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# Evidence for covalent binding of acyl glucuronides to serum albumin via an imine mechanism as revealed by tandem mass spectrometry

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**ABSTRACT** **Acyl glucuronide metabolites of bilirubin and many drugs can react with serum albumin *in vivo* to form covalent adducts. Such adducts may be responsible for some toxic effects of carboxylic nonsteroidal antiinflammatory agents. The mechanism of formation of the adducts and their chemical structures are unknown. In this paper we describe the use of tandem mass spectrometry to locate binding sites and elucidate the binding mechanism involved in the formation of covalent adducts from tolmetin glucuronide and albumin *in vitro*. Human serum albumin and excess tolmetin glucuronide were coincubated in the presence of sodium cyanoborohydride to trap imine intermediates. The total protein product was reduced, carboxymethylated, and digested with trypsin. Six tolmetin-containing peptides (indicated by absorbance at 313 nm) were isolated by high-pressure liquid chromatography and analyzed by liquid secondary-ion mass spectrometry and collision-induced dissociation, using a four-sector tandem mass spectrometer. All six peptides contained tolmetin linked covalently via a glucuronic acid to protein lysine groups. Major attachment sites on the protein were Lys-195, -199, and -525; minor sites were identified as Lys-137, -351, and -541. Our results show unambiguously that the glucuronic acid moiety of acyl glucuronides can be retained within the structure when these reactive metabolites bind covalently to proteins, and they suggest that acyl migration followed by Schiff base (imine) formation is a credible mechanism for the generation of covalent adducts *in vivo*.**

Glucuronidation is a major mechanism for detoxifying and eliminating nucleophilic endogenous substrates and xenobiotics in mammals. Acyl glucuronide metabolites of carboxylic acids are unstable, undergoing isomerization and hydrolysis at physiologic pH (1). In the presence of serum albumin these reactions are accompanied by covalent binding of the parent carboxylic acid to the protein. As recently reviewed (2), covalent binding *in vitro* and *in vivo* has been demonstrated for bilirubin and for several nonsteroidal antiinflammatory agents and may play a role in the toxic side effects of these useful drugs. Two binding mechanisms have been proposed. One involves nucleophilic displacement of the glucuronosyl group by $-NH_2$ (3, 4), -SH (5), or -OH (6, 7) groups of the protein. The products of this reaction would be glucuronic acid and a covalent drug–protein amide adduct containing no glucuronic acid. The second mechanism (8, 9) involves three steps (Fig. 1): migration of the acyl group to the 2, 3, or 4 position of the sugar; ring–chain tautomerism of the pyranose ring; and condensation of the aldehyde group of the ring-opened tautomer with a lysine $\varepsilon$-amino group on the protein to form an imine. The product in this case would be an adduct in which the drug is attached covalently to the protein via a glucuronic acid link. Such a product could potentially undergo slow spontaneous Amadori rearrangement to a more stable 1-amino-1-deoxyketose structure (10). Direct structural evidence for either mechanism has been lacking. Here we report such evidence, obtained by using tandem liquid secondary-ion mass spectrometry (LSIMS) and high-energy collision-induced dissociation (CID) of peptide molecular ions, for the presence of glucuronyl-imine linkages to Lys-199 and five other lysine residues in the irreversible adduct(s) formed from tolmetin glucuronide and human serum albumin (HSA) *in vitro*.

Tolmetin [1-methyl-5-(4-methylbenzoyl)-1*H*-pyrrole-2-acetic acid] is a commercially available nonsteroidal antiinflammatory drug which is metabolized in part to an acyl glucuronide in humans. Its glucuronide (Fig. 1) forms covalent protein adducts *in vitro* (11) and *in vivo* (12), and immunologic reactions to the drug have been described (13).

## EXPERIMENTAL PROCEDURES

**Adduct Formation.** Tolmetin glucuronide (1.5 $\mu$mol), isolated and purified as previously described (11), and HSA (essentially fatty acid free, Sigma) (0.45 $\mu$mol) were incubated in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4) in the presence of excess sodium cyanoborohydride ($NaCNBH_3$, 20 $\mu$mol) to trap any imine formed. After 6 hr at 37°C, the reaction was stopped by centrifugal filtration through a 30-kDa-cutoff membrane and the retained solution was rinsed with water. A control experiment was performed under the same conditions but with 1.5 $\mu$mol of tolmetin instead of tolmetin glucuronide.

