) to either serine 220 (MH^+ -257 = MH^+ -T -H2O; and -S*) or arginine 222 (MH^+ -281, MH^+ -298, MH^+ -312, MH^+ -326 and -R*). Differences in the sequence ion series for either isomer were expected only for the 4- and 5- positions for fragments with the charge retained on the C-terminal side, and for the 2- and 3-positions for fragments with the charge retained on the N-terminal side of the peptide. In fact, fragments of both isomers were found: x4 (573), y4 (547), z4 (530), x5 (701), y5(675) and z5 (658) do not contain the tolmetin group and can only be explained by binding to the serine 220. Consequently, the ions x6 (1027), y6 (1001) and z6 (985) contain additional 239 mu (Fig. 310). Additional fragments in the CID spectrum: y4' (786), z4' (770), y5' (914) and w5' (840) derive from modification of the arginine 222 (shifted by 239 mu). The ions y6' and z6' would not be different to the fragments derived from the other isomer.

The small HPLC fraction eluting at 57.5 min (peak 5, 2% of total absorbance) contained only one peptide (LSIMS: MH^+ = 912.4). The sample was analyzed by gas-phase Edman sequencing which detected Gln-Ile-Lys in cycles 3-5. Cycle one was more difficult to interpret, but the presence of Glu was most likely. This partial sequence matches the amino acid residues 520-524 of HSA (Glu-Arg-Gln-Ile-Lys; theoretical MH^+ = 673), which must contain

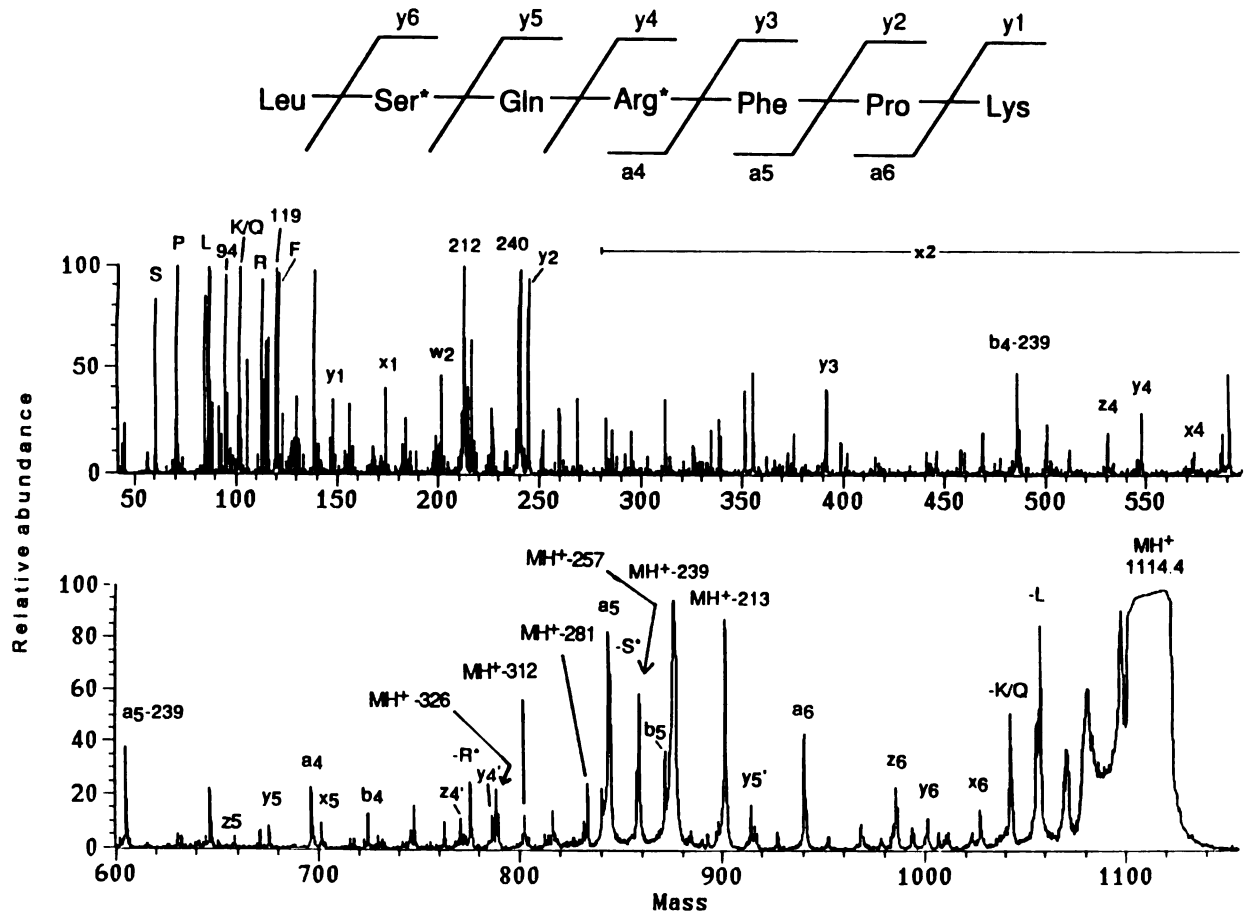

Figure 3.10. The CID mass spectrum for molecular ion MH^+ 1114.4 corresponding to HPLC peak 7. Significant fragments indicating the sequence of the modified peptide are depicted above the spectrum. The fragments containing serine 220 or arginine 222 show a shift of 239 mass units corresponding to the mass of tolmetin. Ions x4, y4, z4, x5, y5, and z5 do not contain the tolmetin group and can only be explained by binding of tolmetin to serine 220. On the other hand the primed ions y4', z4', y5', and w5' can only be explained by binding to arginine 222. Therefore, two isomers must be present in this fraction: one with tolmetin bound to serine 220 and the other with tolmetin bound to arginine 222.

a tolmetin group (239 mu). These data suggest a covalent binding to arginine 521, in analogy to the data discussed earlier.

3.4 DISCUSSION

This work confirms that *in vitro* covalent modification of HSA by TG occurs via two different mechanisms (Schiff base formation and nucleophilic displacement) giving rise to production of two different products: one in which tolmetin glucuronide is bound to HSA and another in which tolmetin is directly bound to HSA (Fig. 3.1). The product containing both tolmetin and glucuronic acid has a component, namely the sugar moiety, which would be a common structural element in HSA adducts generated by reaction of other glucuronides with HSA. This common structural moiety, in addition to the similarities in chemical structures of the drugs, could have a role in causing the cross-reactivity of the antibodies raised against one protein-NSAID adduct toward other protein-NSAID adducts.

The results from the incubations with three different amounts of TG indicate that the product of the covalent binding reaction is influenced by changes in the concentration of TG. The ratio of covalent binding of TG to covalent binding of T is higher for incubation 1 (TG/T=2; 0.3 mM TG) than that for incubation 2 (TG/T=1.8; 1.5 mM TG) and this is in turn higher than the ratio for incubation 3 (TG/T=1.3; 7.5 mM TG) (Table 3.2). Thus at lower concentrations of TG, covalent binding via TG (Schiff base formation mechanism) is more prevalent than binding via T (nucleophilic displacement mechanism). The overall binding yield for incubation 1 (1.2%) is closer to the average binding yield (0.6%) observed for *in vivo* covalent binding of tolmetin to plasma proteins after administration of multiple doses

to human volunteers (Zia-Amirhosseini *et al.*, 1994). Thus the Schiff base mechanism may also be the predominant pathway for the covalent binding reaction *in vivo*.

Glycosylation of a number of proteins has been shown to occur via the Schiff base mechanism. The two steps of the reaction, the imine formation and the Amadori rearrangement, have been well characterized for glycosylation of hemoglobin (Higgins and Bunn, 1981). *In vivo* and *in vitro* glycosylation of HSA (Bailey *et al.*, 1976; Day *et al.*, 1979; Schleicher and Wieland, 1981), collagen (Robins and Bailey, 1972), lens crystalline (Biemann, 1988), basic myelin protein of nerve (Flückiger and Winterhalter, 1978), and red cell membrane protein (Bailey *et al.*, 1976; Miller *et al.*, 1980) appear to occur via the Schiff base formation mechanism.

Several factors could affect the outcome of covalent binding of acyl glucuronides to protein via the Schiff base mechanism: 1) the accessibility of the reactive amino group, 2) the pKa value of the amino group, 3) the nature of the neighboring amino acids, 4) the ease of Amadori rearrangement.

It is obviously necessary that the reactive residue of the protein be accessible to the glucuronide. Some amino acid residues are not well exposed to the solvent due to the three-dimensional folding of the protein. For instance albumin contains 56 lysines; however, as presented in Table 3.2, only a few of these lysines react with tolmetin glucuronide. Similar results were observed for glycosylation of HSA (Iberg and Fluckiger, 1986).

It is also reasonable to hypothesize that the pKa of the reactive protein amino group would affect its reactivity. The amino group has to be uncharged in order to react with an aldehyde to form an imine. Thus the lower the pKa of the amine, the higher the probability of the reaction at pH 7.4. In support of this, Lys 199, one of the glycosylation sites of HSA and one

of the major sites identified in this study, is thought to have a relatively low pKa of 8.0-8.7 (Means and Bender, 1975; Gerig *et al.*, 1978).

It is also possible that some potentially reactive sites are bound (reversibly) or occupied by other ligands. For instance binding of fatty acids to HSA may hinder glycosylation. In fact the rate of non enzymatic glycosylation of HSA *in vitro* is enhanced when fatty acids are removed from the albumin prior to incubation with glucose (Mereish *et al.*, 1982). However, Munafo and colleagues (1990) have observed similar extents of covalent binding for the reaction of TG with HSA and fatty acid free HSA. In contrast, palmitate and oleate inhibited covalent binding of tolmetin glucuronide to fatty acid free HSA by 35.2% and 37.2% respectively, salicylate and acetylsalicylic acid inhibited this reaction by 22.0% and 10.4% respectively (Ojingwa, 1991).

Another possible determining factor is the nature of the neighboring amino acids. Favorable interactions of TG with the residues in the vicinity of the reactive residue, may lead to chemical modification at one site as opposed to others. It may be that neighboring residues, capable of hydrogen binding with the hydroxyl groups of the glucuronic acid or having hydrophobic interactions with the aromatic system of tolmetin, make the modification of one nucleophile of HSA more probable than another.

It has been suggested that Amadori rearrangement to the ketoamine product can be accelerated by acid catalysis (Isbell and Frush, 1958; Feeney *et al.*, 1975). In the case of hemoglobin, catalytic carboxyl groups may be provided by the side groups of acidic amino acid residues and by a propionic carboxyl moiety on the heme (Perutz *et al.*, 1968; Sack *et al.*, 1978).

Similar factors affect the outcome of the nucleophilic displacement mechanism. These are discussed in Chapter 4 ,where covalent modification of albumin by activated esters of tolmetin and zomepirac has been examined.

Covalent binding of proteins by acyl glucuronides has the potential of changing the three-dimensional conformation of the protein. As discussed in Chapter 1 (section 1.4) this is a significant component of our hypothesis because such changes could convert the protein into one that is recognized as nonself by the immune system. Indications of conformational changes in glycosylated albumin both *in vitro* and *in vivo* have been observed (Shaklai *et al.*, 1984). For example when fluorescence spectroscopy was used to characterize HSA and glycosylated HSA, approximately a 30% decrease in the quantum yield (integrated intensity) of the fluorescence spectra of glycosylated HSA relative to unmodified HSA was measured. Moreover, a decrease in the maximum emission wavelength of the glycosylated HSA was observed. Because fluorescence emission characteristics such as wavelength of maximal emission or quantum yields are sensitive to fluorophore environment within the three dimensional structure of the protein (Teale, 1960; Lehrer, 1976), it was concluded that a conformational change had occurred in the glycosylated HSA. HSA has a single tryptophan (214) and 18 tyrosines that have fluorescent activity. Since decreases in quantum yields and maximum emission wavelength were observed for both *in vitro* and *in vivo* glycosylated HSA, it is unlikely that the differences were caused by other factors such as aging or differences in ligand content. More recently, the fluorescence of the products generated via the reaction of BSA with glucose, fructose, glucuronic acid, zomepirac glucuronide (ZG), and suprofen glucuronide (SG) have been examined (Smith and Wang, 1992). The results

suggest that acyl glucuronides cause more extensive modifications in the protein than sugars.

As discussed earlier, covalent binding of tolmetin glucuronide with HSA can occur via the same mechanism as glycosylation (Schiff base mechanism). The addition of a sugar moiety along with a bulky drug group, is thus likely to cause conformational changes in HSA. The altered HSA could potentially act as an immunogen *in vivo*. In support of this, glycosylated lipoproteins and albumin have been shown to be immunogenic in guinea pigs (Witztum *et al.*, 1983). Glycosylation of lipoproteins has been recognized as one of the factors responsible for a pathogenetic interrelationship between diabetes and atherosclerosis. Glycosylation may contribute to the development of atherosclerosis via autoimmune mechanisms (Denisenko, 1990). Thus it is possible that the antibodies generated against the TG-protein conjugate are directed toward a conformational change in the protein. It is equally possible that the antibodies are directed toward tolmetin and/or tolmetin glucuronide. Thus the antigenic determinant may contain segments of the protein, tolmetin, or tolmetin glucuronide. This will be examined in Chapter 5.

4.0 SYNTHESIS AND CHARACTERIZATION OF HUMAN SERUM ALBUMINS MODIFIED BY NSAIDs VIA THE NUCLEOPHILIC DISPLACEMENT MECHANISM

4.1 INTRODUCTION

Human serum albumin (HSA) is the most abundant plasma protein. It consists of 585 amino acids and has a molecular weight of 66,500 Da. HSA contains many acidic and basic residues: 36 aspartic acids, 61 glutamic acids, 23 arginines, and 59 lysines. This results in an electrostatic charge of approximately -17 at physiological pH, which is greater than most of the plasma proteins. HSA contains 35 cysteine residues; 34 of these form disulfide bridges and the other (cysteine 34) is either free or conjugated to other thiol containing compounds such as glutathione. The high charge of albumin aids in its solubility in aqueous media, and the disulfide bridges help maintain the stability and spatial conformation of the protein (Kragh-Hansen, 1990).

The three dimensional structure of HSA (determined by X-ray diffraction studies) contains three structurally homologous domains - domains I, II, and III. Each domain consists of two subdomains: subdomain A and subdomain B. Approximately 67% of HSA is helical and the remainder consists of turns and extended polypeptides. There are 10 principal helices in each of the three domains of HSA (He and Carter, 1992).

As shown in Chapter 3, the covalent binding of TG to HSA may occur via two mechanisms: 1) nucleophilic displacement, and/or 2) Schiff base formation. The final products of these two mechanisms are T-HSA and TG-HSA respectively. As discussed in section 3.4, several factors may influence

the reactivity of various sites on the protein and affect the outcome of the covalent binding.

Here, an activated ester of tolmetin (T) was reacted with HSA to produce a T-HSA conjugate. The modified HSAs were examined by HPLC and high performance tandem mass spectrometry. In addition, a similar reaction with HSA was performed with an activated ester of zomepirac (Z). These experiments were performed to specifically examine the covalent modification of HSA by a nucleophilic mechanism.

4.2 EXPERIMENTAL SECTION

4.2.1 Materials

Trifluoroacetic acid was purchased from Pierce (Rockford, IL). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

4.2.2 Synthesis of Modified HSA

Equimolar amounts (10 mM) of drug and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) were reacted at room temperature for 30 minutes in water with the addition of dilute HCl to maintain a pH of 6.0-6.5; HSA (0.23 mM) was then added and the reaction continued for one hour (Fig. 4.1) (Raso and Stollar, 1975). The solution was concentrated and the modified protein washed with water by seven ultrafiltrations using Amicon centriprep tubes (30 KD cutoff, Amicon Division, Beverly, MA). The concentrated protein solution was then lyophilized to give crude product. The generation of the activated ester and its reaction with protein is shown in Fig. 4.1.

