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MECHANISMS FOR COVALENT BINDING OF BENOXAPROFEN GLUCURONIDE TO HUMAN SERUM ALBUMIN

Studies by Tandem Mass Spectrometry

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ABSTRACT:

Tandem MS has been used to establish the structure and specific binding sites of covalent protein adducts formed upon incubation of the acyl glucuronide of the propionic acid nonsteroidal anti-inflammatory drug benoxaprofen with human serum albumin *in vitro*. Benoxaprofen 1-*O*-β-glucuronide was enzymatically synthesized *in vitro* and incubated with human serum albumin both in the presence and in the absence of $NaCNBH_3$. The modified human serum albumins were digested with trypsin and separated by HPLC. The modified peptides were detected using HPLC-electrospray MS (with selected-ion monitoring) and were structurally characterized by tandem MS using matrix-assisted laser desorption ionization in both the post-source decay and high-energy collision-induced dissociation modes. These studies established that benoxaprofen glucuronide forms covalent adducts with protein nucleophiles both by nucleophilic displacement of glucuronic acid at the anomeric center and by condensation of the rearranged acyl glucuronic acid isomers with ϵ-amino functions of lysine residues after acyl migration of the aglycone from the anomeric center. Lys-159 was identified as the major binding site. Thus, we have established that members of the less reactive propionic acid class of acyl glucuronides, such as the glucuronide of benoxaprofen, are also capable of reacting with protein nucleophiles to form covalent adducts analogous to those of tolmetin glucuronide (tolmetin is an acetic acid nonsteroidal anti-inflammatory drug), *via* the mechanisms previously reported from this laboratory, and that the specific covalent binding site profile appears to be drug dependent.

The formation of acyl glucuronides is a major metabolic pathway for many compounds containing a carboxylic acid function, especially NSAIDs.[1] These acyl glucuronides can undergo hydrolysis as well as spontaneous isomerization from the anomeric center at physiological pH and are capable of reacting with protein nucleophiles, to form covalent adducts, either directly or after isomerization and tautomerism (Ding *et al.*, 1995; Faed, 1984). Some of these drugs cause toxic effects such as hypersensitivity (Zia-Amirhosseini *et al.*, 1995) and idiosyncratic liver toxicity (Boelsterli *et al.*, 1995), raising the question of whether covalent modification of proteins could play an important role in initiating these effects. Several recent studies have been carried out to establish the mechanisms for formation of covalent protein adducts with this class of drug metabolites. One mechanism involves nucleophilic displacement of the glucuronosyl group by $-NH_2$ (van Breemen *et al.*, 1986; McDonagh *et al.*, 1984), -SH (van Breemen and Fenselau, 1985), or -OH (Wells *et al.*, 1987; Ruelius *et al.*, 1986) groups of the protein, to form covalent drug-protein adducts devoid of the glucuronic acid group. Another mechanism (Smith *et al.*, 1986, 1990) involves the spontaneous migration of the acyl group to the 2-, 3-, or 4-hydroxyl groups of the sugar moiety, tautomerization of the pyranose ring to its aldose form, and condensation of the aldehyde group of the ring-opened tautomer with a lysine ϵ-amino on the protein to form an imine. Stabilization of such an imine adduct would be expected to occur slowly *in vivo*, by spontaneous Amadori rearrangement to the more stable 1-amino-1-deoxy-ketose analogue (Hodge, 1955). However, the reversibly formed imine can be converted to the corresponding secondary amine in the presence of $NaCNBH_3$, thus increasing the chemical stability and yield for structural analysis (Ding *et al.*, 1995; Smith *et al.*, 1990; Munafo *et al.*, 1990; Ding *et al.*, 1993).

Previous contributions from this laboratory have reported the structural nature and extent of adduct formation of TG (tolmetin is an acetic acid NSAID) with HSA, both in the presence and in the absence of an imine-reducing agent (Ding *et al.*, 1993, 1995). Those studies established that protein adducts are formed by both mechanisms and that Lys-199 is the major binding site for both adducts, together with a number of less prominent binding sites.