**Trypsin Digestion.** Twenty nmol of the reacted protein mixture was reduced with dithiothreitol (6 $\mu$mol; 60 min at 60°C under argon), alkylated with sodium iodoacetate (10 $\mu$mol; 30 min at room temperature) in 100 $\mu$l of 6 M guanidine hydrochloride/100 mM Tris/1 mM EDTA, pH 8.3, and subsequently dialyzed against 50 mM ammonium bicarbonate buffer (pH 8.0) in a Bethesda Research Laboratories microdialysis apparatus equipped with a 6-kDa-cutoff membrane. The dialyzed protein was then treated with 2% (wt/wt) trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated; Sigma; type XIII) for 4 hr at 37°C. Protein concentrations were determined colorimetrically by using a kit (Pierce).

**HPLC.** The tryptic fragments were separated by reversed phase chromatography on a Beckman HPLC system using a Vydac protein and peptide $C_{18}$ column (250 × 4.6 mm). Elution was performed with 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) within 90 min at a flow rate of 1 ml/min. The elution was monitored at a wavelength of 215 nm for peptides and 313 nm

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Abbreviations: CID, collision-induced dissociation; HSA, human serum albumin; LSIMS, liquid secondary-ion mass spectrometry.

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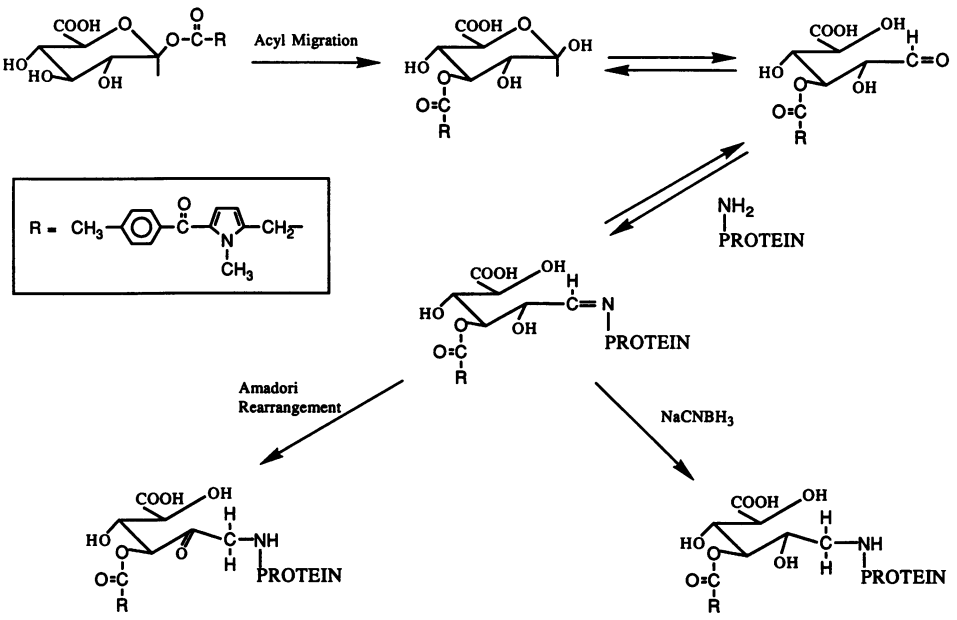


FIG. 1. Imine mechanism for the irreversible binding of acyl glucuronides to proteins.

for peptides containing an attached tolmetin group. The peaks absorbing at 313 nm were collected. Some fractions required an additional HPLC purification step due to high concentrations of coeluted peptides resulting in suppression of the peptide of interest during LSIMS. Here, a Vydac phenyl column (250 × 4.6 mm) and a linear gradient from 5% to 30% solvent B within 50 min were used.