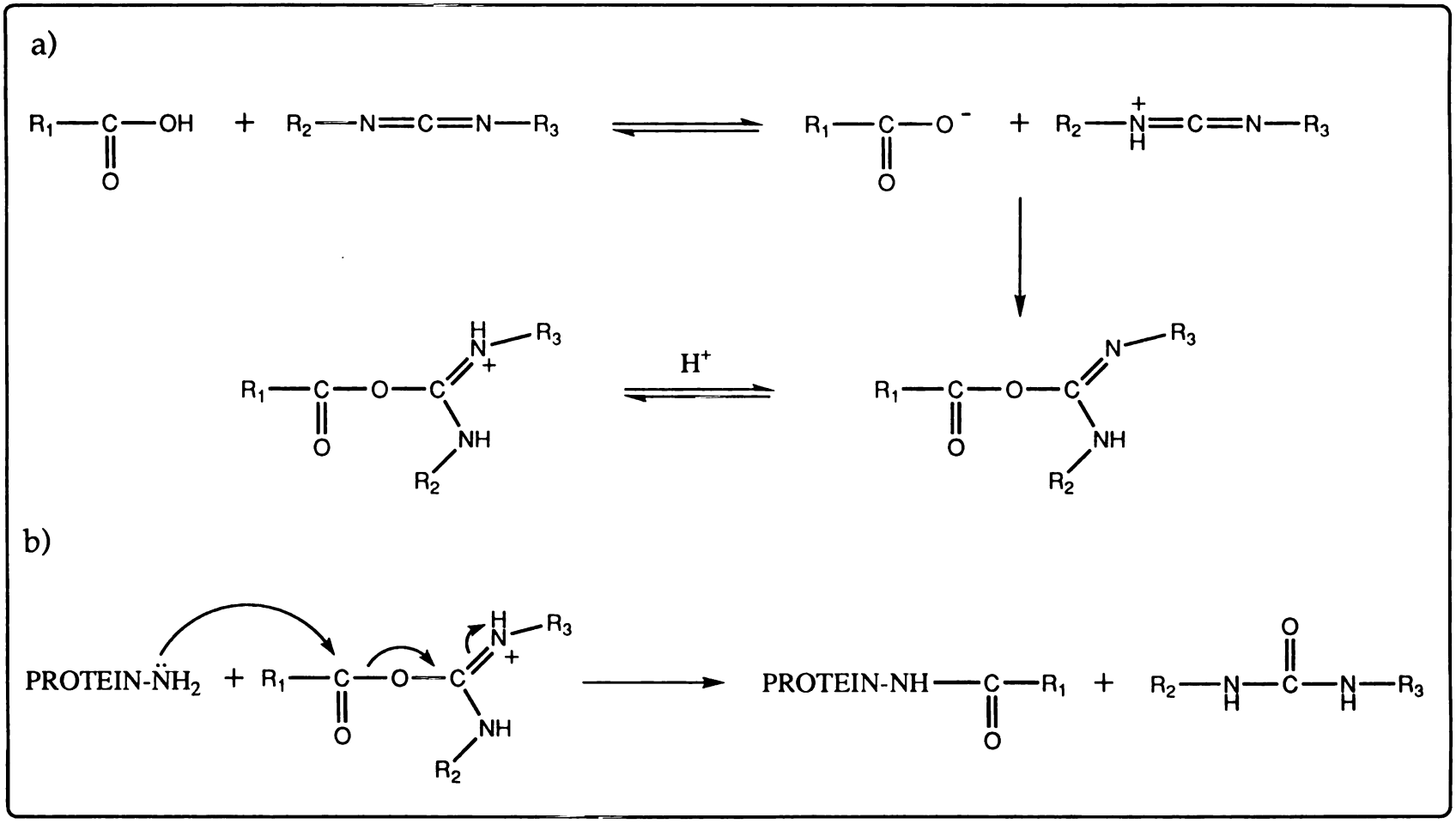

Figure 4.1 The scheme for a) generation of an activated ester of an organic acid, b) reaction of a nucleophile of a protein with the activated ester.

4.2.3 Tryptic Digestion of the Protein

The crude protein (1.5 mg) was dissolved in denaturing buffer (100µl; 100 mM Tris, 1 mM EDTA, 6 M guanidine hydrochloride, pH 8.3) and heated with 3 M dithiothreitol (2µl) under argon for one hour at 55°C to reduce disulfide bonds. The free sulfhydryl groups were alkylated by incubation with iodoacetic acid (4 µl, 0.5 mg/µl solution) at room temperature for 30 minutes. This solution was dialyzed overnight against 50 mM ammonium bicarbonate buffer (pH 8.0) and digested with 2% (wt/wt) trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone treated, Type XIII) at 37°C for 4.5 hours. The peptide digest was acidified with HCl (6 N) to pH 3.0 and stored at -80°C.

4.2.4 Isolation of the Modified Peptides by HPLC

Peptides were separated on a reversed phase Vydac protein and peptide C18 column (250 x 4.6 mm). A linear gradient from 99% solvent A [0.1% (vol/vol) trifluoroacetic acid in water] to 45% solvent B [0.08% trifluoroacetic acid in acetonitrile] over 90 minutes at a flow rate of 1 ml/min was used. The eluent was monitored simultaneously at two wavelengths — 215 nm for peptides and 313 nm for modified peptides. Peptides containing tolmetin or zomepirac (absorbance at 313 nm) were collected. The modified peptides were further purified on a Vydac phenyl column (250 x 4.6 mm) using a linear gradient from 5% to 30% solvent B over 50 minutes.

4.2.5 Mass Spectrometric Analysis of the Modified Peptides

Aliquots (10 to 20 %) of the modified peptide fractions were analyzed by positive ion LSIMS. The masses of MH^+ ions were compared with the molecular masses of peptides predicted from the known sequence of carboxymethylated HSA after digestion with trypsin, but modified by addition

of either 239 (tolmetin) or 273 (zomepirac) mass units. To confirm the amino acid sequence of the peptides and to determine the site of modification, MS/MS experiments were carried out using a Kratos Concept IIHH four-sector tandem mass spectrometer with $E_1B_1E_2B_2$ configuration (Walls *et al.*, 1990) equipped with a cesium ion source (Aberth *et al.*, 1982; Falick *et al.*, 1986) and an electrooptical multichannel array detector (Cottrell and Evans, 1987). The ^{12}C component of the protonated peptide molecules was subjected to collision induced dissociation mass spectrometry.

4.3 RESULTS

Three major peaks (T-1, T-5, T-7) were observed in the HPLC chromatogram of the T-HSA digest (Fig. 4.2b). Peaks T-1 and T-5 represent two of the peptides covalently modified by tolmetin. Peak T-7 was identified as free tolmetin and was the only tolmetin containing peak detected in control incubations containing unactivated tolmetin. Several minor peaks were also observed in the chromatogram of the digest. Binding sites for four of these peptides (T-2, T-3, T-4, T-6) were identified.

The peptide that eluted at 48 min (T-1) gave an abundant protonated molecular ion signal at 986.4 (LSIMS,^{12}C mass scale). This mass corresponds to a protonated peptide containing amino acids 191-197 of HSA {Ala-Ser-Ser-Ala-Lys-Gln-Arg; MH^+ at m/z 747.4} plus a tolmetin moiety (239 mass units). Interpretation of the high energy CID spectrum (Fig. 4.3) confirmed the peptide sequence and revealed the site of covalent modification by the shift in the mass of several sequence ions (x_3, z_3,y_4 , z_4, y_5, z_5, x_6, y_6, z_6) by 239 Da. In addition, the most abundant nonsequence ions (m/z 94, 119, 212, 240, and MH^+ - 213) establish the presence of tolmetin on the modified peptide. The

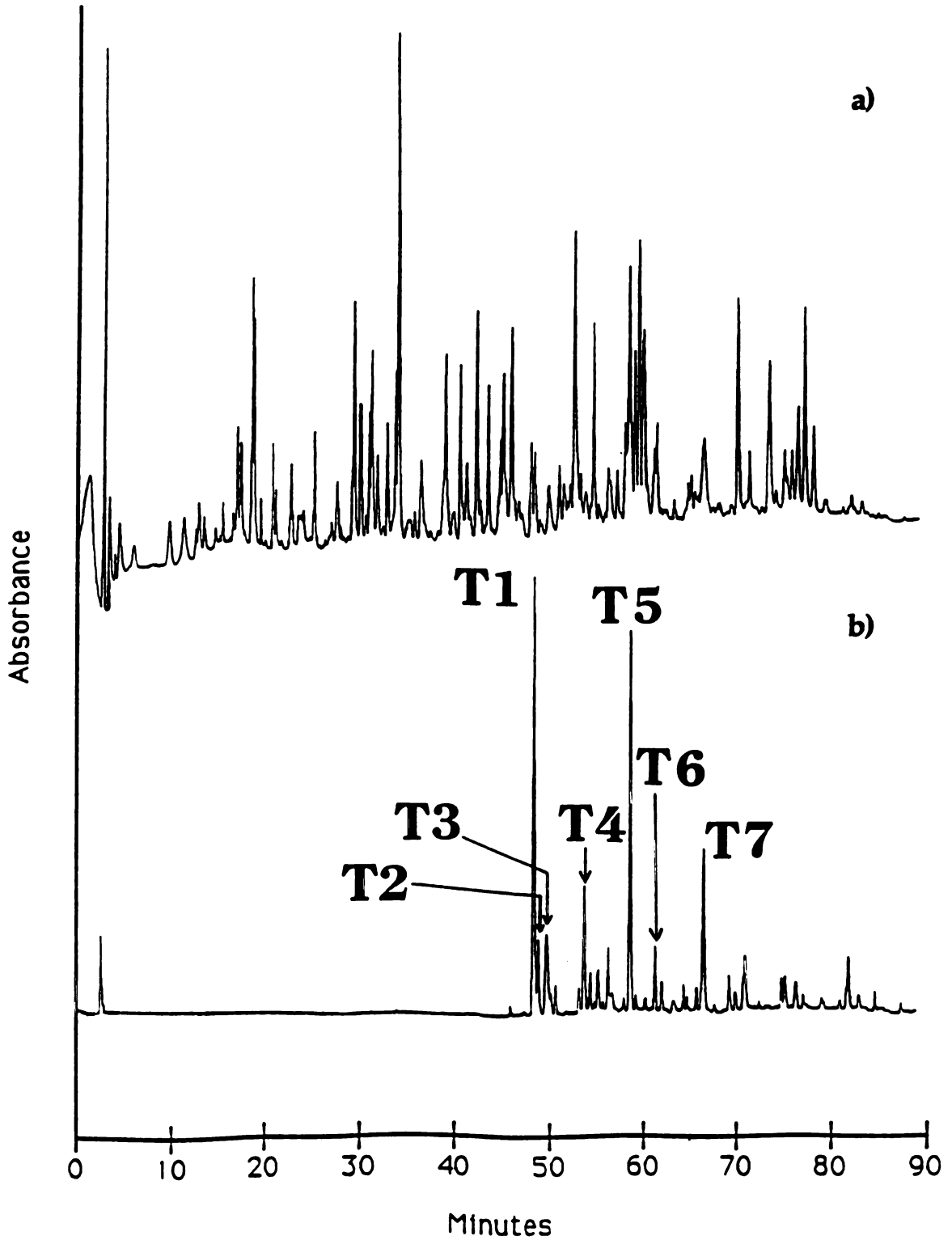

Figure 4.2 HPLC chromatograms of the digested T-HSA adduct: a) 215 nm, the peptide trace, b) 313 nm, the peptides modified by a tolmetin group. Fractions T-1, T-2, T-3, T-4, T-5, and T-6 were subjected to mass spectrometry. Fraction T-7 contained free tolmetin.

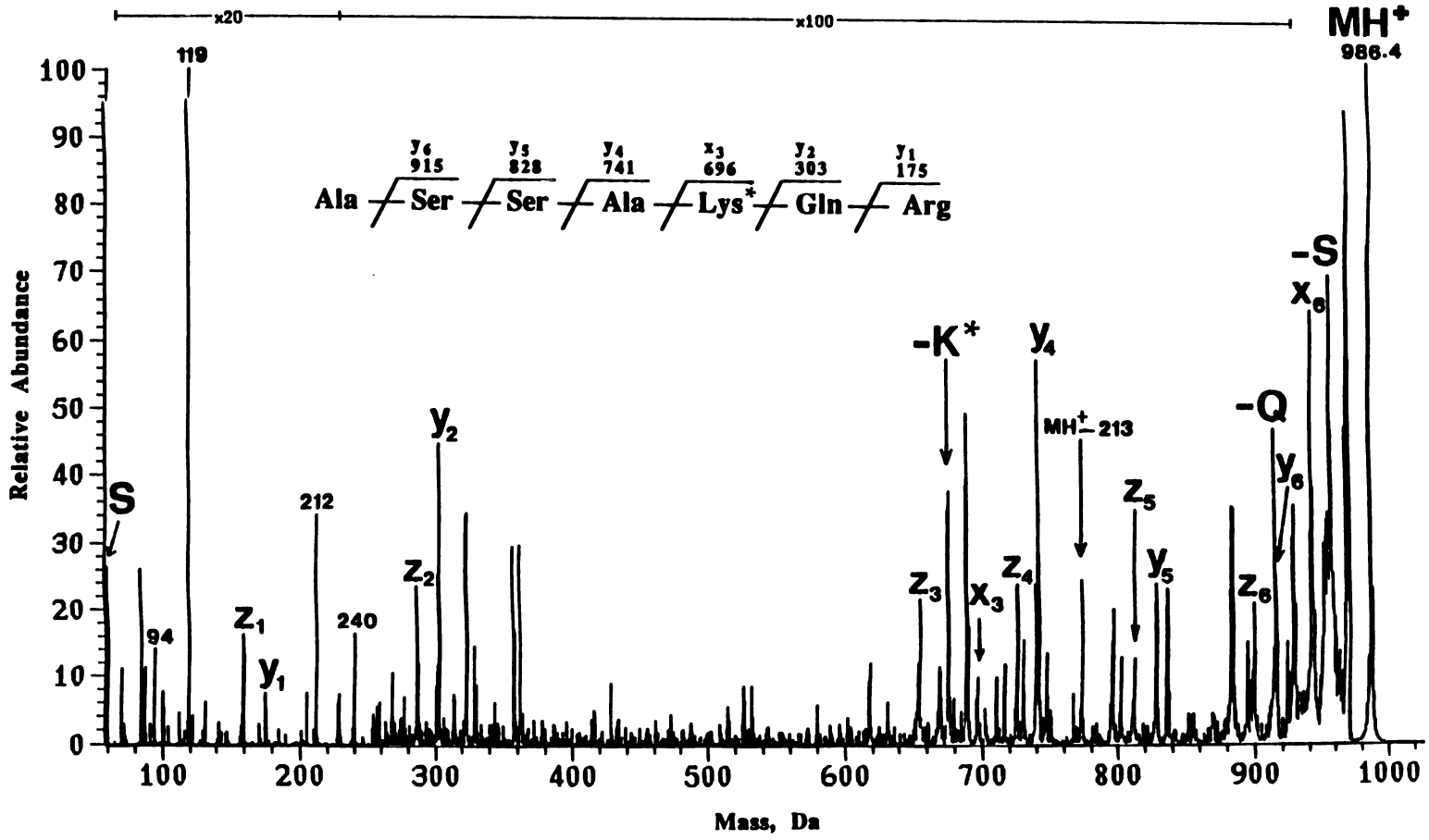

Figure 4.3. The CID mass spectrum of the ^{12}C isotope of molecular ion 986.4 corresponding to HPLC peak T-1. The site of modification is identified by the observed shift in the sequence ions (i.e., $x_3,z_3,y_4,z_4,y_5,z_5,x_6,y_6,z_6$) containing the modified amino acid. The mass for the molecular ion matches with the peptide Ala-Ser-Ser-Ala-Lys*-Gln-Arg (HSA residues 191-197) plus 239 Da. Lysine* 195 is the modified amino acid.

signal from the loss of the modified lysine at (MH^+ - 311) designated as -K^* (Figs. 4.3 & 4.4) proves that tolmetin is directly bound to the ε-amino group of lysine. Similarly, we identified the tryptic digest peaks T-2, which eluted at 48.9 min (MH^+ at 709.4), T-3 which eluted at 49.5 min (MH^+ at 748.4), T-4 which eluted at 54 min (MH^+ at 755.4), T-5 which eluted at 58.5 min (MH^+ at 1187.6), and T-6 which eluted at 61.5 min (MH^+ at 1538.5) (Fig. 4.2b, Table 4.1). These data and the signals correspond to peptides containing the following modified amino acids: Lys 195, Asp 1, Lys 536, Lys 524, Lys 199, Lys 351.