Recently, we extended our studies to include the acyl glucuronides of propionic acid NSAIDs, which are much less chemically reactive than are those of acetic acid NSAIDs, because of steric hindrance and electron-donating effects of the α-methyl group. Now we report results on the reactivity of benoxaprofen [2-(*p*-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid] with HSA.

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[1] Abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; BG, benoxaprofen-1-*O*-β-glucuronide; SIM, selected-ion monitoring; CID, collision-induced dissociation; PSD, post-source decay; HSA, human serum albumin; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; TG, tolmetin glucuronide.

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Benoxaprofen has been clinically effective in the treatment of rheumatoid arthritis and osteoarthritis (Dawson *et al.*, 1982). More than 60% of this drug is metabolized to glucuronides in humans (Chatfield and Green, 1978). Although the drug was withdrawn from the market because of its fatal liver toxicity (Duthie *et al.*, 1982; Goudie *et al.*, 1982), it can be detected easily with high sensitivity because of its fluorescence properties, making it a useful model compound for structural investigation of low-level covalent binding to proteins (Spahn *et al.*, 1990).

Materials and Methods

Chemicals. (*R*/*S*)-Benoxaprofen was obtained from Eli Lilly (Bad Homburg, Germany), before it was withdrawn from the market. Tetrabutylammonium hydroxide was obtained from Aldrich Chemical Co. (Milwaukee, WI). HSA and all other chemicals were from Sigma Chemical Co. (St. Louis, MO). HPLC-grade solvents were obtained from Fisher Scientific (Pittsburgh, PA).

Synthesis and Purification of BG. Enzymatic synthesis and purification procedures were optimized by starting from those previously described (Ojingwa, 1994; Spahn *et al.*, 1989). Briefly, 10 mg/ml sheep liver microsomal protein, 10 mM magnesium chloride, 20 mM saccharic acid-1,4-lactone, 2 mM phenylmethylsulfonyl fluoride, and 100 mM Tris-HCl buffer, pH 6.9, were mixed and preincubated at 37°C for 10 min. Then 15 mM UDP-glucuronic acid and 1 mM benoxaprofen (added as a solution in methanol) were added to the incubation mixture. The final pH of the mixture was adjusted to 6.0 or 7.0 before continued incubation at 37°C. Product formation in the reaction mixture was monitored by HPLC. Aliquots (50 μl) were taken from the mixture at 30-min intervals up to 3 hr. Proteins were precipitated with 950 μl of acetonitrile/50 mM ammonium acetate, pH 4.5. After centrifugation, the supernatant was extracted three times with equal volumes (1 ml) of ethyl acetate. The ethyl acetate phase was dried with sodium sulfate. After filtration, the organic solvent was removed using a rotary evaporator. The residue was reconstituted in HPLC mobile phase and further purified by analytical HPLC (5 mm, C_{18}, 4.6 $\times$ 250 mm; Vydac, Hesperia, CA). The mobile phase contained 28% acetonitrile in 10 mM tetrabutylammonium hydroxide buffer, pH 6.0 (flow rate, 1.5 ml/min). The eluant was monitored on-line with a Shimadzu RF535 fluorescence detector (Shimadzu Corp., Kyoto, Japan), using an excitation wavelength of 313 nm and an emission wavelength of 365 nm, and a Carlo Erba UV detector (UV absorbance at 254 nm). The main fraction containing BGs was lyophilized. The purity of the final product was approximately 92% (assessed by analytical HPLC using benoxaprofen as a standard).

Adduct Formation. *In the Presence of Sodium Cyanoborohydride.* Essentially fatty acid-free HSA (40 nmol) was incubated with BGs (120 nmol) and $NaCNBH_3$ (600 nmol) in 88 μl of 100 mM sodium phosphate buffer, pH 7.4, at 37°C for 96 hr.

Without Sodium Cyanoborohydride. HSA (40 nmol) and BG (2 μmol) were incubated in 88 μl of 100 mM sodium phosphate buffer, pH 7.4, at 37°C for 96 hr. The reaction was terminated by centrifugal filtration (Centricon 30, molecular weight cut-off of 30,000; Amicon, Danvers, MA) to remove the low-molecular weight reactants, followed by five centrifugations of the protein adduct in aliquots (1 ml) of water. Control experiments were performed under the same conditions but using benoxaprofen itself.