**MS.** Aliquots (10%; 0.5 μl) of the concentrated HPLC fractions were added to 1 μl of liquid matrix (1:1 thioglycerol/glycerol with 1% trifluoroacetic acid) for molecular weight determination of all compounds in each fraction. Mass spectra were recorded with a Kratos MS-50S double-focusing mass spectrometer equipped with a cesium ion source (14, 15). The acceleration voltage was set at 6 keV, and the resolution was set to 2000. The masses of the detected $M+H^+$ ions were compared with the molecular masses of peptides predicted from the known sequence of carboxymethylated HSA after trypsin digestion, but modified by addition of either 239 mass units (tolmetin) or 417 mass units (tolmetin glucuronide). To confirm the amino acid sequence of the peptides and to localize 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 (16) equipped with a cesium ion source (14, 15) and an electrooptical multichannel array detector (17). Tandem mass spectra were acquired during operation at a mass resolution of 2000. CID of the $^{12}C$ component of the protonated peptide molecules was performed in a collision cell

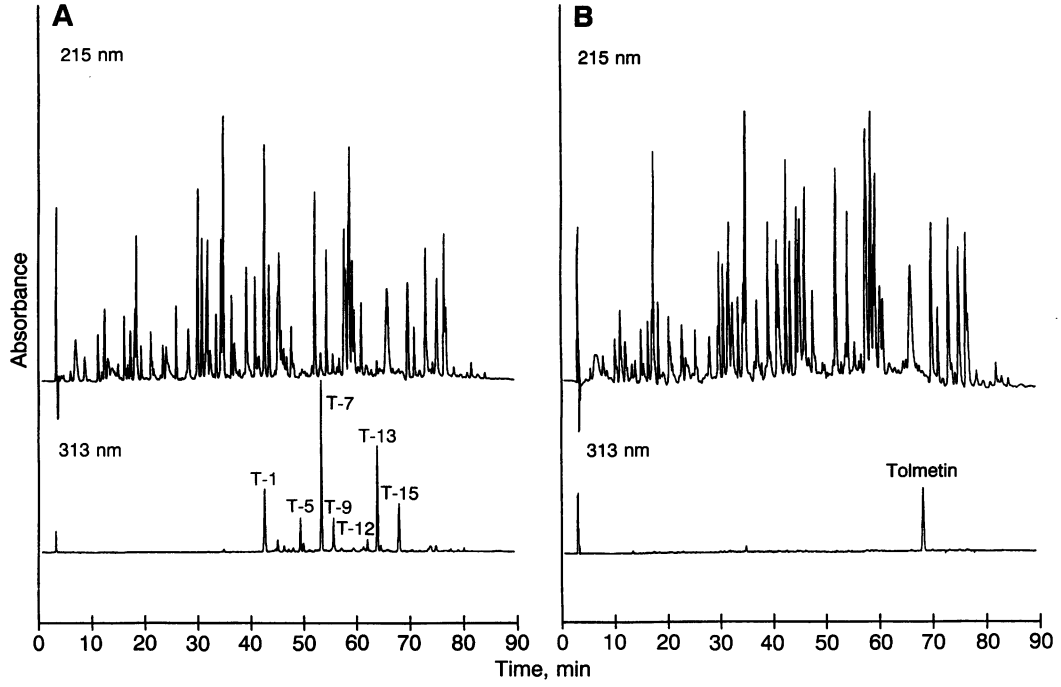


FIG. 2. (*A*) HPLC of the tryptic digest of the reaction product from the incubation of tolmetin glucuronide with HSA in the presence of $NaCNBH_3$, monitored at 215 nm (upper trace) and 313 nm (lower trace, for tolmetin-containing compounds). (*B*) HPLC of product from control experiment in which tolmetin was substituted for tolmetin glucuronide. The peak at 313 nm is tolmetin, which is equivalent to peak T-15 in *A*.