Three binding sites in Z-HSA were identified. These were similar to those observed for the T-HSA product, namely lysines 195, 199, and 351. The HPLC chromatogram of the tryptic digest is shown in Fig. 4.5. The CID chromatogram (Fig. 4.6) of the peak labeled Z-2 in Fig. 4.5 shows an MH^+ at 1221.6 which corresponds to the molecular ion of the peptide containing lysine 199. The non-sequence ions (m/z 108, 136, 139, 246, 274) indicate the presence of zomepirac attached to the peptide (Figs. 4.6 & 4.7). The shift in the masses of several sequence ions (b_2, b_3, y_6, y_7, x_7) by 273 indicates that zomepirac is directly bound to lysine 199 (Fig. 4.6). The molecular ions corresponding to the other two identified modified peptides are MH^+ at 1020.4 (Z-1, Fig. 4.5b) and 1569.8 (Z-3, Fig. 4.5b) (Table 4.1). HPLC peak Z-4 is due to the absorbance of unbound zomepirac (Fig. 4.5).

The molar binding ratios were 0.6 and 0.5 mole of drug per mole of HSA for T-HSA and Z-HSA respectively. The identified binding sites represent 68% and 37% of the total bound drug for T-HSA and Z-HSA respectively (Table 4.1), assuming no loss of bound drug during protein digestion.

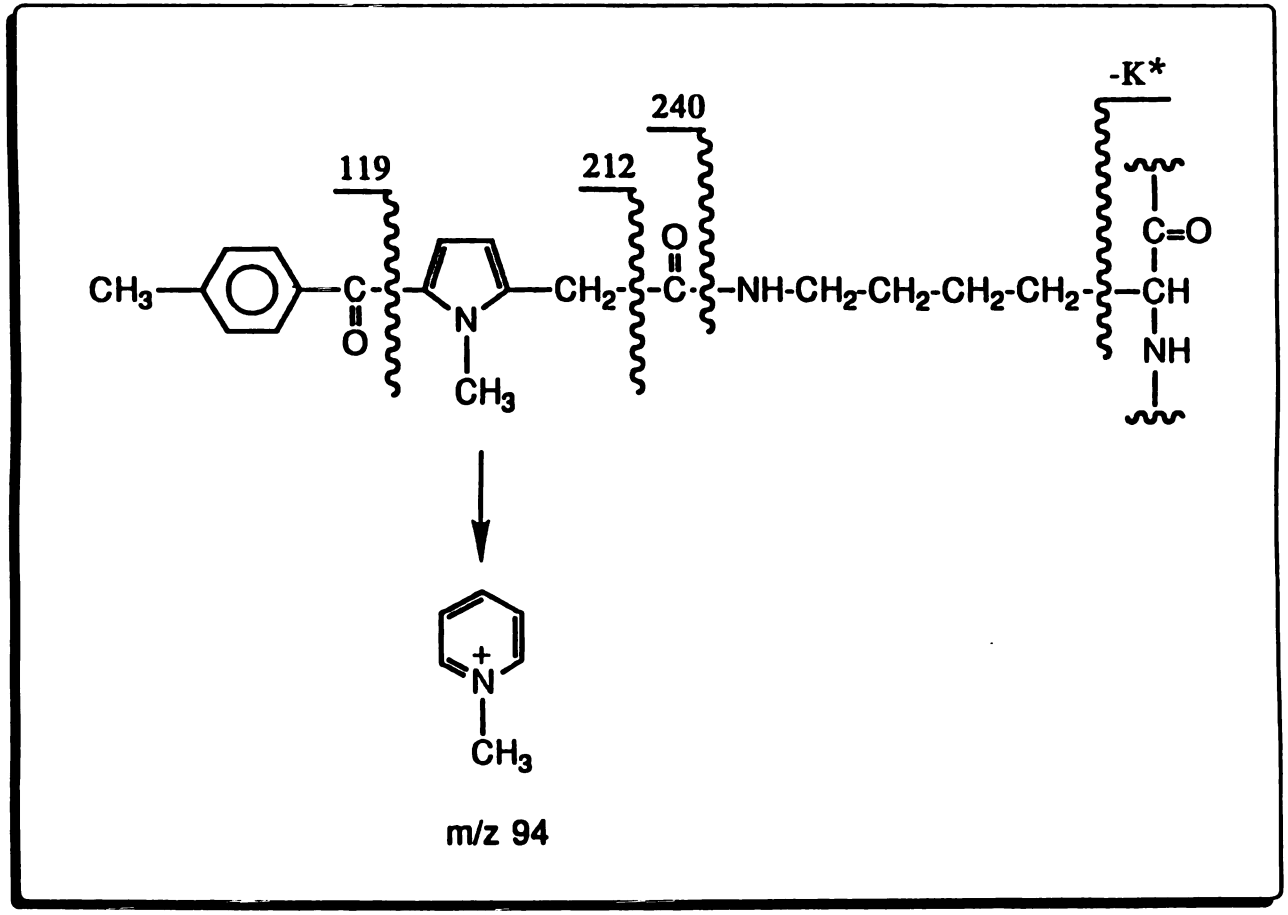

Figure 4.4. Fragment ions consistent with tolmetin bound to lysine.

Table 4.1. Drug-containing fractions (monitored at 313 nm) with assigned binding sites.

HPLC Peak No.	HPLC Retention Time (min)	%Bound[a]	MH^+	Sequence[b]	HSA residues	Binding Site
T-1	48	25	986.4	ASSAK*QR	191-197	Lys-195
T-2	48.9	4	709.4	D*AHK	1-4	Asp-1
T-3	49.5	7	748.4	HK*PK	535-538	Lys-536
T-4	54	7	755.4	QIK*K	522-525	Lys-524
T-5	58.5	22	1187.6	LK*C(Cm)ASLQK	198-205	Lys-199
T-6	61.5	3	1535.8	LAK*TYETTLEK	349-359	Lys-351
Z-1	54.5	14	1020.4	ASSAK‡QR	191-197	Lys-195
Z-2	63.5	19	1221.6	LK‡C(Cm)ASLQK	198-205	Lys-199
Z-3	65.7	4	1569.8	LAK‡TYETTLEK	349-359	Lys-351

[a] Relative binding, calculated as % of total absorbance at 313 nm minus absorbance of free drug (T-7, Z-4).
[b] Cm represents carboxymethylation of cysteine (C).

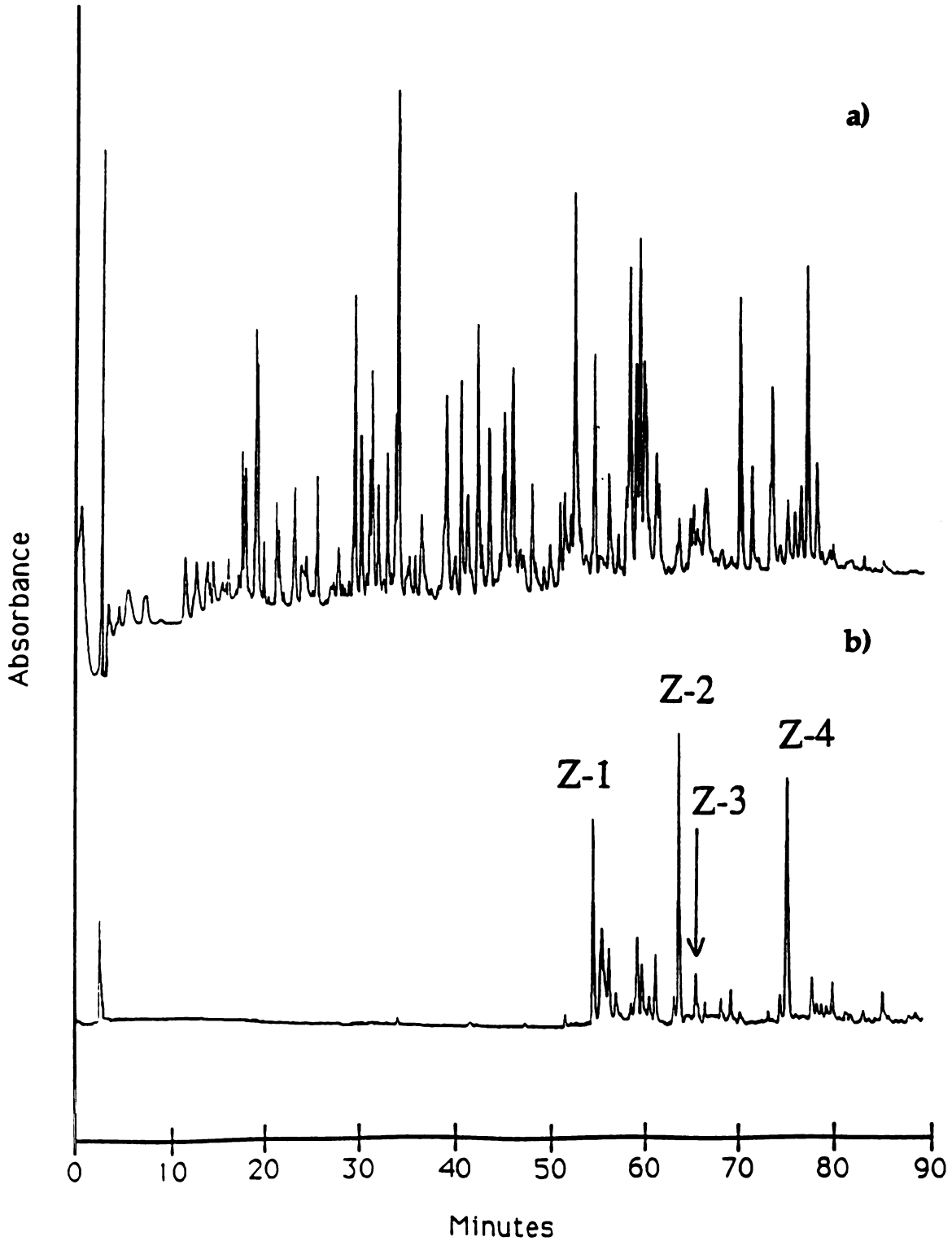

Figure 4.5. HPLC chromatograms of the digested Z-HSA adduct: a) 215 nm, the peptide trace, b) 313 nm, the peptides that contain zomepirac. Fractions Z-1, Z-2, and Z-3 were subjected to mass spectrometry. Fraction Z-4 contained free zomepirac.

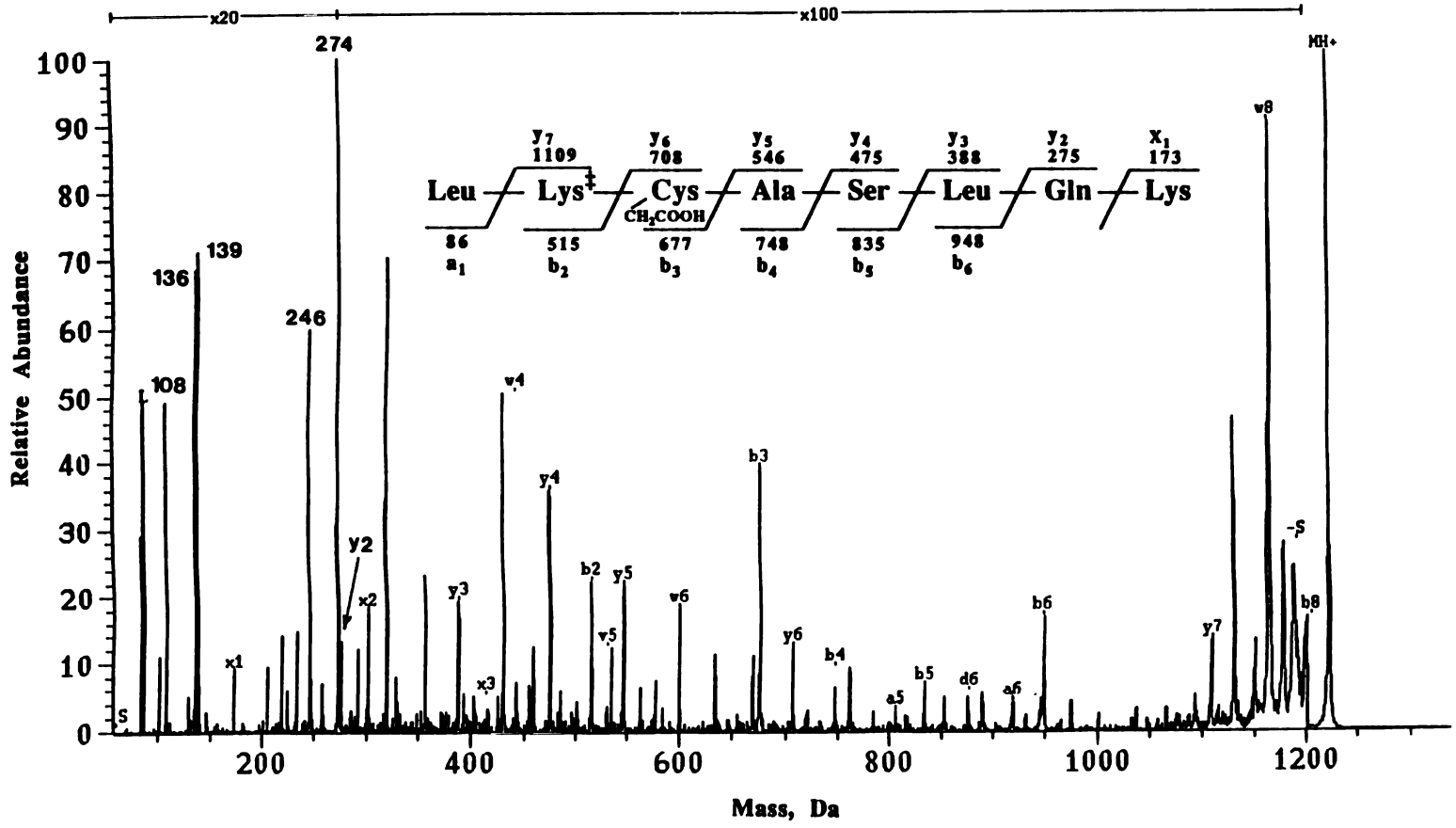

Figure 4.6. The CID mass spectrum of the $^{12}C^{35}Cl$ isotope (m/z 1221.6) of the molecular ion stable isotope cluster corresponding to HPLC peak Z-2. The site of modification is identified by the observed shift in the sequence ions (i.e., b_2, b_3, y_6, y_7, x_7) containing the modified amino acid. The mass for the molecular ion matches with the peptide Leu-Lys‡-Cys-Ala-Ser-Leu-Gln-Lys (HSA residues 198-205) plus 273 Da. Lysine‡ 199 is the modified amino acid.