Trypsin Digestion of the Protein. The protein adduct mixtures (40 nmol) were reduced with dithiothreitol (24 μmol) in 250 μl of buffer A (6 M guanidine-HCl, 100 mM Tris, 1 mM EDTA, pH 8.3) for 1 hr at 60°C, alkylated with iodoacetic acid (52.4 μmol) at room temperature in the dark for 30 min, and then dialyzed overnight against a 100 mM ammonium bicarbonate buffer, using a microdialysis apparatus (Bethesda Research Labs) equipped with a membrane with a molecular weight cut-off of 8000. The dialyzed protein was then digested with 2% (w/w) trypsin (Promega, Madison, WI) overnight at 37°C.

HPLC. A portion of the tryptic digests was reserved for on-line HPLC-electrospray MS and SIM analysis. The remaining 95% of the tryptic peptides were separated by reverse-phase chromatography on a Beckman HPLC system using a Vydac C_{18} column (250 $\times$ 4.6 mm). The gradient used was 98% solvent A (0.1% trifluoroacetic acid in water)/2% solvent B (0.08% trifluoroacetic acid in acetonitrile) to 55% solvent A/45% solvent B over 90 min, at a flow rate of 1 ml/min. The elution was monitored on-line both with a Shimadzu RF535 fluorescence detector, using an excitation wavelength of 313 nm and an emission wavelength of 365 nm, and with a Carlo Erba detector (UV absorbance at 215 nm). Fractions showing a fluorescence peak at 365 nm were collected, and each fraction was concentrated to 10 μl for MS analysis.

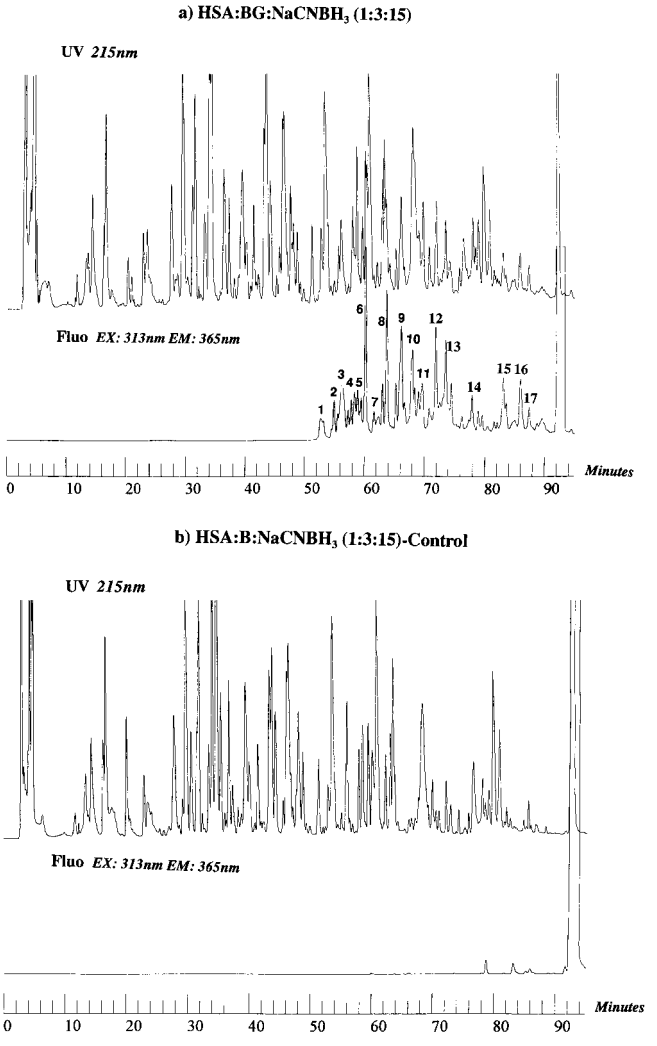

FIG. 1. *HPLC chromatograms of tryptic digests obtained after incubation of HSA with BG and $NaCNBH_3$ (at a HSA/BG/$NaCNBH_3$ molar ratio of 1:3:15) for 96 hr (a) and HPLC chromatograms of tryptic digests from a control experiment in which incubation conditions were the same as in* a *but with benoxaprofen instead of BG (*b*).*

a, *Upper*, profile of UV absorbance at 215 nm; *lower*, profile of fluorescence emission at 365 nm with excitation at 313 nm.