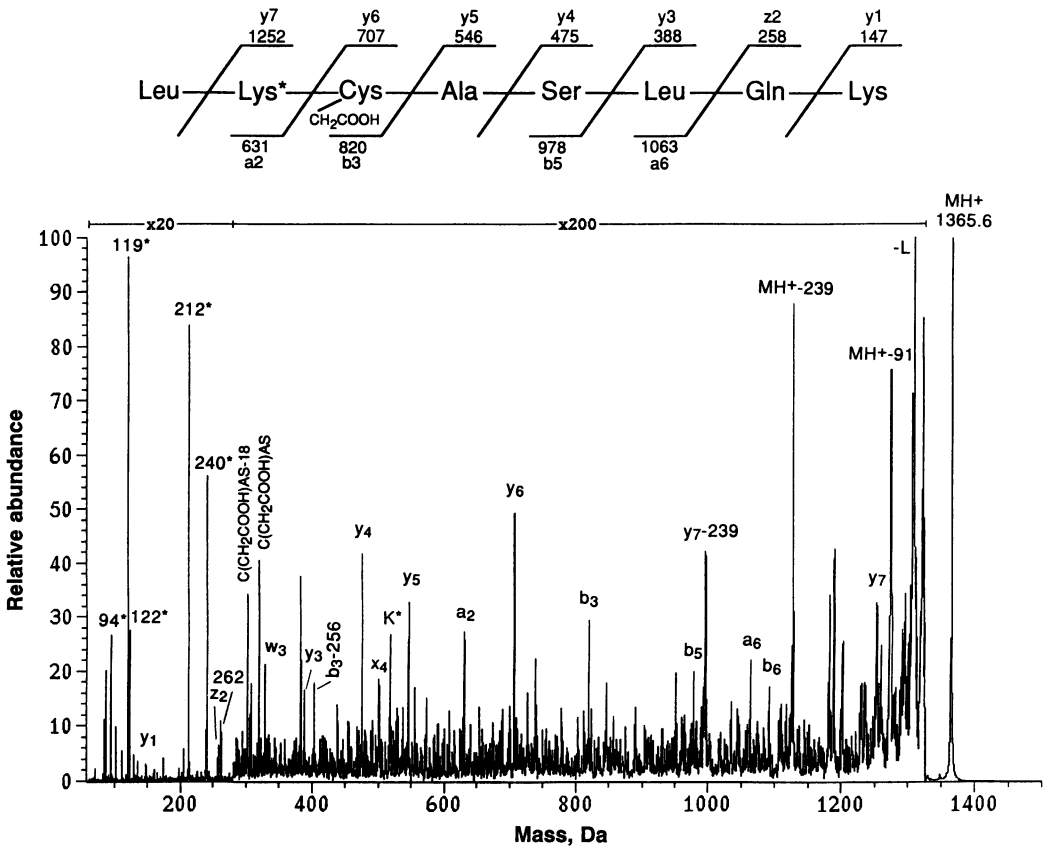


FIG. 3. CID mass spectrum for molecular ion ($M+H^+$) of $m/z$ 1365.6 in HPLC fraction T-7, which was eluted at 52.5 min. Significant ions indicating the sequence of the modified peptide are depicted above the spectrum. Fragments containing Lys-199 (Lys*) show a shift of 417 Da for tolmetin glucuronide, indicating that this lysine is the site of covalent binding. Starred items in the spectrum indicate fragments from the tolmetin moiety.

situated at the focal point between mass spectrometers I and II. The collision cell was floated at 4 keV and filled with sufficient helium so that the parent ion intensity was attenuated to one-third of its initial value. Again, 1–2 μl of the sample was used with the same matrix as above. The CID nomenclature for peptide dissociation processes follows that of Roepstorff and Fohlman (18) as modified by Biemann (19).

## RESULTS

In the HPLC separation of the digest deriving from incubation with tolmetin glucuronide, we observed three relatively intense peaks (T-1, T-7, and T-13) and several minor peaks (T-5, T-9, T-12, and T-15) at 313 nm (Fig. 2*A*). Only one peak at 313 nm was observed in the digest from the control incubation with tolmetin (Fig. 2*B*). This peak was identical with peak T-15, representing unbound tolmetin. The remaining six tolmetin-containing peaks were directly identified by LSIMS/CID.