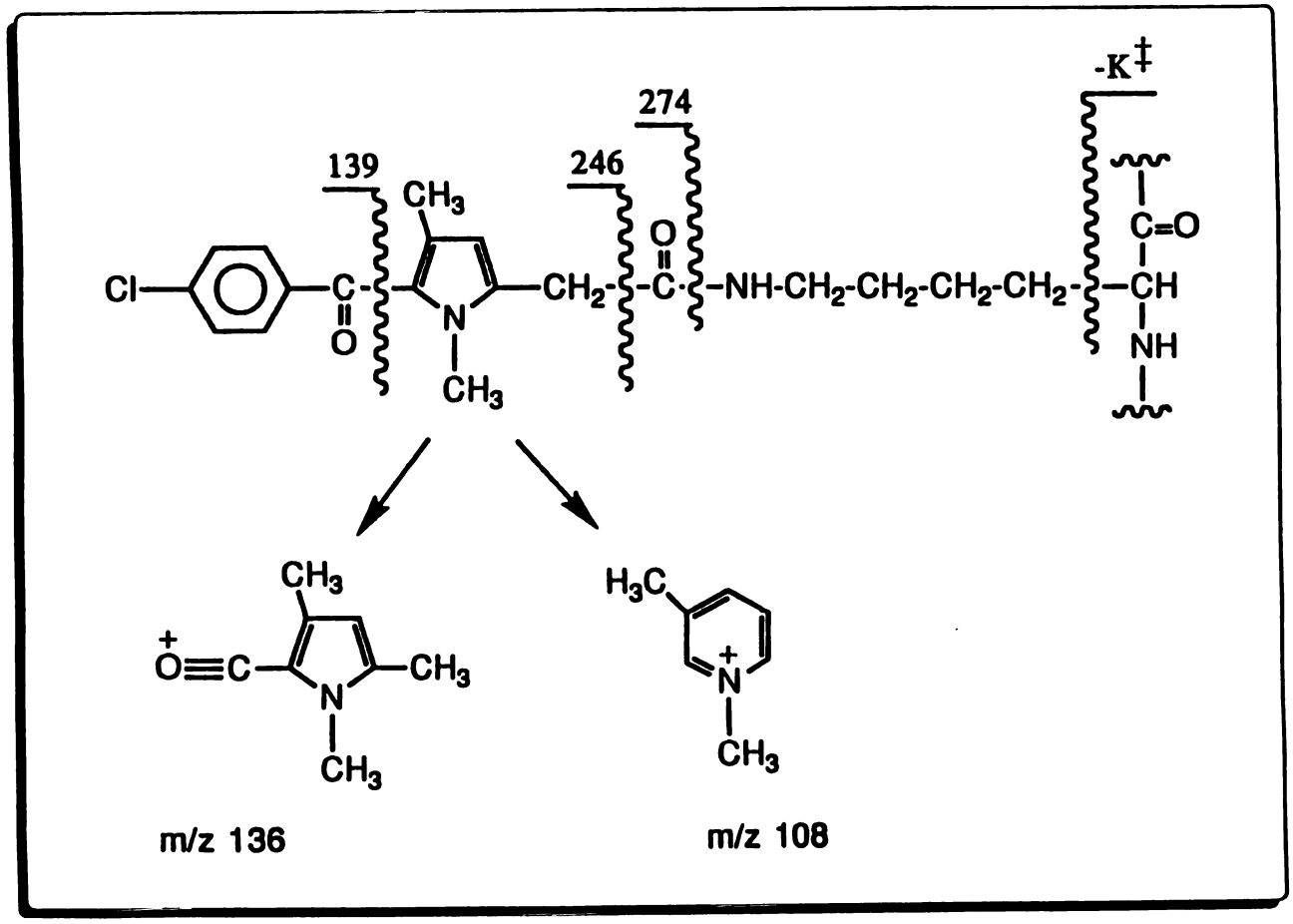

Figure 4.7. Fragment ions consistent with zomepirac bound to lysine.

4.4 DISCUSSION

Reaction of an activated ester of a carboxylic acid containing drug with a protein leads to direct binding of drug to nucleophiles of various amino acids. Here binding was observed to lysines 199, 195, 524, 536, and 351, and aspartic acid 1. Binding to other nucleophile containing amino acids (e.g. arginines, cysteines, serines, and tyrosines) was not observed in the examined HPLC fractions. Since only the major HPLC peaks (in terms of relative absorbance) were examined, binding of tolmetin or zomepirac to these other amino acids can not be ruled out. In fact when tolmetin glucuronide was reacted with HSA, direct binding of tolmetin at arginines 222 and 521, and serines 220, 232, and 480 was observed (Table 3.2).

The predominant reaction sites on HSA for activated esters of T and Z were the ε-amino groups of lysines 195 and 199. Reaction with hydroxyl or thiol groups was not detected but can not be ruled out because only the most abundant HPLC fractions containing T or Z were analyzed. However, lysines 195 and 199 together accounted for 47% and 33% of the covalently bound T and Z respectively (Table 4.1). These lysines are both located in the hydrophobic pocket of subdomain IIA of HSA. Lysine 199 is a favored site for interaction of small molecules with HSA and is the site for covalent binding of aspirin, glucose, and benzylpenicilloyl groups (Walker, 1976; Iberg and Fluckiger, 1986; Yvon and Wal, 1988). Interestingly, lysines 195 and 199 account for 50-60% of the reaction of tolmetin glucuronide with HSA in the presence of $NaCNBH_3$, even though this occurs via the Schiff base mechanism (Ding *et al.*, 1993). In the absence of a reducing agent, 20-30% of the total binding occurs at lysines 195 and 199 as TG modification of the

protein (Schiff base formation), while 5-7% of total binding occurs via direct T binding (nucleophilic displacement) (Table 3.2).

Curiously we observed binding of tolmetin to Lys 524 (7% of overall binding) yet not to Lys 525. Both of these residues are located in subdomain IIIB of HSA. Lys 525 is a major site for non-enzymatic glycosylation of albumin (33% of overall binding) (Iberg and Fluckiger, 1986) and a major site (14-20% of overall binding) for reaction with tolmetin glucuronide in the presence of the reducing agent $NaCNBH_3$ (Ding *et al.*, 1993). However, Lys 525 is a minor binding site (3% of total binding) for tolmetin glucuronide in the absence of a reducing agent (Table 3.2). Both glycosylation and drug-glucuronide binding occur via formation of a Schiff base. The preferential reaction of tolmetin activated esters with Lys 524 instead of Lys 525 may reflect differences in the molecular dimensions of the tolmetin activated ester versus tolmetin glucuronide.

Of the observed minor reaction sites, Lys 351, in subdomain IIB, is also a reactive site for glucose (Iberg and Fluckiger, 1986) and tolmetin glucuronide (Ding *et al.*, 1993). In the presence of a reducing agent, 4% of total binding occurs at Lys 351 via binding of a TG unit (Schiff base formation) (Table 3.3). However, Lys 536, which is in subdomain IIIB, the domain for reversible binding of many drugs (aspirin, clofibrate, ibuprofen, and warfarin) (He and Carter, 1992), has not previously been reported as a reactive site for covalent binding of drugs to HSA.

This study shows that reaction of carbodiimide-activated esters of the structurally similar drugs tolmetin and zomepirac with HSA is regioselective but, by no means, regiospecific. It also shows that alkyl amides of albumin survive denaturation, dithiothreitol reduction, iodoacetic acid alkylation and tryptic digestion.

5.0 PRODUCTION OF ANTIBODIES AGAINST PROTEIN CONJUGATES OF TOLMETIN GLUCURONIDE AND REACTIVITY OF THESE ANTIBODIES AGAINST SEVERAL DRUGS AND THEIR GLUCURONIDES

5.1 INTRODUCTION

Hypersensitivity reactions to drugs are categorized into four types: 1) immediate (Type I), 2) cytotoxic (Type II), 3) immune complex mediated (Type III), and 4) cell mediated (Type IV) (Table 5.1). They have a diverse range of immunologic pathologies and symptoms (Abbas *et al.*, 1991; Sullivan, 1993).

As discussed in Chapter 1, hypersensitivity reactions to non-steroidal anti-inflammatory drugs have been observed. According to the records of the Comprehensive Hospital Drug Monitoring (CHDM) in Berne and St. Gallen, between 1974 and 1989, of 19,082 patients who were treated with an NSAID, 95 had an allergic or pseudoallergic response to one or two of the drugs (Oberholzer *et al.*, 1993). Also 8.1% of 200 rheumatic patients experienced an allergic response after the intake of NSAIDs, primarily pyrazolone compounds (Anggelutsa *et al.*, 1989). Currently the package insert for tolmetin lists the frequency of allergic reactions to this drug to be less than 1% of the exposed population. However, the Food and Drug Administration has received more frequent reports of anaphylactoid (Type I) reactions to tolmetin than to ibuprofen or naproxen (Rinsler *et al.*, 1994). As mentioned in Chapter 1, zomepirac was withdrawn from the market due to causing severe allergic like reactions, some of which lead to death (U.S. Congress *et al.*, 1982).

The majority of the hypersensitivity reactions to zomepirac and tolmetin have occurred within 15-20 minutes after the intake of drug, and

Table 5.1 Immunopathological classification of hypersensitivity reactions. (Adapted from *Abbas et al.* 1991 and *Sullivan* 1993)

Type of Hypersensitivity	Pathologic Immune Mechanisms	Mechanisms of Tissue Injury and Disease	Clinical Manifestations
Type I: Immediate	IgE antibody	Mast cells and their mediators (vasoactive amines, arachidonic acid metabolites, cytokines)	anaphylaxis, urticaria, angioedema, some exanthems
Type II: Cytotoxic (antibody mediated)	IgM, IgG antibodies against tissue or cell surface antigen	1. Complement activation 2. Recruitment and activation of leukocytes (neutrophils, macrophages) 3. Abnormalities in receptor functions	cytopenias, some vasculitis, some organ inflammation
Type III: Immune complex mediated	Immune complexes of circulating antigens and IgM or IgG antibodies	1. Complement activation 2. Recruitment and activation of leukocytes	serum sickness, some vasculitis
Type IV: Cell mediated	1. $CD4^+$ T cells (delayed type hypersensitivity) 2. $CD8^+$ Cytotoxic T lymphocytes (T cell mediated cytolysis)	1. Activated macrophages, cytokines 2. Direct target cell lysis	contact sensitivity, some exanthems, some organ inflammation

have had clinical manifestations similar to those observed for Type I (immediate hypersensitivity) reactions. Immediate hypersensitivity (allergic) reactions are mediated by IgE antibodies and mast cells. Events that are thought to lead to this type of reaction are depicted in Fig. 5.1. Binding of antigen (immunogen) to antigen specific antibodies on B cells (or other antigen presenting cells) results in the presentation of the antigen to helper T cells. This process starts a cascade of events that leads to secretion of IgE antibodies. These antibodies sensitize mast cells by binding to their IgE receptors. The cross-linking of at least two IgE antibodies on the surface of mast cells triggers the release of mediators of allergic reactions (histamine, leukotrienes C_4, D_4, and E_4, and cytokines). This type of immunologic reaction is characterized by rapid vascular leakage of plasma, vasodilation, and bronchoconstriction, followed by inflammation. In the most severe cases (anaphylaxis), the patient may die due to asphyxiation and circulatory collapse. Patients who are prone to allergic reactions generally have more IgE, more mast cells, and more IgE receptors per mast cell than the non-allergic individuals. Synthesis of IgE is regulated by heredity, exposure to antigen, and T cell products (Abbas *et al.*, 1991).

Can a small molecular weight drug act as an immunogen? It is generally believed that even though a small molecule may be capable of binding to surface antibodies on antigen presenting cells, these molecules can not be presented to T cells. In order for this process to occur, the small molecule has to be covalently bound to a macromolecule (i.e. protein or a polypeptide) (Park *et al.*, 1987; Sullivan, 1993). The smaller size molecule is generally called a hapten and the protein is called a carrier. This process of drug binding to a carrier is referred to as haptenation.

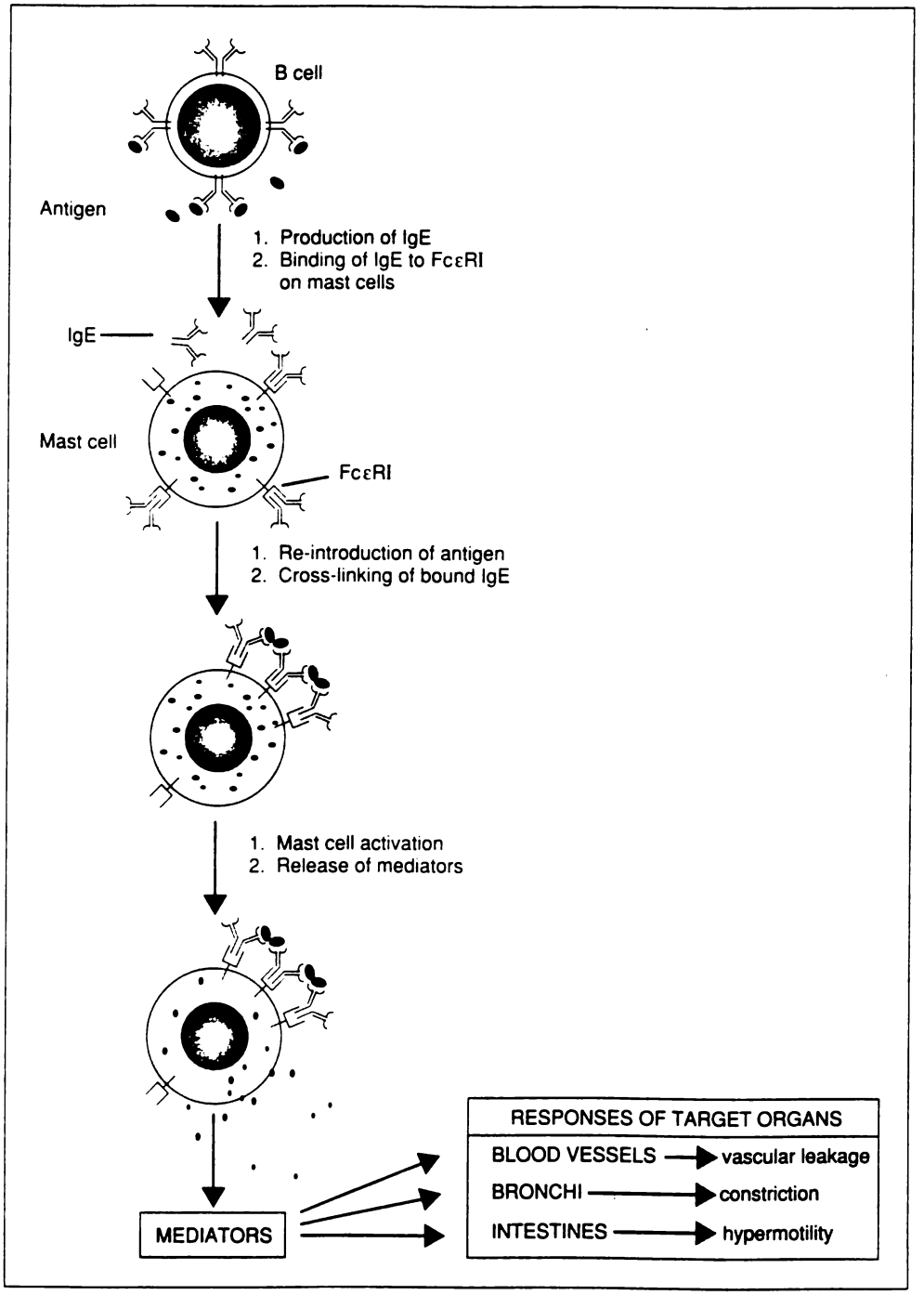

Fig. 5.1. Events leading to an immediate hypersensitivity (allergic) reaction. (Reproduced from *Abbas et al.* 1991)

A few classes of compounds are believed to exert their immunologic toxicity by haptenation. The family of β-lactam containing drugs such as penicillin, cephalosporins, carbapenems, and monobactams are among these compounds. In solution, penicillin is converted to a number of derivatives that can covalently bind to proteins (Levine and Redmond, 1969; Saxon *et al.*, 1987; Shepherd, 1991). Immunologic toxicity has also been observed with the ingestion of sulfonamides (Shear *et al.*, 1986; Rieder *et al.*, 1991). These drugs are oxidized by cytochrome P450 to a reactive metabolite which covalently binds to proteins. Drug specific IgG and IgE antibodies have been found in sulfonamide sensitive patients.

