Synthesis and mass-spectrometric characterization of human serum albumins modified by covalent binding of two non-steroidal anti-inflammatory drugs: tolmetin and zomepirac

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Human serum albumins modified by covalently bound tolmetin or zomepirac were synthesized as models for similar products formed *in vivo* from acyl glucuronides. Activated esters of both drugs were prepared with 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide, and then allowed to react with human serum albumin. Tryptic digests of both protein products were analysed

by HPLC to identify peptides containing covalently bound drugs, and binding sites on albumin were identified by high-performance tandem MS. Three binding sites were common to both products, i.e. lysine-195, -199 and -351. Three further modified residues were identified for the tolmetin-albumin product, i.e. aspartic acid 1, and lysine-524 and -536.

INTRODUCTION

Many non-steroidal anti-inflammatory drugs (NSAIDs), including tolmetin (T) and zomepirac (Z), contain carboxylic acid functional groups. A common metabolite of these drugs, the acyl glucuronide conjugate, has been shown to react with plasma proteins *in vitro* and *in vivo* to form protein adducts [1–6]. Two mechanisms have been proposed for this reaction [4]. One is a displacement mechanism in which a nucleophilic group (NH₂, OH, SH) of the protein attacks the acyl carbon of the aglycone, displacing the glucuronic acid and forming an amide, ester, or thioester bond between the protein (P) and the drug (D) (D–P). The other involves formation of a Schiff base (imine) intermediate with amino groups of the protein. In this mechanism, glucuronic acid (G) is not displaced but instead acts as a bridge between the protein and drug moiety (D–G–P). We have shown that protein adducts formed between tolmetin glucuronide and albumin *in vitro* contain tolmetin bound to amino acid residues via a glucuronic acid bridge (T–G–P) and tolmetin bound directly to residues (T–P) [2]. Thus, tolmetin glucuronide reacts with HSA *in vitro* via both the Schiff base and the nucleophilic displacement mechanisms. Presumably, one or both of these mechanisms may be involved in the formation of protein adducts *in vivo*.

A number of acidic NSAIDs (including tolmetin and zomepirac) have elicited potentially fatal hypersensitivity reactions (including anaphylaxis, bronchospasm and hepatotoxicity) [7–10]. We have hypothesized that the allergic-like reactions and the organ toxicities may occur via an immune-based mechanism whereby the acyl glucuronide metabolite of acidic NSAIDs acts as a hapten upon covalent binding to a protein. Previously, we developed a useful method to generate the product of the Schiff base mechanism (T–G–P) [1] and demonstrated that this protein adduct is immunogenic in mice and induces antibodies specific for both the drug and the drug–glucuronide [11]. It is possible, however, that the protein adducts are formed primarily through the nucleophilic displacement mechanism *in vivo*. Our next aim, therefore, was to determine the immunogenic ability of a protein

conjugate formed primarily via the nucleophilic displacement mechanism (D–P). Thus, in the present study, we have developed a useful method for generating the albumin adducts of tolmetin and zomepirac (T–P and Z–P) via the nucleophilic displacement reaction. In addition, we have characterized the albumin residues involved in adduct formation.

MATERIALS AND METHODS

Materials

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

Synthesis of modified human serum albumin (HSA)

Equimolar amounts (10 mM) of drug and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC·HCl) were allowed to react for 30 min in water with addition of dilute HCl to maintain a pH of 6.0–6.5 (at room temperature); HSA (0.23 mM) was then added and the reaction continued for 1 h [12]. The solution was centrifuged and the supernatant was concentrated using Amicon Centriprep tubes (30 kDa-molecular-mass cut-off; Amicon Division, Beverly, MA, U.S.A.). The modified protein solution was subjected to seven ultrafiltration cycles after dilution with water each time using the Centriprep tubes. The protein solution was then freeze-dried to give crude product.

Tryptic digestion

The method of Ding et al. [1] was followed. The crude protein (1.5 mg) was dissolved in denaturing buffer (100 µl; 100 mM Tris, 1 mM EDTA, 6 M guanidine hydrochloride, pH 8.3) and heated with 3 M dithiothreitol (2 µl) under argon at 55 °C for 1 h to reduce disulphide bonds. The free thiol groups were alkylated by incubation with iodoacetic acid (4 µl of a 0.5 mg/µl

Abbreviations used: NSAIDs, non-steroidal anti-inflammatory drugs; HSA, human serum albumin; LSIMS, liquid secondary-ion MS; CID, collision-induced dissociation.

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source [14,15] and an electro-optical multichannel array detector [16]. The ^{12}C component of the protonated peptide molecules was subjected to collision-induced-dissociation (CID) MS.

RESULTS

Three intense peaks (T-1, T-5, T-7) were observed in the HPLC chromatogram of the digest from the T-HSA preparation (Figure 1b). 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 313 nm peak detected in control experiments with 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 (Figure 1b).