The component that was eluted at 52.5 min (T-7) gave a strong molecular ion ($M+H^+$) of 1365.6 Da on LSIMS using the $^{12}C$ mass scale. This mass corresponds to a tryptic peptide containing amino acids 198–205 of HSA [Leu-Lys-Cys($CH_2CO_2H$)-Ala-Ser-Leu-Gln-Lys; $M+H^+$ at 948.5] plus

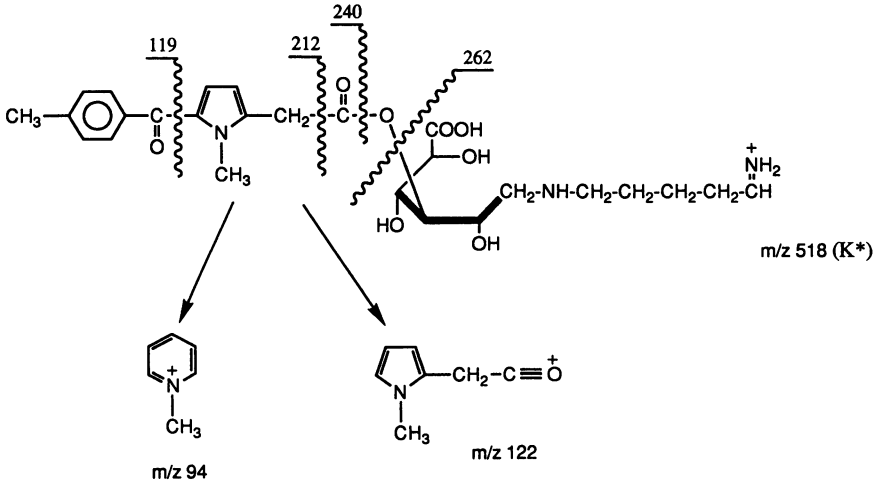


FIG. 4. Characteristic fragment ions indicating tolmetin glucuronide bound to a lysine of a peptide. The tolmetin group is depicted attached to the 3 position of the glucuronic acid moiety, for convenience. Our results do not allow determination of the exact position.

Table 1. Tolmetin-containing fractions (monitored at 313 nm) with assigned binding sites

| Fraction | % bound† | M+H⁺, *m/z* | Sequence‡ | HSA residues | Binding site |
|---|---|---|---|---|---|
| T-1 | 20, 14 | 1164.6 | ASSAK*QR | 191–197 | Lys-195 |
| T-5 | 6, 6 | 1234.6 | ATK*EQLK | 539–545 | Lys-541 |
| T-7 | 40, 33 | 1365.6 | LK*C(Cm)ASLQK | 198–205 | Lys-199 |
| T-9 | 9, 5 | 1713.8 | LAK*TYETTLEK | 349–359 | Lys-351 |
| T-12 | 2, 2 | 1472.6 | K*YLYEIAR§ | 137–144 | Lys-137 |
| T-13 | 14, 20 | 1545.8 | K*QTALVELVK | 525–534 | Lys-525 |

†Relative binding, calculated as percentage of total absorbance at 313 nm minus absorbance of free tolmetin (T-15), determined from two similar incubation experiments.
‡K* represents the lysine residue with an attached tolmetin glucuronide (additional 417 Da). Cm represents carboxymethylation of cysteine (C).
§Sequence assignment from M+H⁺ only (see Fig. 5).

a covalently bound tolmetin glucuronide moiety (417.1 Da). Interpretation of the CID spectrum (Fig. 3) confirmed this peptide sequence and revealed the site of covalent modification by the shift in the mass of several of the sequence ions ($a_2$, $b_3$, $b_5$, $a_6$, $b_6$, and $y_7$) by 417 Da. In addition, the most abundant nonsequence ions (*m/z* 94, 119, 122, 212, 240, M+H⁺ − 239, and M+H⁺ − 91) including the modified lysine immonium ions at *m/z* 262 and 518 (K*) establish the structural integrity of tolmetin glucuronide bound to the ε-amino group of lysine (Fig. 4). Modification of Lys-199 by a large bulky group is consistent with the exclusive isolation of the modified 198–205 tryptic peptide sequence.

Similarly, we identified the tryptic digest peak T-13, eluted at 63.5 min (M+H⁺ at 1545.8) and the three other repurified fractions T-1, T-5, and T-9 (Table 1). The amount of repurified fraction T-12 was too small to allow complete confirmation of its sequence. Fig. 5 depicts the LSIMS spectrum for peak T-12 and the low-mass part of the CID spectrum deriving from the molecular ion at *m/z* 1472.6, suggesting that the sequence includes amino acids 137–144 of HSA and that binding occurs at Lys-137.