MS Analysis of the Modified Peptides. *Electrospray MS and SIM.* A microbore HPLC system (Applied Biosystems model 140B) was interfaced with a VG Platform II single-quadrupole mass spectrometer (Fisons/VG) equipped with an electrospray source. The mass spectrometer was scanned, in the noncontinuum mode, from *m/z* 295 to *m/z* 2000 in 5 sec. For the SIM experiment, the voltage of the sampling cone was increased from 40 to 200 V to induce fragmentation. A fragment-ion chlorine isotope doublet characteristic of the presence of the benoxaprofen moiety (*m/z* 256 for ^{35}Cl and 258 for ^{37}Cl) was chosen for detection of covalently modified peptides. This mass value of the most abundant ^{35}Cl isobar was scanned over a window of 0.5 Da above and below this mass, with a scan time of 0.50 sec and an interchannel delay of 0.05 sec. The HPLC mobile phase and gradient used were the same as those described above for the analytical separation.

MALDI and PSD MS. The molecular weight values of the tryptic peptides were determined by analyzing 1 μl of each concentrated HPLC fraction (typically containing approximately 1 pmol of peptide) with a VG TofSpec SE

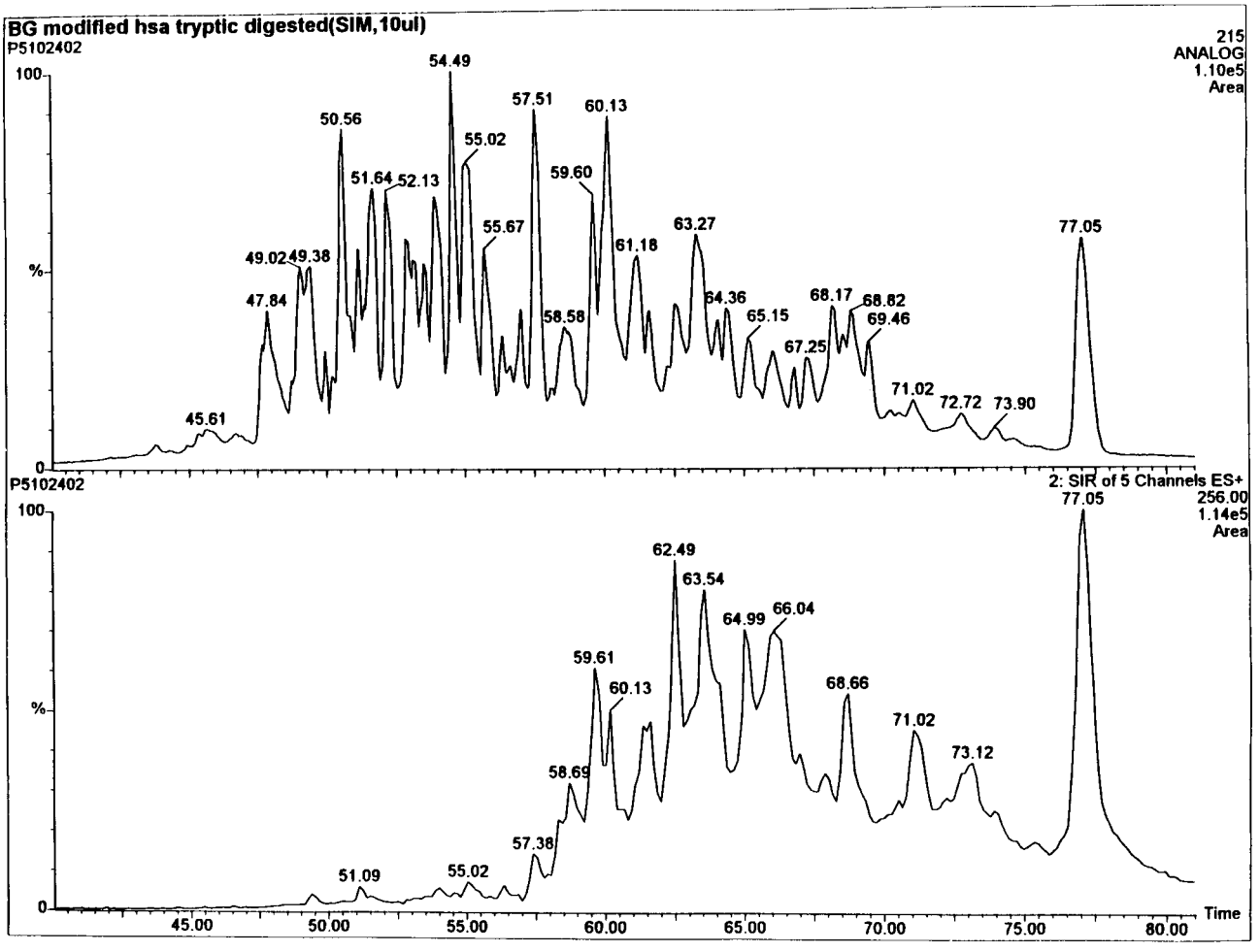

FIG. 2. *SIM chromatograms of tryptic digests obtained after incubation of HSA with BG and $NaCNBH_3$ (at a HSA/BG/$NaCNBH_3$ molar ratio of 1:3:15) for 96 hr. Upper*, profile of UV absorbance at 215 nm; *lower*, profile of total ion current with monitoring at *m/z* 256 and a mass window of 1.0 Da.

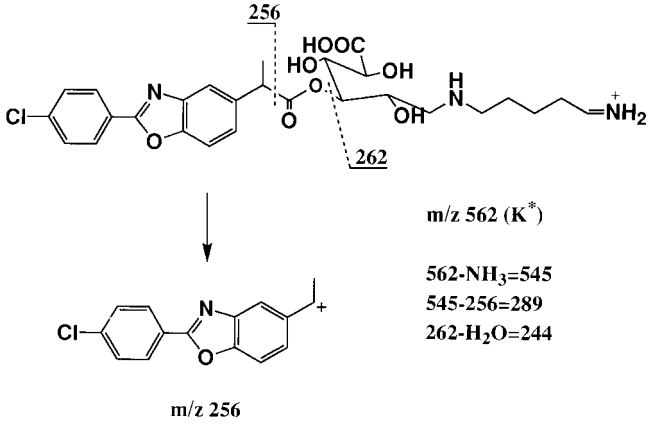

FIG. 3. *Immonium and related ions of the BG-modified lysine (reduced by $NaCNBH_3$).*

Mass values corresponding to the $^{12}C^{35}Cl$ isobar are shown.

MALDI mass spectrometer equipped with a nitrogen laser (337-nm wavelength) and operated in the reflectron mode. Peptides were cocrystallized with equal volumes of matrices consisting of saturated solutions of either 2,4-dihydroxybenzoic acid or α-cyano-4-hydroxycinnamic acid in 0.1% aqueous trifluoroacetic acid. All MALDI spectra were externally calibrated by