As described above, cross-linking of mast cell bound IgE antibodies is required for the occurrence of an immediate hypersensitivity reaction. Thus mainly multivalent hapten-protein conjugates give rise to such reactions (Park *et al.*, 1987; Sullivan, 1993). Therefore, as described in section 1.5 of this thesis, we have theorized that tolmetin glucuronide is a hapten which upon binding to a protein becomes immunogenic (causes an antibody response). To test this hypothesis, the immunogenicity of mouse serum albumin conjugate of tolmetin glucuronide has been examined in mice. Furthermore, to address the phenomenon of cross-reactivities among non-steroidal anti-inflammatory drugs, the reactivity of the generated antisera against other NSAIDs and their glucuronides has been examined.

5.2 EXPERIMENTAL SECTION

5.2.1 Materials

Human and mouse serum albumin, complete and incomplete Freund's adjuvants (CFA, IFA), tolmetin, naproxen, fenoprofen, valproic acid, and

zomepirac were purchased from Sigma Chemical Co. (St. Louis, MO). Tolectin® (capsule, 400mg) was purchased from McNeil Pharmaceutical (Spring House, PA), and Nalfon® (capsule, 300 mg) was purchased from Eli Lilly (Indianapolis, IN). All the necessary chemicals for performing ELISA were obtained from Pierce (Rockford, IL). HPLC solvents were purchased from Fisher Scientific (Pittsburgh, PA). Tolmetin glucuronide and fenoprofen glucuronide were isolated from human urine.

5.2.2 Isolation of glucuronides from urine

The procedures for isolation of tolmetin glucuronide from urine were previously described in section 3.2.2 of this thesis.

Fenoprofen glucuronide was purified from the urine of a volunteer who had ingested 600 mg of fenoprofen. To prevent degradation of the β-1-O-acyl glucuronide, urine pH was adjusted to 2.5 by addition of concentrated phosphoric acid. After a gravity filtration step, the urine was extracted twice with ethyl acetate. The organic solvent was dried over sodium sulfate, filtered, and evaporated. The urine extract was further purified by semi-preparative HPLC. For this purpose a reverse phase C-18 column (10 mm x 25 cm, 5 μm), a mobile phase of 40% acetonitrile-10 mM tetrabutylammonium hydrogen sulfate (pH 2.5), and a flow rate of 2.5 ml/min was used. The eluent was monitored at 272 nm. Fenoprofen glucuronide eluted at 11 min and fenoprofen at 42 min. The collected glucuronide fractions were mixed with ethyl acetate, and phosphoric acid was added to this mixture to promote transfer of the glucuronide into the organic phase. The ethyl acetate layer was sequentially washed with water and a saturated solution of sodium chloride, and then dried over sodium sulfate.

The residue obtained after evaporating the filtered ethyl acetate contained 97% pure fenoprofen glucuronide.

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) were used to confirm the identity of the isolated glucuronides.

5.2.3 Synthesis of glucuronides

Zomepirac, naproxen, and valproic glucuronides were synthesized and purified using a method described in the literature (Panfil *et al.*, 1992). The α and β anomers of 1-O-zomepirac glucuronide and 1-O-(R & S)-naproxen glucuronide were separated on a silica gel column (hexane:ethyl acetate, 8:1), although R- and S-naproxen glucuronides could not be separated for each anomeric pair. The α & β anomers of valproic glucuronide could not be separated by silica gel chromatography. The identity of the glucuronides was established by NMR and MS.

5.2.4 Preparation of antigens (protein conjugates)

Mouse serum albumin (MSA) was chosen as a model self-protein. MSA (0.69 mM) was reacted with TG (6.9 mM) to produce TG-MSA conjugate in the presence of $NaCNBH_3$ (30 mM). For the control group, MSA (0.69 mM) was incubated with T (6.9 mM) in the presence of NaCNBH3 (30 mM). Tolmetin is incapable of chemically modifying proteins. Thus, the control group was immunized with unmodified MSA. TG-HSA conjugate or HSA antigen were prepared in a manner analogous to the MSA antigens. The reaction was carried out at 37°C in phosphate buffer (1 ml, 0.1M, pH 7.4) for six hours. One fourth of the mixture (250 µl) was used to quantitate bound tolmetin after hydrolysis of the modified protein according to the procedures described in section 3.2.4. In order to separate the modified protein from the

smaller molecules (e.g. tolmetin or tolmetin glucuronide) in the remaining sample (750 µl), this mixture was subjected to six rounds of centrifugal filtration using Amicon centricons (30KD molecular weight cutoff). The mixture was diluted with water before each filtration step and lyophilized after the last step. The residue containing modified and unmodified protein was used to immunize mice.

5.2.5 Immunizations

Female Balb/c mice (20-23 g, B&K Inc., Fremont, CA) were immunized with 250µg of antigen dissolved in 100µl of phosphate buffered saline (PBS, pH 7.4) in combination with CFA for the first immunization and IFA for subsequent immunizations. The antigens were injected into the peritoneal cavity. One group of 12 mice received the TG-MSA conjugate and the control group of 12 mice received the mixture of MSA plus tolmetin. The injections were done once every two weeks and blood samples were collected seven days after each immunization. Blood was incubated at 37°C for 2.5 hours and centrifuged (3000 rpm, 4°C) for 20 minutes. The sera were stored in 30 and 5 µl aliquots at -20°C.

5.2.6 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was utilized to test the reactivity of the anti-sera against MSA- and HSA-conjugates of tolmetin. The wells of the plate (Applied Scientific, San Francisco, CA) were coated with 100µl of a solution of TG-HSA conjugate or HSA (100µg/ml) in sodium bicarbonate buffer (0.2 M, pH 9.4) and shaken overnight at 4°C. All the other incubation steps were performed at room temperature for either one hour (anti-serum), two hours (secondary antibody), or 15-20 minutes (substrate). A 1% BSA solution in PBS (0.01%

thimerosal) was used as the blocker. Alkaline phosphatase labeled anti-mouse IgG (H+L) was used as the secondary antibody (1:600 dilution) dissolved in the blocker solution. *p*-Nitrophenyl phosphate disodium salt (5mg in 10 ml of a 1M diethanolamine solution) was used as the enzyme substrate and absorbance (absorb.) of the colored *p*-nitrophenol product was measured at 410 nm. The wells were washed after each incubation step with PBS (0.05 % Tween-20).

5.2.7 Inhibition Studies

The reactivities of the anti-sera against the protein conjugates were determined after a 15 minute pre-incubation of the sera with the following (potential inhibitors): protein conjugate, tolmetin, tolmetin glucuronide, zomepirac, zomepirac glucuronide, naproxen, naproxen glucuronide, fenoprofen, fenoprofen glucuronide, valproic acid, valproic acid glucuronide, glucuronic acid, glucose, and chloroacetic acid. The same ELISA system described in section 5.2.6 was used for these studies. The anti-sera were diluted with the blocker solution (1/1000). Solutions of the compounds were prepared in phosphate buffer (0.1 M, pH 7.4). Then equal volumes of the diluted anti-sera and the inhibitor solutions were mixed to give a 1/2000 dilution of anti-serum and the desired final concentrations of the inhibitors.

Percent inhibition at each inhibitor concentration was calculated using the following relationship:

$$\% \text{ Inhibition} = \frac{\text{Absorb. (inhibitor absent)} - \text{Absorb. (inhibitor present)}}{\text{Absorb. (inhibitor absent)}} \cdot 100$$

5.3 RESULTS

As expected, no conjugate specific antibodies were observed in any of the control mice. The reactivity of serum obtained from one of these control mice against TG-HSA conjugate and (T+HSA) is shown in Fig. 5.2. The curve showing the reactivity of this control serum against TG-HSA conjugate (closed circles) is superimposable on the curve showing the reactivity against (T+HSA). This indicates that conjugate specific antibodies were not produced in the control mice. Three of the 12 mice immunized with the TG-MSA conjugate produced TG-protein conjugate specific antibodies after four immunizations (Fig. 5.3a-c). The antisera of the remaining nine mice did not contain conjugate specific antibodies after four immunizations. As seen in the plots, the antisera of the three responder mice have higher reactivity against the protein conjugate than the unmodified protein. This specificity of the antibodies for TG-protein conjugate was confirmed in all three antisera by an observed inhibition of the antibody-antigen interaction when the sera were pre-incubated with TG-MSA conjugate (Fig. 5.4).

The results of the inhibition studies with tolmetin and tolmetin glucuronide indicate that the antibodies recognize different segments of the TG-MSA conjugate. As shown in Fig. 5.5a-c, both tolmetin and tolmetin glucuronide are good inhibitors of the interaction between the antibodies and the antigen. In all three mice, a great deal of the inhibition is due to tolmetin. Therefore, tolmetin specific antibodies are present in these anti-sera. The tolmetin and tolmetin glucuronide inhibition curves are not superimposable (Fig. 5.5a-c). Thus, some antibodies also recognize tolmetin glucuronide as a unit.

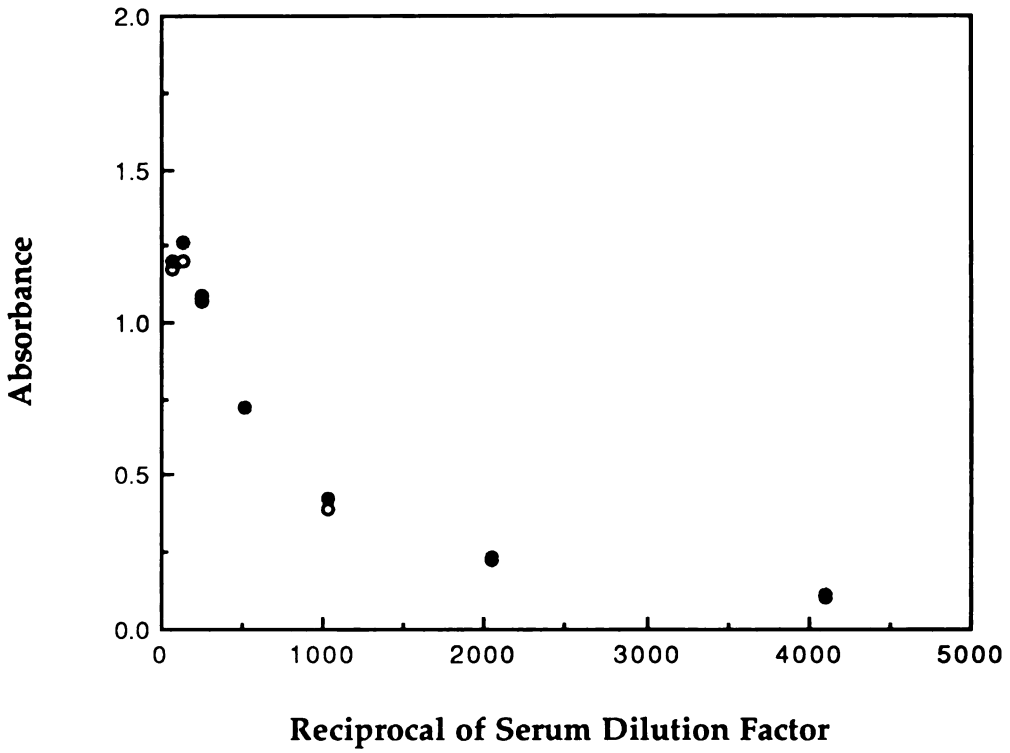

Fig. 5.2. Reactivity of serum obtained from a mouse that was immunized four times with (T+MSA) mixture. The open circles show the reactivity against (T+HSA), the closed circles show the reactivity against TG-HSA conjugate.

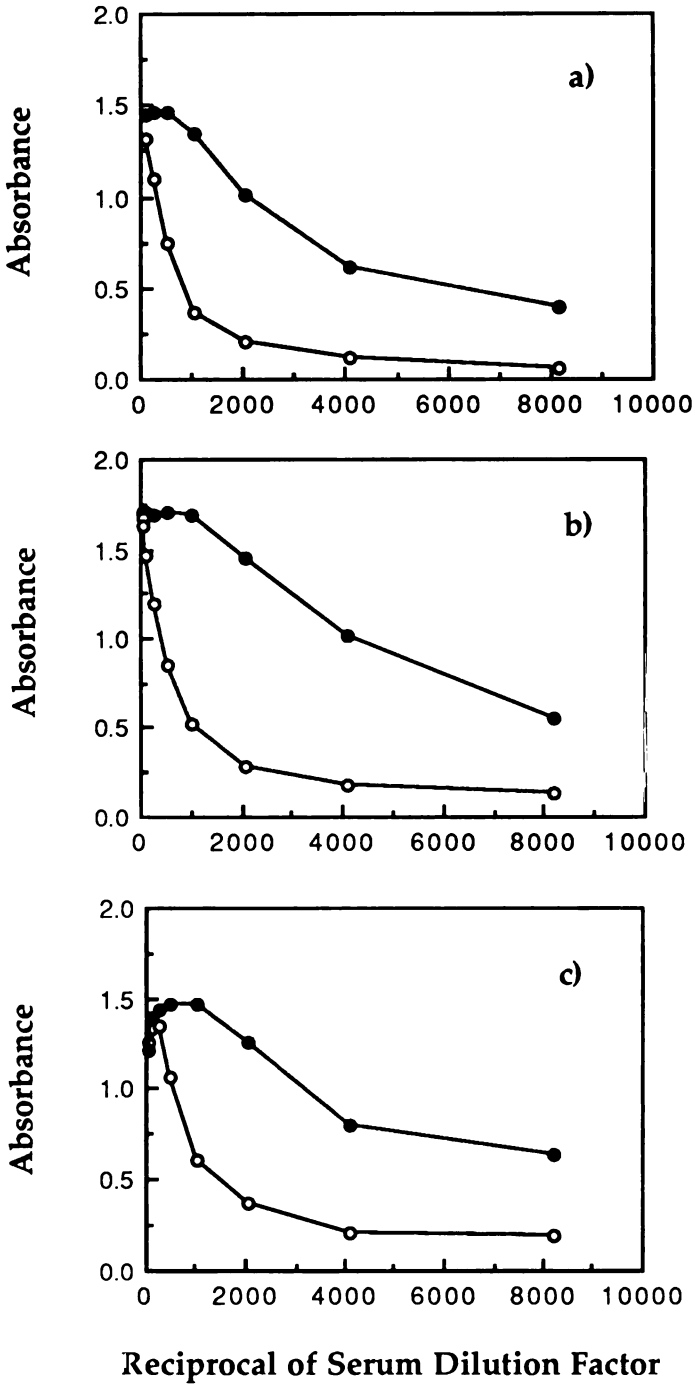

Fig. 5.3. Reactivity of antisera against TG-HSA (closed circles) and T+HSA (open circles). The antisera were obtained from three different mice that were immunized four times with TG-MSA conjugate: a)mouse 3, b) mouse 4, c) mouse 5.