The molar binding ratio, under the conditions used in this *in vitro* experiment, was between 0.2 and 0.4 mol of tolmetin per mol of HSA, as calculated from the total absorbance at 313 nm with tolmetin as an external standard. The six identified binding sites are responsible for 91% and 80% of the total binding in the two incubation experiments described here (Table 1).

## DISCUSSION

The data provide conclusive evidence for the covalent binding of tolmetin glucuronide to HSA by Schiff base formation with lysine ε-amino groups. In all of the identified tolmetin-containing peptides, the glucuronic acid moiety was present. Tolmetin itself did not bind covalently to HSA under the conditions tested. Our results indicate, furthermore, that several different tolmetin glucuronide binding sites, rather than only one specific site, are involved during *in vitro* incubation. The most prominent binding area, however, includes Lys-195 and -199. Lys-199, located strategically in the hydrophobic pocket of subdomain IIA (20), seems to be one of the most reactive and the best investigated sites for xenobiotic modifications. It is a target for covalent binding of acetylsalicylate (21) and benzylpenicilloyl groups (22) and for nonenzymatic glycation (23). It has also been concluded on the basis of several displacement studies that Lys-195 and Lys-199, as well as the lone tryptophan (at 214), are part of the warfarin binding area (reviewed in ref. 24).

Lys-525 has been identified as the primary site for nonenzymatic glycation (23, 25), with Lys-351 also involved to some extent. We report here the covalent binding of a xenobiotic at Lys-525. It seems likely that the presence of the glucuronic acid moiety facilitates the linkage of tolmetin to this site.

Our data do not provide any evidence for binding to subdomain IIIA, the second principal binding region on HSA (20), a binding pocket with Arg-410 and Tyr-411 in its center.

The results are consistent with our earlier observations that the yields of covalently bound adducts *in vitro* are enhanced