using a standard peptide mixture. The mass values obtained for the modified HSA tryptic peptides were compared with those observed in control experiments. All mass values that did not match known or anticipated tryptic peptides were subjected to PSD analysis using the same instrument, to obtain information on the peptide sequence and to localize the site of modification, if possible.

High-Energy CID MS. One microliter of each selected HPLC fraction and 1 μl of saturated 2,4-dihydroxybenzoic acid solution in 40% acetonitrile/water, acidified with 5% trifluoroacetic acid were premixed and loaded onto the sample probe for MALDI-CID analysis. These experiments were performed using a tandem electric sector-magnetic sector-electric sector (EBE)-orthogonal acceleration TOF mass spectrometer (Micromass AutoSpec 5000). The nitrogen laser (337-nm wavelength) was operated at a 10-Hz pulse rate. The $^{12}C^{35}Cl$ isobar for the precursor mass was selected manually in MS-1 and subjected to collision with xenon gas, using a collision energy of 800 eV. The resulting mixture of precursor and product ions passed through the orthogonal acceleration chamber, in which an electric field orthogonal to the optical axis was applied at the appropriate time to accelerate each packet of precursor and product ions into a linear TOF mass analyzer (with an effective path length of approximately 0.48 m). For this mode of operation, the sampling efficiency was 100%. The microchannel plate (MCP) detector of the TOF analyzer always automatically recorded the full product-ion spectrum, irrespective of the precursor ion mass selected in MS-1. A detailed description of *de novo* peptide sequence determination using this instrumentation can be found elsewhere (Medzihradszky *et al.*, 1996, 1997). Peptide fragments were labeled according to the nomenclature suggested by Roepstorff and Fohlman (1984) and modified by Biemann (1988). Manual interpretation of the high-energy CID spectra obtained was carried out according to the method of Medzihradszky and Burlingame (1994).