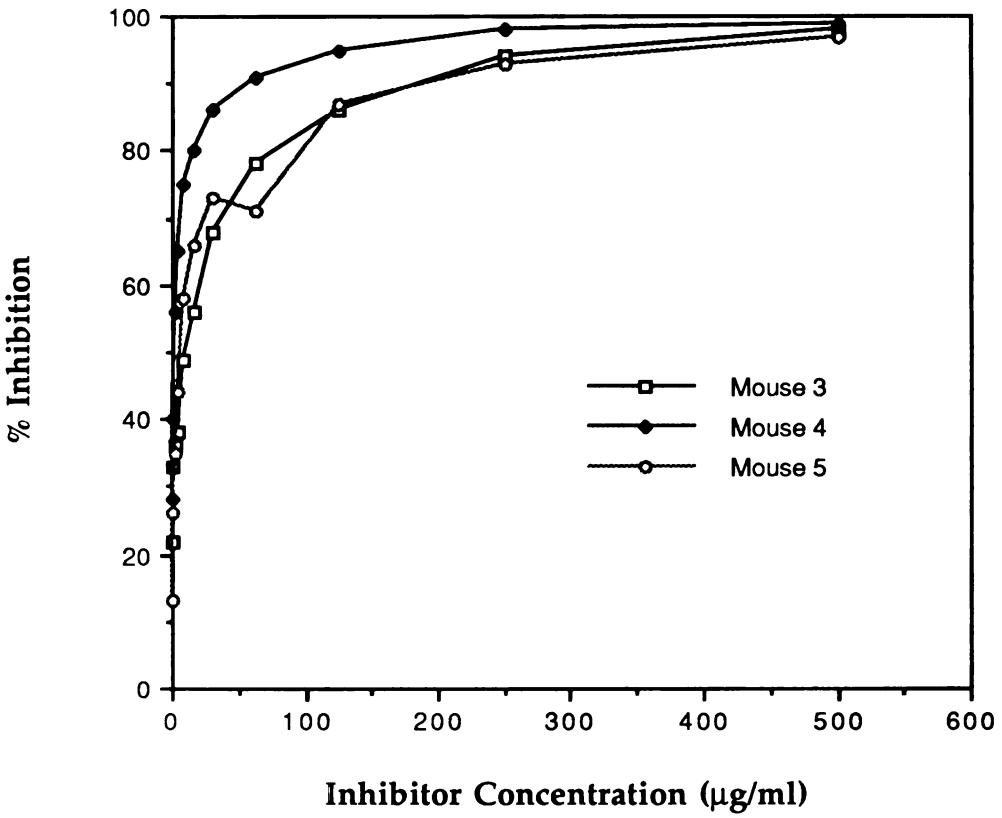

Fig. 5.4. Inhibition of antibody-antigen interaction by TG-MSA conjugate. The inhibition curves for mouse 3 (squares), mouse 4 (diamonds), and mouse 5 (circles) are shown.

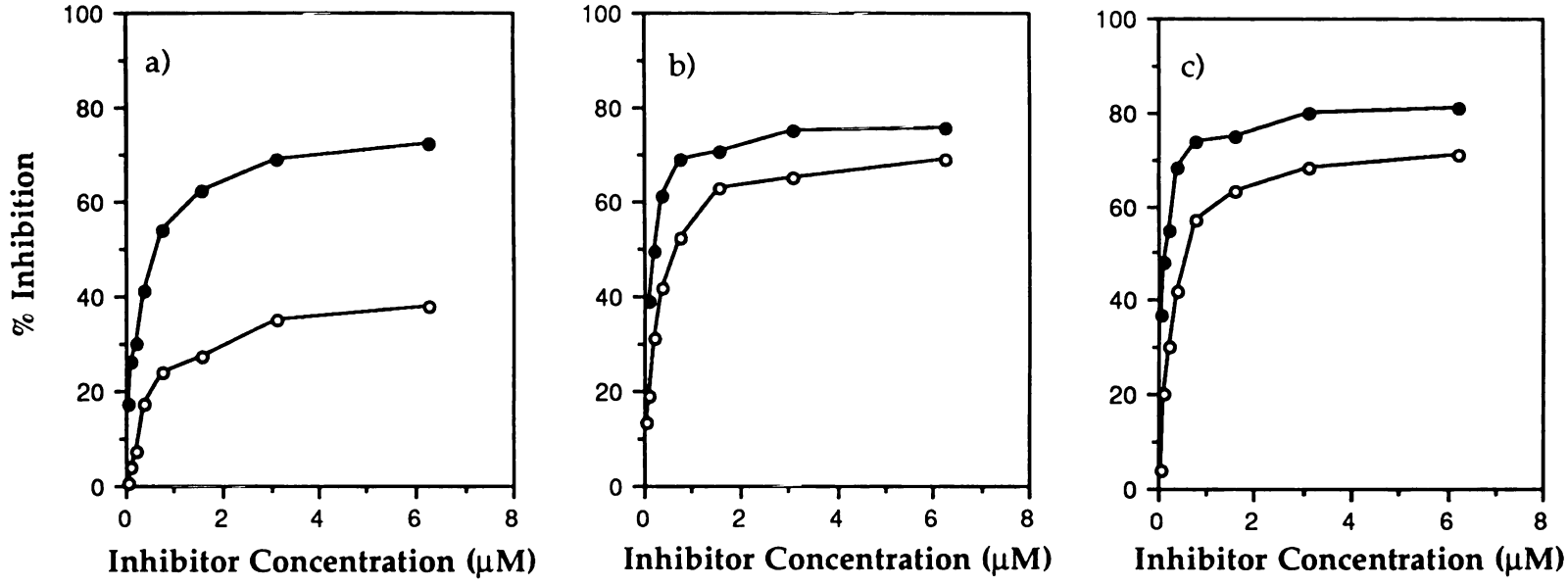

Fig. 5.5. Inhibition of antibody-antigen interaction by tolmetin (open circles) and tolmetin glucuronide (closed circles). The three plots show the results from mice that were immunized four times with TG-MSA conjugate: a) mouse 3, b) mouse 4, c) mouse 5.

The inhibition studies with other drugs and their glucuronides are consistent with the observation that glucuronide specific antibodies are present in the tested anti-sera. It appears that the reactivity of these antibodies towards other drugs varies greatly depending on the structure of the compound. As can be seen in Fig. 5.6a-c, there is a relatively good recognition of zomepirac and its glucuronide by the antibodies (at 0.05-6.25 μM inhibitor concentrations). The maximal extent of inhibition observed with zomepirac ranged between 1/2 to 2/3 of that observed for tolmetin. This indicates that a sizable fraction (1/2 to 1/3) of the antibodies did not cross-react with zomepirac. Cross-reactivity to fenoprofen and naproxen and their glucuronides was observed at much higher concentrations (1.95-125 μM) of these compounds - presumably reflecting structural differences of fenoprofen and naproxen with tolmetin (Fig. 5.7a-c). This indicates that the antibodies have low affinity for fenoprofen and naproxen.

To determine the cross-reactivity of the antibodies against an acidic drug that is not a non-steroidal anti-inflammatory drug, inhibition studies were performed with valproic acid which is an anti-epileptic agent (Rall and Schleifer, 1990). The antibodies did not recognize valproic acid or valproic glucuronide (data not shown).

The antibodies did not cross-react with glucose, glucuronic acid, or chloroacetic acid as determined by inhibition studies.

5.4 DISCUSSION

As described in Chapter 1, our theory states that drug glucuronide-protein conjugates can trigger an immune response. In support of this theory, my work shows that TG-MSA conjugate is indeed immunogenic in

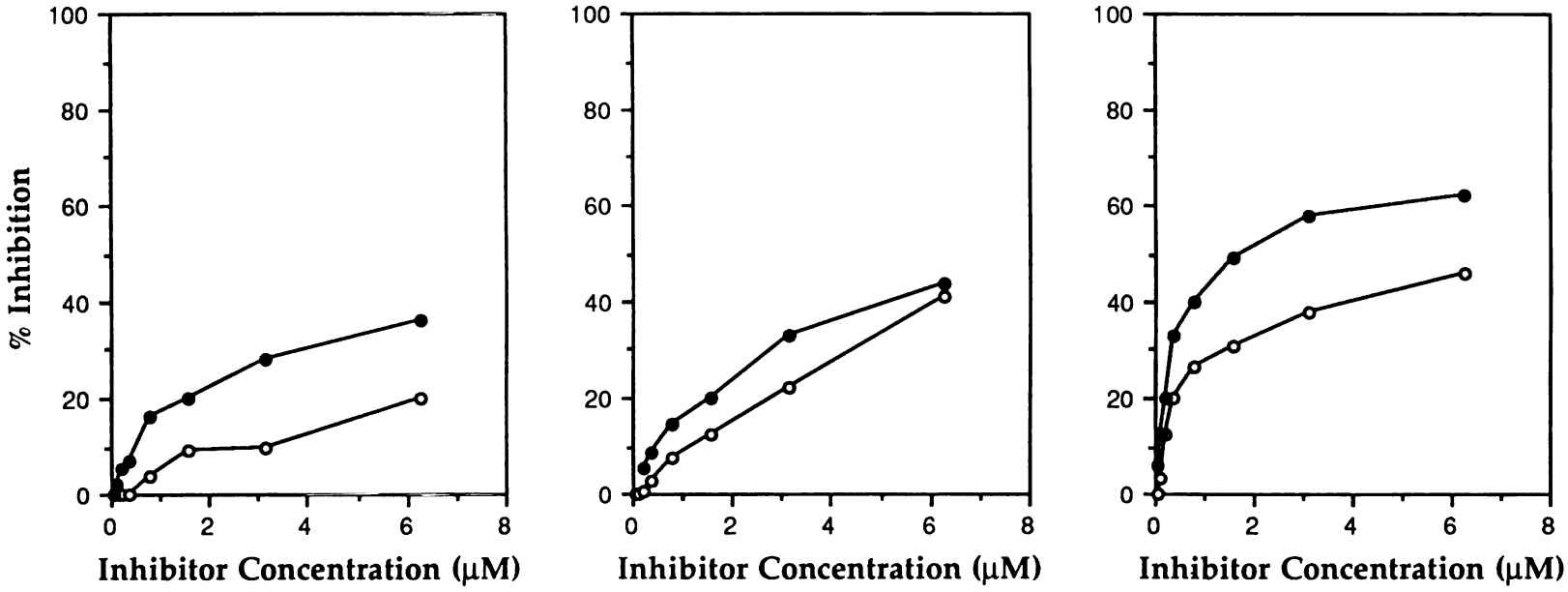

Fig. 5.6. Inhibition of antibody-antigen interaction by zomepirac (open circles) and zomepirac glucuronide (closed circles). The three plots show the results from mice that were immunized four times with TG-MSA conjugate: a) mouse 3, b) mouse 4, c) mouse 5.

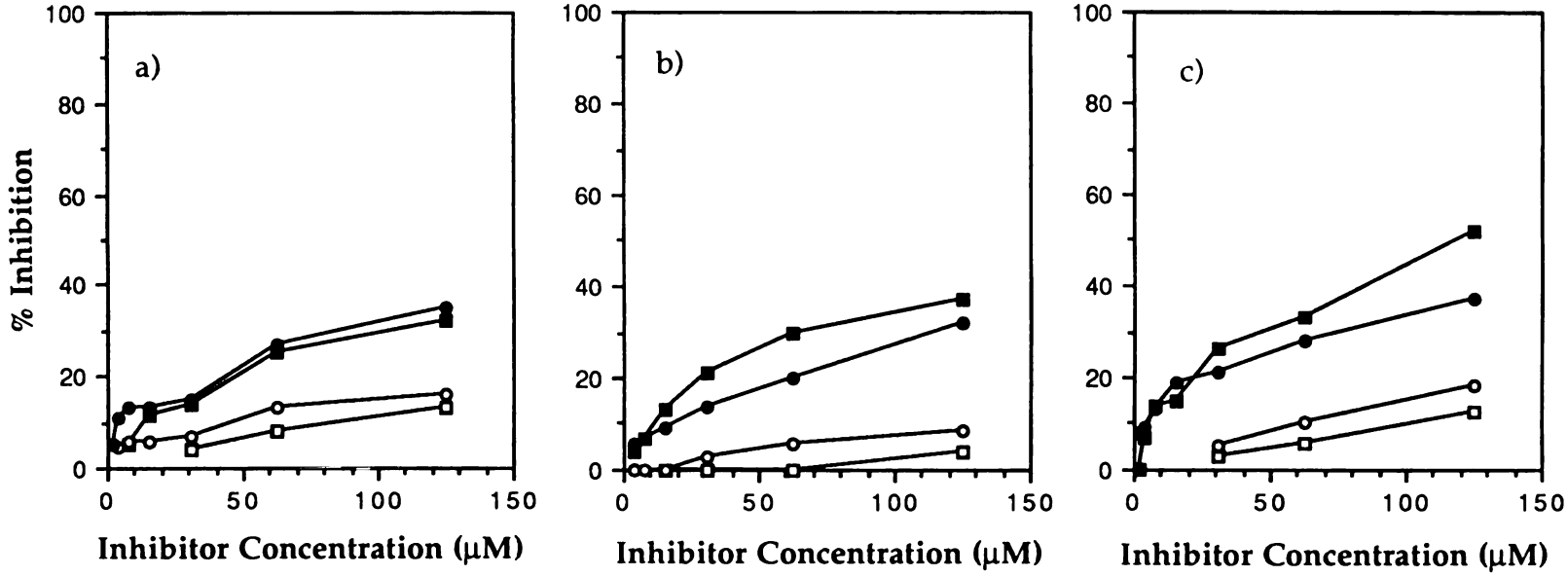

Fig. 5.7. Inhibition of antibody-antigen interaction by fenoprofen (open circles), fenoprofen glucuronide (closed circles), naproxen (open squares), naproxen glucuronide (closed squares). The different plots represent results form mice that were immunized four times with TG-MSA conjugate: a)mouse 3, b) mouse 4, c) mouse 5.

mice. The fact that the frequency of the occurrence of an antibody response is low (3 out of 12 immunized animals) is consistent with the low frequency of allergic reactions in humans.

TG-MSA adduct could completely inhibit the antigen-antibody interaction. However, tolmetin and tolmetin glucuronide inhibited 30 to 75% of antigen-antibody interaction on the ELISA plate. This result suggests that the antibodies in the antisera of the responder mice contained three components. One which was specific for tolmetin alone, another which recognized tolmetin glucuronide as a unit, and a third smaller component reacted against parts of the protein.

The antibodies generated against TG-MSA recognized different non-steroidal anti-inflammatory agents and their glucuronide metabolites to varying degrees. Zomepirac showed a relatively good inhibition of antibody-antigen interaction. This result is expected since zomepirac is structurally so similar to tolmetin (Fig. 5.8). The fact that zomepirac glucuronide was a better inhibitor than zomepirac (Fig. 5.6) confirms the presence of drug-glucuronide specific antibodies in the examined sera.