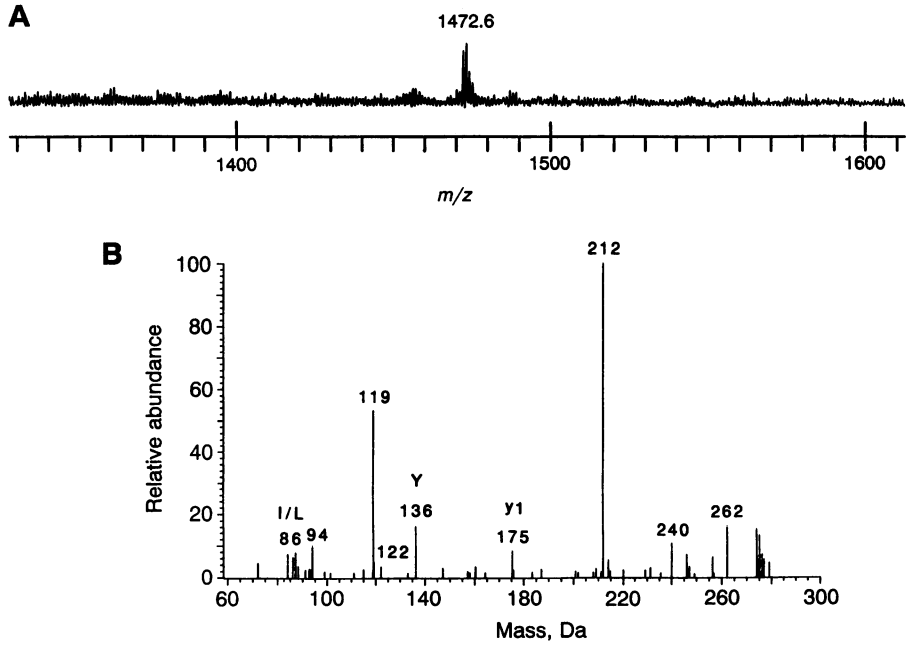


FIG. 5. (*A*) Liquid secondary-ion mass spectrum of HPLC fraction T-12. (*B*) Part of the CID mass spectrum for molecular ion of *m/z* 1472.6 in HPLC fraction T-12. The limited data, due to the low sample amount (<25 pmol), prove the presence of tolmetin glucuronide (compare with Fig. 4) and show evidence for some amino acids ($y_1$ for Arg, immonium ions for Tyr and Ile/Leu). The mass for the molecular ion matches with the peptide Lys*-Tyr-Leu-Tyr-Glu-Ile-Ala-Arg (HSA residues 137–144) plus 417 Da. Lys-137, the only possible binding site in this peptide, is responsible for about 2% of the total binding of tolmetin glucuronide to HSA.

in the presence of NaCN or $NaCNBH_3$ (9, 11, 26) and that preincubation with acetylsalicylic acid, which is known to acetylate Lys-199, decreases the covalent binding of zomepirac glucuronide to HSA *in vitro* (9). Though our findings do not rule out the possibility that covalent binding can also occur via nucleophilic displacement, they do establish that imine formation is a major reaction pathway in the formation of albumin adducts with acyl glucuronides *in vitro*. As depicted in Fig. 1, condensation of the aldehyde group of the ring-opened tautomer with a lysine ε-amino moiety must be preceded by acyl migration (8, 9). Therefore, linkage of the protein to the glucuronide in the adducts we have detected is via the C-1 carbon of the sugar. However, the attachment of the tolmetin group in the final adducts could be to the 2, 3, or 4 position of the sugar because of acyl migration.

Rearrangement of acyl glucuronides to 2-, 3-, and 4-positional isomers was first proposed by Goebel (27) in 1938, to explain the mutarotation of benzoic acid glucuronide, and was confirmed by spectroscopic techniques for bilirubin glucuronides in 1978 (28). Pharmacologists, however, have only recently begun to recognize that isomerization is a general reaction of acyl glucuronides and that acyl glucuronides, when they accumulate in the body, may exist as mixtures of stereoisomers with different reactivities, binding properties, pharmacokinetics, and stabilities. The present findings reveal an additional layer of complexity. They show that both cyclic and open-chain tautomers of acyl glucuronide isomers can also occur and that the latter may play a key, and previously overlooked, role in the biological reactivity of glucuronic acid conjugates of carboxylated substrates. Thus, both cyclic and open-chain conformers of acyl glucuronide stereoisomers may need to be taken into account when the biochemistry of glucuronide conjugates is considered.

The advantages and power of tandem MS in locating binding sites and elucidating binding mechanisms have been demonstrated in this study. Key tryptic fragments from modified albumin molecules which were present as only a small proportion of the total protein were readily identified. Direct structural characterization by the method described here contrasts favorably with the rather incomplete information derivable by techniques such as Edman sequencing. Moreover, MS-based strategies require much less extensive purification of the compounds of interest. Analogous applications of this MS methodology have been reported recently, from this and other laboratories, in studies of binding of styrene oxide to hemoglobin (29), epoxycreatine to creatine kinase (30), and acrylodan to interleukin 1β (31).

The confirmation of covalent binding of tolmetin to protein with retention of the glucuronic acid moiety within the adduct is consistent with *in vitro* and *in vivo* binding data previously reported for other carboxylic acid drugs investigated in our laboratory (2, 32). It was suggested (2) that this identification of a common constituent within the adducts of many drugs might explain the immunologic cross reactivities observed with several nonsteroidal antiinflammatory drugs. The mechanism (Fig. 1) consistent with the results described here leads to an identical functional group—i.e., the glucuronic acid moiety—in the potential hapten. Critical testing of this hypothesis will require immunologic studies.