TABLE 1

Identified binding sites for covalent binding of BG to HSA (in the presence of $NaCNBH_3$)

HPLC Peak[a]	HSA Residues	Molecular Weights of Peptides[b]	Sequence Information	Modifications[c]
1	191–197	1208.5, 1190.5	PSD, CID	BG-K-195[d]
2	187–195	1353.5, 1335.5	PSD, CID	BG-K-190
3	517–521	1109.4	PSD	BG-K-519
	440–445	1198.4	PSD, CID	BG-K-444
4	411–414	1000.3, 982.3	PSD, CID	BG-K-413
5	433–439	1301.5, 1283.5	PSD	BG-K-436
	182–195	1979.8, 1961.8	PSD, CID	BG-K-190
6	539–545	1278.5, 1260.5	PSD, CID	BG-K-541[d]
7	157–160	982.3, 964.3	PSD, CID	BG-K-159
8	157–160	982.3, 964.3	PSD, CID	BG-K-159
	198–205	1409.6	PSD, CID	BG-K-199[d]
9	461–472	1902.8	PSD	BG-K-466
10	205–209	1097.4	PSD, CID	BG-K-205
11	508–521	2168.8, 2150.8	PSD, CID	BG-K-519
12	156–160	1129.4, 1111.4	PSD	BG-K-159
13	71–81	1695.7	PSD	BG-K-73
14	210–218	1480.6, 1462.6	PSD, CID	BG-K-212
15	65–81	2394.1	PSD, CID	BG-K-73
16	145–160	2516.2	PSD	BG-K-159
17	146–160	2360.1	PSD, CID	BG-K-159

[a] HPLC peak numbers correspond to the labeled peaks in fig. 1.
[b] Lower molecular weights (−18 mass units) correspond to water-loss ions generated by MALDI.
[c] K, lysine.
[d] Modification sites also found for TG.

Results

Enzymatic Synthesis and Purification of BG. Under the conditions described above, the highest yields of BG were obtained after incubation for 2.5 hr, at both pH 6.0 and pH 7.0. The combined yields of (*R*/*S*)-BG were 68% at pH 6.0 and 72% at pH 7.0, based on their HPLC peak areas (data not shown). The BG synthesis was carried out at pH 6.0, because glucuronides are generally more stable at weakly acidic pH values. Using analytical HPLC with the modified mobile phase, complete separation of the diastereomers of BG was observed after 25 min of elution, whereas the much more hydrophobic starting material benoxaprofen eluted after 32 min. Using both UV and fluoresence detection ensured that the target product was well separated from other molecules. After protein precipitation, ethyl acetate extraction, HPLC, and lyophilization, the final product obtained was a mixture of (*R*)- and (*S*)-BG (with an *R*/*S* ratio of 1:1) and had a purity of approximately 92%, based on HPLC peak areas (benoxaprofen was used as a standard compound).