Higher concentrations (Fig. 5.7) of naproxen and fenoprofen were needed to observe some degree of interaction with the antibodies. This finding is consistent with the fact that tolmetin has a somewhat different structure than naproxen and fenoprofen. Thus these antibodies cross-react with other non-steroidal anti-inflammatory drugs and this interaction depends a great deal on the chemical structure of the drug. As was the case with tolmetin and zomepirac, both naproxen and fenoprofen glucuronides inhibited antibody-antigen interactions to a larger extent than the drugs themselves. Again this finding is consistent with the presence of drug-glucuronide specific antibodies in these antisera. It also supports our theory

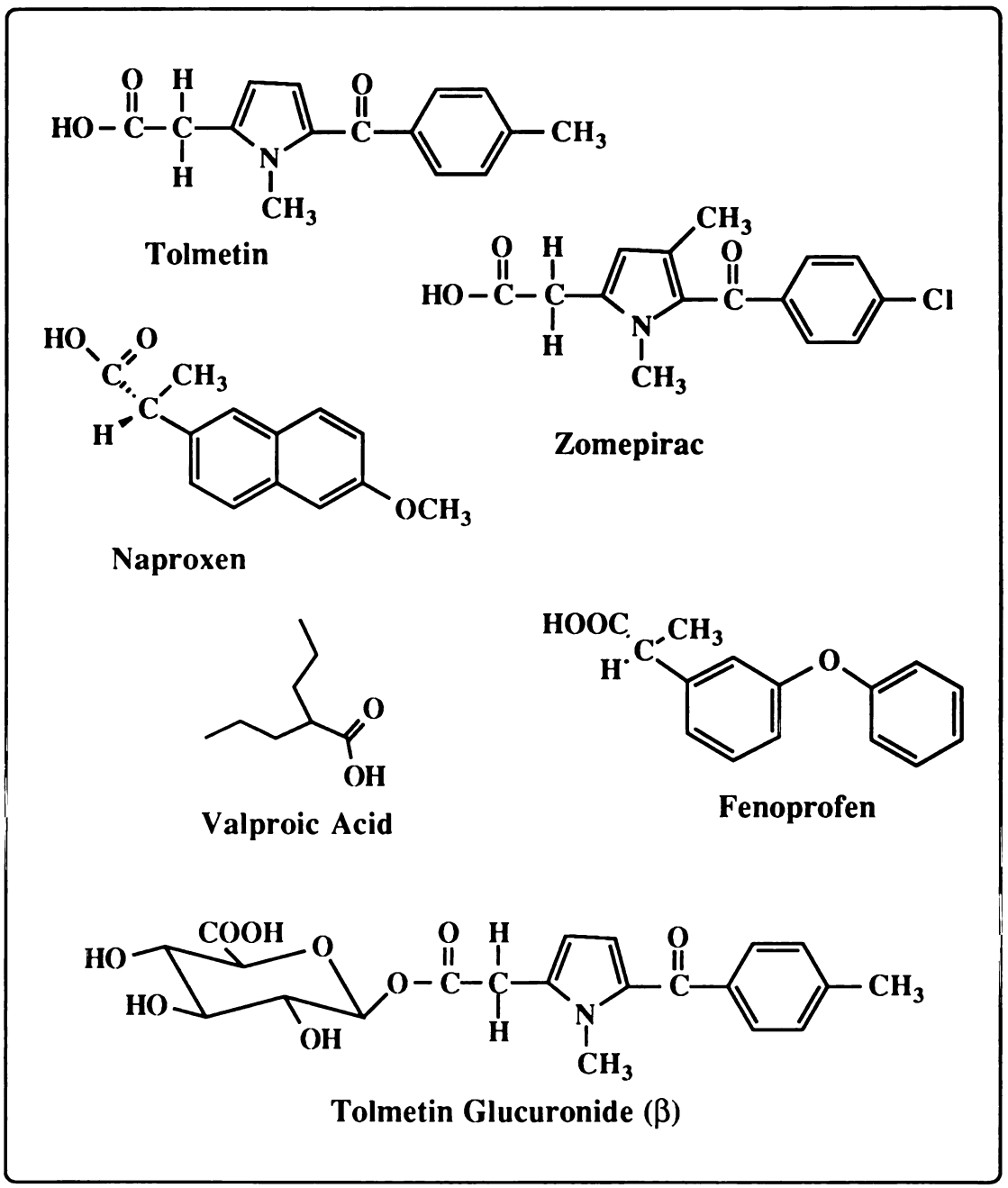

Fig. 5.8. Some of the inhibitors that were used in the inhibition experiments.

that when the glucuronic acid moiety is retained as a part of the protein conjugate, the generated antibodies against this conjugate would have a broader specificity toward other drug-glucuronides.

Valproic acid and its glucuronide were not recognized by tolmetin and tolmetin glucuronide specific antibodies. As shown in Fig. 5.8, valproic acid is structurally very different from tolmetin. Thus, there were no antibodies specific for the glucuronic acid moiety alone; some degree of drug structural similarity is necessary for drug and drug glucuronide specific antibodies to cross-react with other acidic drugs and their acyl glucuronides. The antibodies did not cross-react with glucuronic acid or glucose. These findings are consistent with the fact that people who are allergic to NSAIDs are not prevented from taking carboxylic acid containing drugs of other pharmacologic classes. The results presented here could potentially lead to the design of safer NSAIDs and more appropriate drug therapy.

6.0 SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

6.1 Formation and Stability of Plasma Protein Adducts of Tolmetin in Humans

Tolmetin glucuronide covalently binds to plasma proteins *in vivo*. Covalently bound levels are detectable in human plasma within one half hour after the administration of a single dose of Tolectin®. After a ten day multiple dosing regimen, maximum covalently bound levels of tolmetin are almost an order of magnitude higher than that observed after a single dose. Thus the plasma protein conjugates accumulate in the body upon repeated exposure to the drug. Covalently bound tolmetin has an average half-life of 4.8 days. Since the drug-protein conjugate is long lived in humans, it has a greater potential for causing a toxic effect.

6.2 Mechanisms of Covalent Binding of Tolmetin Glucuronide to Human Serum Albumin

Both direct binding of tolmetin to HSA (nucleophilic displacement) and binding of tolmetin to HSA via a glucuronic acid bridge (Schiff base formation) were observed in reaction mixtures of TG and HSA. The following amino acids were modified by binding of a tolmetin unit: Ser 220, Arg 222, Lys 541, Lys 199, Ser 232, Ser 480, and Arg 521. The following amino acids were modified by a tolmetin glucuronide unit: Lys 199, Lys 541, Lys 195, and Lys 525.

Modification of HSA by T or TG appeared to depend on the concentration of TG in the reaction mixture. Four sites (TG-Lys 525, T-Arg

525, T-Lys 199, T-Ser 480) were only modified in the reaction mixture containing the highest concentration of TG. There was a tendency for a higher proportion of modifications to be via a TG unit as the concentration of TG was lowered. At the lowest examined concentration, 49% of the structurally assigned binding (74% of binding could be identified) was due to TG, whereas at the highest examined concentration 39% of the structurally assigned binding (68% of binding could be identified) was due to TG binding. The overall binding (% T equivalents/moles HSA, 1.2%) in the reaction mixture containing the lowest concentration of TG is close to the observed maximal binding (0.6%) in human plasma. Thus we predict that a great deal of the covalent binding of tolmetin to plasma proteins in healthy humans occurs via a glucuronic acid bridge (i.e. Schiff base formation mechanism). The presence of this common chemical moiety (i.e. glucuronic acid) could increase the probability of the occurrence of a cross-reaction to other carboxylic acid containing NSAIDs.

6.3 Human Serum Albumins Modified by NSAIDs via the Nucleophilic Displacement Mechanism

Activated esters of tolmetin and zomepirac react with HSA to generate several modified sites on the protein. The following residues were identified to be modified by the addition of a tolmetin unit: Lys 195, Lys 199, Lys 524, Lys 536, Asp-1, and Lys 351. Three residues -Lys 199, Lys 195, Lys 351- were identified to be modified by a zomepirac unit. The majority of the identified binding occurred at Lysines 195 and 199 which are located in a hydrophobic pocket of HSA. No serines, tyrosines, cysteines, or arginines were among the identified modified residues.

6.4 Immunogenicity of Protein Conjugates of Tolmetin Glucuronide and Reactivity of the Generated Antibodies Against Several Drugs and Their Glucuronides

Tolmetin glucuronide-mouse serum albumin conjugates are capable of triggering production of conjugate specific antibodies in mice. This observation is in support of our theory that protein conjugates of acyl glucuronides may give rise to an antibody response in hypersensitive patients.

The antigenic determinants in the antisera of responder mice included both tolmetin and tolmetin glucuronide. The antibodies cross-reacted with zomepirac, a close chemical congener of tolmetin, and zomepirac glucuronide. The antibodies cross-reacted to a lesser extent with naproxen, fenoprofen, and their glucuronides. This finding is consistent with the fact that these two NSAIDs have somewhat different structures than that of tolmetin. Valproic acid which has a very different structure than tolmetin did not cross-react with the antibodies, and neither did valproic glucuronide, glucuronic acid, and glucose. It is encouraging that these antibodies did not cross-react with the glucuronide of a totally unrelated acidic drug, otherwise the hypersensitive patients would not have been able to use a large number of drugs presently on the market.

6.5 Future Directions

6.5.1 Investigating the mechanism of covalent binding of tolmetin glucuronide to HSA *in vivo*

It is necessary to determine how tolmetin glucuronide binds to proteins *in vivo*. One should collect plasma from volunteers who are on tolmetin therapy and examine the mechanism of binding of tolmetin glucuronide to human serum albumin in these samples by similar methods presented in Chapters 3 and 4 of this thesis. HSA may be isolated using affinity chromatography.

6.5.2 Determining the immunogenicity of a series of drug glucuronide-protein conjugates

In order to classify the NSAIDs in terms of their immunologic reactivity in an animal model, one should examine the ability of protein conjugates of several NSAIDs to induce an immunologic response in mice. Here, care should be taken so that the conjugates used have roughly the same binding yield (equivalents T bound/mole protein). Moreover, the same strain of mice should be used for all of these studies. Subsequently, the specificity and reactivity of the generated anti-sera against other NSAIDs and their metabolites should be examined.

6.5.3 Finding a measure of immunologic reactivity of certain NSAIDs in humans

In order for our hypothesis, which states that drug glucuronide-protein conjugates trigger an immune response, to be valid, patients who have recently had an allergic reaction to an NSAID should have circulating drug specific antibodies in their sera. To test this point one needs to examine these sera for presence of such antibodies using ELISA. Inhibition experiments similar to those described in Chapter 5 could be performed to further pinpoint

the specificity and cross-reactivity of the antibodies against other NSAIDs and their glucuronides.

If only sera from hypersensitive patients who have had a reaction a long time ago is available, then a different approach needs to be taken. In this case one may try to develop an ELISPOT, whereby the reactivity of the patient's B lymphocytes are tested against drug glucuronide-protein conjugate (van der Meide *et al.*, 1991). Alternatively, after concentrating the B lymphocytes, fluorescence-activated cell sorting may be used to detect B cells that contain drug glucuronide-HSA conjugate specific antibodies on their surface (Abbas *et al.*, 1991).

6.5.4 Exploring the possible role of Human Leukocyte Antigen (HLA) in causing the immunologic toxicity

HLA typing of large groups of patients with various autoimmune diseases has shown that some HLA alleles are present at higher frequencies in patients with particular diseases than in the general population. For example rheumatoid arthritis is strongly associated with HLA-DR4, and insulin-dependent diabetes mellitus is associated with HLA-DR2, DR3, and DR4 (Abbas *et al.*, 1991). Thus the relative risk of developing a disease with a known HLA allele may be estimated. Moreover, if such association were found, the finding would indicate participation of T-cells in triggering the allergic reaction.

7.0 REFERENCES

Abbas, A.K., Lichtman, A.H., and Pober, J.S. (1991). "Cellular and Molecular Immunology". PP. 284, 294-296, 354-357, 373-374. W.B. Saunders Company, Philadelphia.

Aberth, W., Straub, K., and Burlingame, A.L. (1982). Secondary ion mass spectrometry with cesium ion primary beam and liquid target matrix for analysis of bioorganic compounds. *Anal. Chem.* **54**, 2029-2034.

Amos, H.E., Wilson, D.V., Taussig, M.J., and Carlton, S.J. (1971). Hypersensitivity reactions to acetylsalicylic acid. *Clin. Exp. Immunol.* **8**, 563-572.

Anggelutsa, P.A., Brinzinskii, M.A., and Ter-Vartanian, S.K.H. (1989). Allergic complications in patients with rheumatism. *Vrachebnoe Delo* **9**, 9-12.

Australian Adverse Drug Reactions Bulletin (1993). Severe hypersensitivity reactions to NSAIDs , Tiaprofenic acid and urinary symptoms, Angioedema, Sumatriptan and chest pain. **12**, 2.

Bailey, A.J., Robins, S.P., and Tanner, M.J.A. (1976). Reducible components in the proteins of human erythrocyte membrane. *Biochim. Biophys. Acta* **434**, 51-57.

Bakke, O.M., Wardell, W.M., and Lasagna, L. (1984). Drug discontinuations in the United Kingdom and the United States, 1964 to 1983: issues of safety. *Clin. Pharmacol. Ther.* **35**, 559-567.

Benet, L.Z., Spahn-Langguth, H., Iwakawa, S., Volland, C., Mizuma, T., Mayer, S., Mutschler, E., and Lin, E.T. (1993). Predictability of the covalent binding of acidic drugs in man. *Life Sci.* **53**, PL141-PL146.

Biemann, K. (1988). Contributions of mass spectrometry to peptide and protein structure. *Biomed. Environ. Mass Spectrom.* **16**, 99-111.

Bretza, M. (1985). Anaphylactoid reactions to tolmetin. *West J. Med.* **143**, 55-59.

Cook, D.J., Achong, M.R., and Murphy, F.R. (1988). Three cases of diflunisal hypersensitivity. *Can. Med. Assoc. J.* **138**, 1029-1030.

Cottrell, J.S., and Evans, S. (1987). Characteristics of a multichannel electro-optical detection system and its application to the analysis of large molecules by fast atom bombardment mass spectrometry. *Anal. Chem.* **59**, 1990-1995.

Day, J.F., Thorpe, S.R., and Baynes, J.W. (1979). Nonenzymatically glycosylated albumin: In vitro preparation and isolation from normal human serum. *J. Biol. Chem.* **254**, 595-597.

Denisenko, T.V. (1990). [Glycosylated lipoproteins as atherogenic factor in diabetes (review of the literature)]. *Voprosy Meditsinskoi Khimii* **36**, 5-10.

Dickinson, R.G., and King, A.R. (1993). Studies on the reactivity of acyl glucuronides V: Glucuronide-derived covalent binding of diflunisal to bladder tissue of rats and its modulation by urinary pH and β-glucuronidase. *Biochem. Pharmacol.* **46**, 1175-1182.

Ding, A., Ojingwa, J.C., McDonagh, A.F., Burlingame, A.L., and Benet, L.Z. (1993). Evidence for the covalent binding of acyl glucuronides to serum albumin via an imine mechanism as revealed by tandem mass spectrometry. *Proc. Natl. Acad. Sci. (USA)* **90**, 3797-3801.

Faed, E.M. (1984). Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab. Rev.* **15**, 1213-1249.

Falick, A.M., Walls, F.C., and Laine, R.A. (1986). Cooled sample introduction probe for liquid secondary ionization mass spectrometry. *Anal. Biochem.* **159**, 132-137.

Feeney, R.E., Blankengorn, G., and Dixon, H.B.F. (1975). Carbonyl-amine reactions in protein chemistry. *Adv. Protein Chem.* **29**, 135-203.

Fehske, K.J., Muller, W.E., and Woller, U. (1980). Direct demonstration of the highly reactive tyrosine residue of human serum albumin located in fragment 299-585. *Arch. Biochem. Biophys.* **205**, 217-221.

Flückiger, R., and Winterhalter, K.H. (1978). Glycosylated hemoglobins. *In* "Biochemical and Clinical Aspects of Hemoglobin Abnormalities" (W. S. Caughey, eds.), pp. 205-214. Academic Press, New York.

Gerig, J.T., Katz, K.E., and Reinheimer, J.D. (1978). Reactions of 2,6-dinitro-4-trifluoromethyl benzenesulfonate with human serum albumin. *Biochim. Biophys. Acta* **534**, 196-209.

Haines, A.H. (1976). Relative reactivities of hydroxyl groups in carbohydrates: migration of substituents-1, acyl migration. *Adv. Carbohydr. Chem. Biochem.* **33**, 101-107.