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1. Faed, E. M. (1984) *Drug Metab. Rev.* **15**, 1213–1249.
2. Spahn-Langguth, H. & Benet, L. Z. (1992) *Drug Metab. Rev.* **24**, 5–48.
3. McDonagh, A. F., Palma, L. A., Lauff, J. J. & Wu, T.-W. (1984) *J. Clin. Invest.* **74**, 763–770.
4. van Breemen, R. B., Fenselau, C., Mogilvesky, W. & Odell, G. B. (1986) *J. Chromatogr.* **383**, 387–392.
5. van Breemen, R. B. & Fenselau, C. (1985) *Drug Metab. Dispos.* **13**, 318–320.
6. Ruelius, H. W., Kirkman, S. K., Young, E. M. & Janssen, F. W. (1986) *Adv. Exp. Med. Biol.* **197**, 431–441.
7. Wells, D. S., Janssen, F. W. & Ruelius, H. W. (1987) *Xenobiotica* **17**, 1437–1449.
8. Smith, P. C., McDonagh, A. F. & Benet, L. Z. (1986) *J. Clin. Invest.* **77**, 934–939.
9. Smith, P. C., Benet, L. Z. & McDonagh, A. F. (1990) *Drug Metab. Dispos.* **18**, 639–644.
10. Hodge, J. E. (1955) *Adv. Carbohydr. Res.* **10**, 169–206.
11. Munafo, A., McDonagh, A. F., Smith, P. C. & Benet, L. Z. (1990) *Pharm. Res.* **7**, 21–27.
12. Hyneck, M. L., Smith, P. C., Munafo, A., McDonagh, A. F. & Benet, L. Z. (1988) *Clin. Pharmacol. Ther.* **44**, 107–114.
13. Bretza, J. A. & Novey, H. S. (1985) *West. J. Med.* **143**, 55–59.
14. Aberth, W., Straub, K. M., Walls, F. C. & Burlingame, A. L. (1982) *Anal. Chem.* **54**, 2029–2034.
15. Falick, A. M., Wang, G. H. & Walls, F. C. (1986) *Anal. Chem.* **58**, 1308–1311.
16. Walls, F. C., Baldwin, M. A., Falick, A. M., Gibson, B. W., Kaur, S., Maltby, D. A., Gillece-Castro, B. L., Medzihradszky, K. F., Evans, S. & Burlingame, A. L. (1990) in *Biological Mass Spectrometry*, eds. Burlingame, A. L. & McCloskey, J. A. (Elsevier, Amsterdam), pp. 197–216.
17. Cottrell, J. S. & Evans, S. (1987) *Rapid Commun. Mass Spectrom.* **1**, 1–2.
18. Roepstorff, P. & Fohlman, J. (1984) *Biomed. Mass Spectrom.* **11**, 601–603.
19. Biemann, K. (1988) *Biomed. Environ. Mass Spectrom.* **16**, 99–111.
20. He, W. M. & Carter, D. C. (1992) *Nature (London)* **358**, 209–215.
21. Walker, J. E. (1976) *FEBS Lett.* **66**, 173–175.
22. Yvon, M. & Wal, J.-M. (1988) *FEBS Lett.* **239**, 237–240.
23. Iberg, N. & Flückiger, R. (1986) *J. Biol. Chem.* **261**, 13542–13545.
24. Kragh-Hansen, U. (1990) *Dan. Med. Bull.* **37**, 57–84.
25. Garlick, R. L. & Mazer, J. M. (1983) *J. Biol. Chem.* **258**, 6142–6146.
26. Ojingwa, J. C. & Benet, L. Z. (1990) *Pharm. Res.* **7**, S-257 (abstr.).
27. Goebel, W. F. (1938) *J. Biol. Chem.* **122**, 649–653.
28. Compernolle, F., Van Hees, G. P., Blanckaert, N. & Heirwegh, K. P. M. (1978) *Biochem. J.* **171**, 185–201.
29. Kaur, S., Hollander, D., Haas, R. & Burlingame, A. L. (1989) *J. Biol. Chem.* **264**, 16981–16984.
30. Buechter, D. D., Medzihradszky, K. F., Burlingame, A. L. & Kenyon, G. L. (1992) *J. Biol. Chem.* **267**, 2173–2178.
31. Yem, A. W., Epps, D. E., Mathews, W. R., Guido, D. M., Richard, K. A., Staite, N. D. & Deibel, M. R., Jr. (1992) *J. Biol. Chem.* **267**, 3122–3128.
32. Benet, L. Z., Spahn-Langguth, H., Iwakawa, S., Volland, C., Mizuma, T., Mayer, S., Mutschler, E. & Lin, E. T. (1993) *Life Sci.*, in press.