The peptide that eluted at 48 min (T-1) gave an abundant protonated molecular ion signal using LSIMS ionization at 986.4 Da (^{12}C mass scale). This mass corresponds to a protonated tryptic peptide containing amino acids 191–197 of HSA (Ala-Ser-Ser-Ala-Lys-Gln-Arg; $M + \text{H}^+$ at m/z 747.4) plus a covalently bound tolmetin moiety (239 mass units). Interpretation of the high-energy CID spectrum (Figure 2) confirmed this peptide sequence and revealed the site of covalent modification by the

Figure 3 Fragment ions consistent with tolmetin bound to a lysine of a peptide

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

Fraction	Percentage bound*	$M + \text{H}^+$	Sequence†	HSA residues	Binding site
T-1	25	986.4	ASSAKQR	191–197	Lys-195
T-2	4	709.4	DAHK	1–4	Asp-1
T-3	7	748.4	HKPK	535–538	Lys-536
T-4	7	755.4	QIKK	522–525	Lys-524
T-5	22	1187.6	LKC(Cm)ASLQK	198–205	Lys-199
T-6	3	1535.8	LAKTYETLLEK	349–359	Lys-351
Z-1	14	1020.4	ASSAKQR	191–197	Lys-195
Z-2	19	1221.6	LKC(Cm)ASLQK	198–205	Lys-199
Z-3	4	1569.8	LAKTYETLLEK	349–359	Lys-351

* Relative binding, calculated as percentage of total absorbance at 313 nm minus absorbance of free drug (T-7, Z-4).

† Cm represents carboxymethylation of cysteine (C).

Figure 4 HPLC chromatograms of the digested Z-HSA product: (a) 215 nm, the peptide trace, (b) 313 nm, the modified peptides containing zomepirac

Fractions Z-1, Z-2 and Z-3 were subjected to MS. Fraction Z-4 contained free zomepirac.

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 non-consequence ions (m/z 94, 119, 212, 240 and $M - \text{H}^+ - 213$) establish the presence of tolmetin on the modified peptide. The signal from the loss of the modified lysine at ($M + \text{H}^+ - 311$) designated as $-K^A$ (Figures 2 and 3) 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 ($M + \text{H}^+$ at 709.4), T-3, which eluted at 49.5 min ($M + \text{H}^+$ at 748.4), T-4, which eluted at 54 min ($M + \text{H}^+$ at 755.4), T-5, which eluted at 58.5 min ($M + \text{H}^+$ at 1187.6), and T-6, which eluted at 61.5 min ($M + \text{H}^+$ at 1538.5) (Figure 1b, Table 1). These results and the signals correspond to peptides containing the following modified amino acids: Lys-195, Asp-1, Lys-536, Lys-524, Lys-199, Lys-351. The modification of Asp-1 occurs at the N-terminus.

Three binding sites in the Z-HSA product 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 depicted in Figure 4. The CID chromatogram (Figure 5) of the peptide corresponding to the peak labelled Z-2 in Figure 4 shows an $M + \text{H}^+$ 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 and 274) indicate the presence of zomepirac attached to the peptide (Figures 5 and 6). 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 (Figure 5). The molecular ions corresponding to the other two identified modified peptides are $M + \text{H}^+$ at 1020.4 (Z-1, Figure 4b) and 1569.8 (Z-3, Figure 4b) (Table 1).

Figure 5 The CID mass spectrum of the $^{12}\text{C}^{35}\text{Cl}$ isotope (m/z 1221.6) of the molecular-ion stable isotope cluster from fraction 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. Lys†-199 is the modified amino acid.

Figure 6 Fragment ions consistent with zomepirac bound to lysine of a peptide

The molar binding ratio for the T-HSA product was 0.6 mol of drug per mol of HSA and 0.5 for the Z-HSA product. The identified binding sites represent 68% and 37% of the total binding of T-HSA and Z-HSA respectively (Table 1), assuming no loss of bound drug during protein digestion.

DISCUSSION

The predominant reaction sites on HSA for activated esters of tolmetin and zomepirac were the ϵ -amino groups of lysine-195 and -199. Reaction with hydroxyl or thiol groups were not detected, but can not be ruled out because only the most abundant HPLC fractions containing T or Z were examined. However, lysine-195 and -199 together accounted for 47% and 33% of the covalently bound T and Z respectively (Table 1). These lysines

are both located in the hydrophobic pocket of subdomain IIA of HSA. Lysine-199 is a favoured site for interaction of small molecules with HSA and is the site for covalent binding of aspirin, glucose and benzylpenicilloyl groups [17–19]. Interestingly, lysine-195 and -199 account for 50–60% of the reaction of tolmetin glucuronide with HSA, even though this occurs via a different mechanism.

Curiously we observed binding of tolmetin to lysine-524 (7% of overall binding) yet not to lysine-525. Both of these residues are located in subdomain IIIB of HSA. Lysine-525 is a major site for non-enzymic glycosylation of albumin (33% of overall binding) [18] and a major site (14–20% of overall binding) for reaction with tolmetin glucuronide [1]. Both glycosylation and drug-glucuronide binding occur initially via formation of the respective Schiff base. The preferential reaction of tolmetin-activated esters with Lys-524 may reflect differences in the molecular dimensions of tolmetin glucuronide and the tolmetin-activated ester, or it may reflect differences in the pK_a values of lysines 524 and 525.

Of the observed minor reaction sites, Lys-351, in subdomain IIB, is also a reactive site for glucose [18] and tolmetin glucuronide [1]. However, lysine-536, which is in subdomain IIIB, has not previously been reported as a reactive site for covalent binding of compounds.

This study shows that reaction of carbodi-imide-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. The modified amino acids on the protein can be identified by MS/MS using very small quantities of partially purified material. Thus, this technique is a sensitive method for detecting and identifying nucleophilic displacement reaction products formed between albumin and acyl glucuronides. In addition, this work provides a useful method for generating drug-protein

(D-P) adducts. We are currently investigating the immunogenicity of these adducts.

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