Han, K.K., Delacourte, A., and Hemon, B. (1987). Chemical modification of thiol group(s) in protein: application to the study of anti-microtubular drugs binding. *Comp. Biochem. Physiol.* **88B**, 1057-1065.

Hart, D., Ward, M., and Lifschitz, M.D. (1987). Suprofen-related nephrotoxicity - a distinct clinical syndrome. *Ann. Intern. Med.* **106**, 235-238.

Hasegawa, J., Smith, P.C., and Benet, L.Z. (1982). Apparent intramolecular acyl migration of zomepirac glucuronide. *Drug Metab. Dispos.* **10**, 469-473.

He, X.M., and Carter, D.C. (1992). Atomic structure and chemistry of human serum albumin. *Nature (London)* **358**, 209-215.

Hecker, M., Foegh, M.L., and Ramwell, P.W. (1992). The eicosanoids: prostaglandins, thromboxanes, leukotrienes, and related compounds. *In*

"Basic and Clinical Pharmacology" (B. G. Katzung, eds.), pp. 263-277. Appleton & Lange, Norwalk, Connecticut.

Higgins, P.J., and Bunn, H.F. (1981). Kinetic analysis of the nonenzymatic glycosylation of hemoglobin. *J. Biol. Chem.* **256**, 5204-5208.

Hunkapiller, M.W. (1985). "Users Bulletin 14". PP. 1-50. Applied Biosystems Inc., Foster City.

Hyneck, M.L., Munafo, A., and Benet, L.Z. (1988). Effect of pH on acyl migration and hydrolysis of tolmetin glucuronide. *Drug Metab. Dispos.* **10**, 322-324.

Hyneck, M.L., Smith, P.C., Munafo, A., McDonagh, A.F., and Benet, L.Z. (1988). Disposition and irreversible plasma protein binding of tolmetin in humans. *Clin. Pharmacol. Ther.* **44**, 107-114.

Hyneck, M.L., Smith, P.C., Unseld, E., and Benet, L.Z. (1987). HPLC determination of tolmetin, tolmetin glucuronide and its isomeric conjugates in plasma and urine. *J. Chromatogr.* **420**, 349-366.

Iberg, N., and Fluckiger, R. (1986). Nonenzymatic glycosylation of albumin in vivo. *J. Biol. Chem.* **261**, 13542-13545.

Insel, P.A. (1990). Analgesic-antipyretic and antiinflammatory agents; drugs employed in the treatment of rheumatoid arthritis and gout. *In* "Goodman and Gilman's The Pharmacological Basis of Therapeutics" (A. G. Gilman, T.

W. Rall, A. S. Nies and P. Taylor, eds.), pp. 638-669. Pergamon Press, Inc., Elmsford, New York.

Isbell, H.S., and Frush, H.L. (1958). Mutarotation, hydrolysis, and rearrangement reactions of glycosylamines. *J. Org. Chem.* **23**, 1309-1319.

Iwakawa, S., Spahn, H., Benet, L.Z., and Lin, E.T. (1988). Carprofen glucuronides: stereoselective degradation and interaction with human serum albumin. *Pharm. Res.* **5(Suppl.)**, S-214.

Kickler, T.S., Buck, S., Ness, P., Shirey, R.S., and Sholar, P.W. (1986). Probenecid induced immune hemolytic anemia. *J. Rheumatol.* **13**, 208-209.

King, A.R., and Dickinson, R.G. (1993). Studies on the reactivity of acyl glucuronides IV: Covalent binding of diflunisal to tissues of the rat. *Biochem. Pharmacol.* **45**, 1043-1047.

Kragh-Hansen, U. (1990). Structure and ligand binding properties of human serum albumin. *Danish Med. Bull.* **37**, 57-84.

Lehrer, S.S. (1976). Perturbation of intrinsic protein fluorescence. *In* "Biochemical Fluorescence" (R. F. Chen and H. Edelhoch, eds.), pp. 515-550. Marcel Dekker, Inc., New York.

Levine, B.B., and Redmond, A.P. (1969). Minor haptenic determinant-specific reagins of penicillin hypersensitivity in man. *Int. Arch. Allergy Appl. Immunol.* **35**, 445.

McKinnon, G.E., and Dickinson, R.G. (1989). Covalent binding of diflunisal and probenecid to plasma protein in humans: persistence of the adducts in the circulation. *Res. Commun. Chem. Path. Pharmacol.* **66**, 339-354.

Means, G.E., and Bender, M.L. (1975). Acetylation of human serum albumin by *p*-nitrophenyl acetate. *Biochemistry* **14**, 4989-4994.

Mereish, K.A., Rosenberg, H., and Cobby, J. (1982). Glucosylated albumin and its influence on salicylate binding. *J. Pharm. Sci.* **71**, 235-238.

Miller, J.A., Gravallese, E., and Bunn, H.F. (1980). Nonenzymatic glycosylation of erythrocytes membrane protein. Relevance to diabetes. *J. Clin. Invest.* **65**, 896-901.

Munafo, A., Hyneck, M.L., and Benet, L.Z. (1993). Pharmacokinetics and irreversible binding of tolmetin and its glucuronic acid esters in the elderly. *Pharmacology* **47**, 309-317.

Munafo, A., McDonagh, A.F., Smith, P.C., and Benet, L.Z. (1990). Irreversible binding of tolmetin glucuronic acid esters to albumin in vitro. *Pharm. Res.* **7**, 21-27.

Oberholzer, B., Hoigne, R., Hartmann, K., Capaul, R., Egli, A., Wymann, R., Galeazzi, R.L., Kuhn, M., Kunzi, U.P., and Maibach, R. (1993). Incidence of drug side effects by symptoms and syndromes. From the experiences of the Comprehensive Hospital Drug Monitoring and the Swiss Drug Side Effect

Center. As an example: allergic and pseudo-allergic reactions with mild analgesics and NSAID. *Therapeutische Umscham* **50**, 13-19.

Ojingwa, J.C. (1991). Reactivity of acyl glucuronides: studies with tolmetin and zomepirac glucuronides. University of California, San Francisco.

Ojingwa, J.C., Spahn-Langguth, H., and Benet, L.Z. (1994). Irreversible binding of tolmetin to macromolecules via its glucuronide: binding to blood constituents, tissue homogenates and subcellular fractions in vitro. *Xenobiotica* **in press**,

Panfil, I., Lehman, P.A., Zimniak, P., Ernst, B., Franz, T., Lester, R., and Radominska, A. (1992). Biosynthesis and chemical synthesis of carboxyl-linked glucuronide of lithocholic acid. *Biochim. Biophys. Acta* **1126**, 221-228.

Park, B.K., Coleman, J.W., and Kitteringham, N.R. (1987). Drug disposition and drug hypersensitivity. *Biochem. Pharmacol.* **36**, 581-590.

Perutz, M.F., Muihead, H., Cox, J.M., and Goaman, L.C.G. (1968). Three dimensional fourier synthesis of horse oxyhaemoglobin at 2.8Å resolution: The atomic model. *Nature* **219**, 131-139.

Pumford, N.R., Myers, T.T., Davila, J.C., Highet, R.J., and Pohl, L.R. (1993). Immunochemical detection of liver protein adducts of the nonsteroidal antiinflammatory drug diclofenac. *Chem. Res. Toxicol.* **6**, 147-150.

Rainsford, K.D. (1988). Current concepts of the mechanisms of side effects of nonsteroidal antiinflammatory drugs as a basis for establishing research priorities. An experimentalist's view. *J. Rheumatol.* **15**, 63-70.

Rall, T.W., and Schleifer, L.S. (1990). Drugs effective in the therapy of the epilepsies. *In* "Goodman and Gilman's The Pharmacological Basis of Therapeutics" (A. G. Gilman, T. W. Rall, A. S. Nies and P. Taylor, eds.), pp. 450-453. Pergamon Press, Inc., Elmsford, New York.

Raso, V., and Stollar, D. (1975). The antibody-enzyme analogy. Characterization of antibodies to phosphopyridoxyltyrosine derivatives. *Biochemistry* **14**, 584-591.

Rieder, M.J., Shear, N.H., Kanee, A., Tang, B.K., and Spielberg, S.P. (1991). Prominence of slow acetylator phenotype among patients with sulfonamide hypersensitivity reaction. *Clin. Pharmacol. Ther.* **49**, 13-17.

Rinsler, S., Love, P.Y., Stadel, B.V., Stein, R.A., and Harter, J.G. (1994). Categorization of NSAID associated ADRs reported to the FDA, as anaphylactoid reactions. *Clin. Pharmacol. Ther.* **55**, 156.

Robins, S.P., and Bailey, A.J. (1972). Age-related changes in collagen: The identification of reducible lysine-carbohydrate condensation products. *Biochem. Biophys. Res. Commun.* **48**, 76-84.

Roepstorff, P., and Fohlman, J. (1984). Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom.* **11,** 601-603.

Ruelius, H.W., Kirkman, S.K., Young, E.M., and Janssen, F.W. (1986). Reactions of oxaprozin-1-O-acyl glucuronide in solutions of human plasma and albumin. *Adv. Exp. Med. Biol.* **197,** 431-441.

Sack, J.S., Andrews, L.C., Magnus, K.A.H., J.C., Rubin, J., and Love, W.E. (1978). Location of amino acid residues in human deoxyhemoglobin. *Hemoglobin* **2,** 153-169.

Saxon, A., Beall, G.N., Rhor, A.S., and Adelman, D. (1987). Immediate hypersensitivity reactions to beta-lactam antibiotics. *Ann. Intern. Med.* **107,** 204.

Schleicher, E., and Wieland, D.H. (1981). Specific quantitation by HPLC of protein (lysine) bound glucose in human serum albumin and other glycosylated proteins. *J. Clin. Chem. Clin. Biochem.* **19,** 81-87.

Shaklai, M., Garlick, R.L., and Bunn, H.F. (1984). Nonenzymatic glycosylation of human serum albumin alters its conformation and function. *J. Biol. Chem.* **259,** 3812-3817.

Shear, N.H., Spielberg, S.P., Grant, D.M., Tang, B.K., and Kalow, W. (1986). Differences in metabolism of sulfonamides predisposing to idiosyncratic toxicity. *Ann. Intern. Med.* **105,** 179.

Shepherd, G.M. (1991). Allergy to beta-lactam antibiotics. *Immunol. Allergy Clin. North. Am.* **11**, 611-634.

Smith, P.C., Benet, L.Z., and McDonagh, A.F. (1990). Covalent binding of zomepirac glucuronide to proteins: evidence for a Schiff base mechanism. *Drug Metab. Dispos.* **18**, 639-644.

Smith, P.C., Hasegawa, J., Langendijk, P.N.J., and Benet, L.Z. (1985). Stability of acyl glucuronides in blood, plasma, and urine: studies with zomepirac. *Drug Metab. Dispos.* **13**, 110-112.

Smith, P.C., and Liu, J.H. (1993). Covalent binding of suprofen acyl glucuronide to albumin in vitro. *Xenobiotica* **23**, 337-348.

Smith, P.C., McDonagh, A.F., and Benet, L.Z. (1986). Irreversible binding of zomepirac to plasma protein in vitro and in vivo. *J. Clin. Invest.* **77**, 934-939.

Smith, P.C., and Wang, C. (1992). Nonenzymic glycation of albumin by acyl glucuronides in vitro. *Biochem. Pharmacol.* **44**, 1661-1668.

Spahn-Langguth, H., and Benet, L.Z. (1992). Acyl glucuronides revisited: is the glucuronidation process a toxification as well as a detoxification mechansim? *Drug Metab. Rev.* **24**, 5-48.

Stogniew, M., and Fenselau, C. (1982). Electrophilic reactions of acyl-linked glucuronides: Formation of clofibrate mercapturate in humans. *Drug Metab. Dispos.* **10**, 609-613.

Strom, B.L., West, S.L., Sim, E., and Carson, J.L. (1989). The epidemiology of the acute flank pain syndrome from suprofen. *Clin. Pharmacol. Ther.* **46**, 693-699.

Sullivan, T.J. (1993). Drug allergy. *In* "Allergy Principles and Practice" (E. J. Middleton, C. E. Reed, E. F. Ellis, N. F. J. Adkinson, J. W. Yunginger and W. W. Busse, eds.), pp. 1726-1746. Mosby, St. Louis, Missouri.

Szczeklik, A. (1986). Analgesics, allergy and asthma. *Drugs* **32**, 148-163.

Teale, F.W.J. (1960). The ultraviolet fluorescence of proteins in neutral solution. *Biochem. J.* **76**, 381-388.

U.S. Congress, Committee on Government Operations, Intergovernment Relations and Human Resources, I.R.a.H. (1982). FDA's regulation of Zomax®. 98th Congress.

van Breemen, R.B., and Fenselau, C. (1985). Acylation of albumin by 1-O-acyl glucuronides. *Drug Metab. Dispos.* **13**, 318-320.

van der Meide, P.H., Joosten, A.M., Hermans, P., Kloosterman, T.C., Olsson, T., and de Labie, M.C. (1991). Assessment of the inhibitory effect of

immunosuppressive agents on rat T cell interferon-gamma production using an ELISPOT assay. *J. Immunol. Methods* **144**, 203-213.

Volland, C., Sun, H., Dammeyer, J., and Benet, L.Z. (1991). Stereoselective degradation of the fenoprofen acyl glucuronide enantiomers and irreversible binding to plasma protein. *Drug Metab. Disp.* **19**, 1080-1086.

Walker, J.E. (1976). Lysine residue 199 of human serum albumin is modified by acetylsalicylic acid. *FEBS Lett.* **66**, 173-175.

Walls, F.C., Baldwin, M.A., Falick, A.M., Gibson, B.W., Kaur, S., Maltby, D.A., Gillece-Castro, B.L., Medzihradszky, F.K., Evans, S., and Burlingame, A.L. (1990). Experience with multichannel array detection in tandem mass spectrometric characterization of biopolymers. *In* "Biological Mass Spectrometry" (A. L. Burlingame and J. A. McCloskey, eds.), pp. 197-216. Elsevier, Amsterdam.

Watt, J.A., and Dickinson, R.G. (1990). Reactivity of diflunisal acyl glucuronide in human and rat plasma and albumin solutions. *Biochem. Pharmacol.* **39**, 1067-1075.

Wells, D.S., Janssen, F.W., and Ruelius, H.W. (1987). Interactions between oxaprozin glucuronide and human serum albumin. *Xenobiotica* **17**, 1437-1449.

Williams, A.M., Worrall, S., De Jersey, J., and Dickinson, R.G. (1992). Glucuronide-derived adduct of valproic acid and plasma protein and anti-adduct anibodies in humans. *Biochem. Pharmacol.* **43**, 745-755.

Witztum, J.L., Steinbrecher, U.P., Fisher, M., and Kesaniemi, A. (1983). Nonenzymatic glucosylation of homologous low density lipoprotein and albumin renders them immunogenic in the guinea pig. *Proc. Natl. Acad. Sci. USA* **80**, 2757-2761.

Yvon, M., and Wal, J.M. (1988). Identification of lysine residue 199 of human serum albumin as a binding site for benzylpenicilloyl groups. *FEBS Lett.* **230**, 237-240.

Zia-Amirhosseini, P., Ojingwa, J.C., Spahn-Langguth, H., McDonagh, A.F., and Benet, L.Z. (1994). Enhanced covalent binding of tolmetin to proteins in humans after multiple dosing. *Clin. Pharmacol. Ther.* **55**, 21-27.

Zimmerman, H.J. (1990). Update of hepatotoxicity due to classes of drugs in common clinical use: non-steroidal drugs, anti-inflammatory drugs, antibiotics, antihypertensives, and cardiac and psychotropic agents. *Semin. Liver Dis.* **10**, 322-338.