Incubation Studies in the Presence of $NaCNBH_3$. *Identification of Types of Adducts.* BG was incubated with HSA and $NaCNBH_3$ at a BG/HSA/$NaCNBH_3$ molar ratio of 1:3:15 and was then subjected to tryptic digestion. The HPLC chromatograms (UV detection, 215 nm; fluorescence detection, excitation at 313 nm and emission at 365 nm) of the digest and control are shown in fig. 1. The largest peak in both UV and fluorescence chromatograms (92.5 min) represents signals

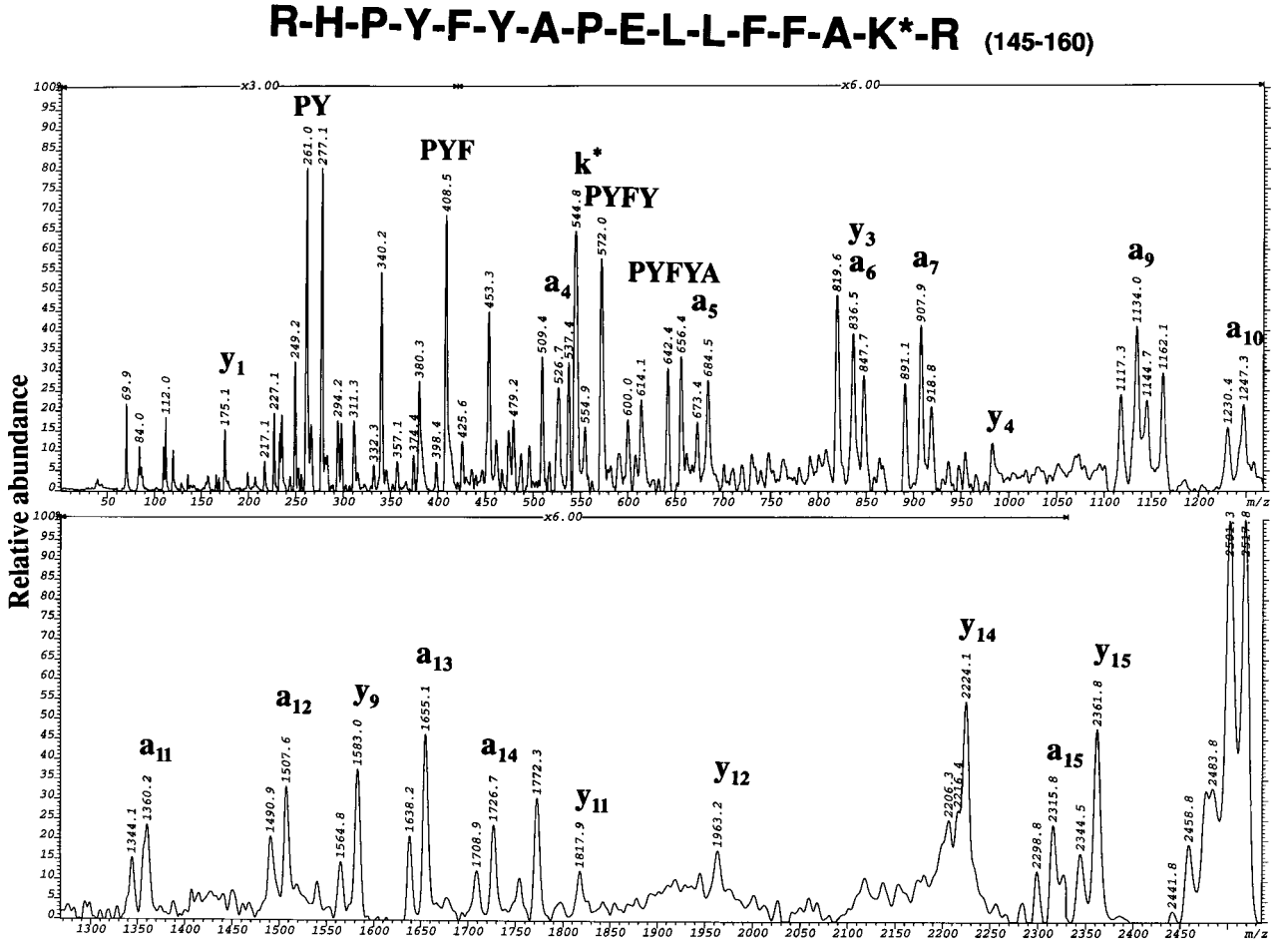

FIG. 4. *MALDI-PSD spectrum of a tryptic peptide (residues 145–160) of HSA with Lys-159 covalently modified by BG at its ϵ-amino group in the presence of $NaCNBH_3$ (+461 mass units).*

*K**, BG-modified lysine residue; *k**, immonium and related ions of BG-modified lysine (reduced).

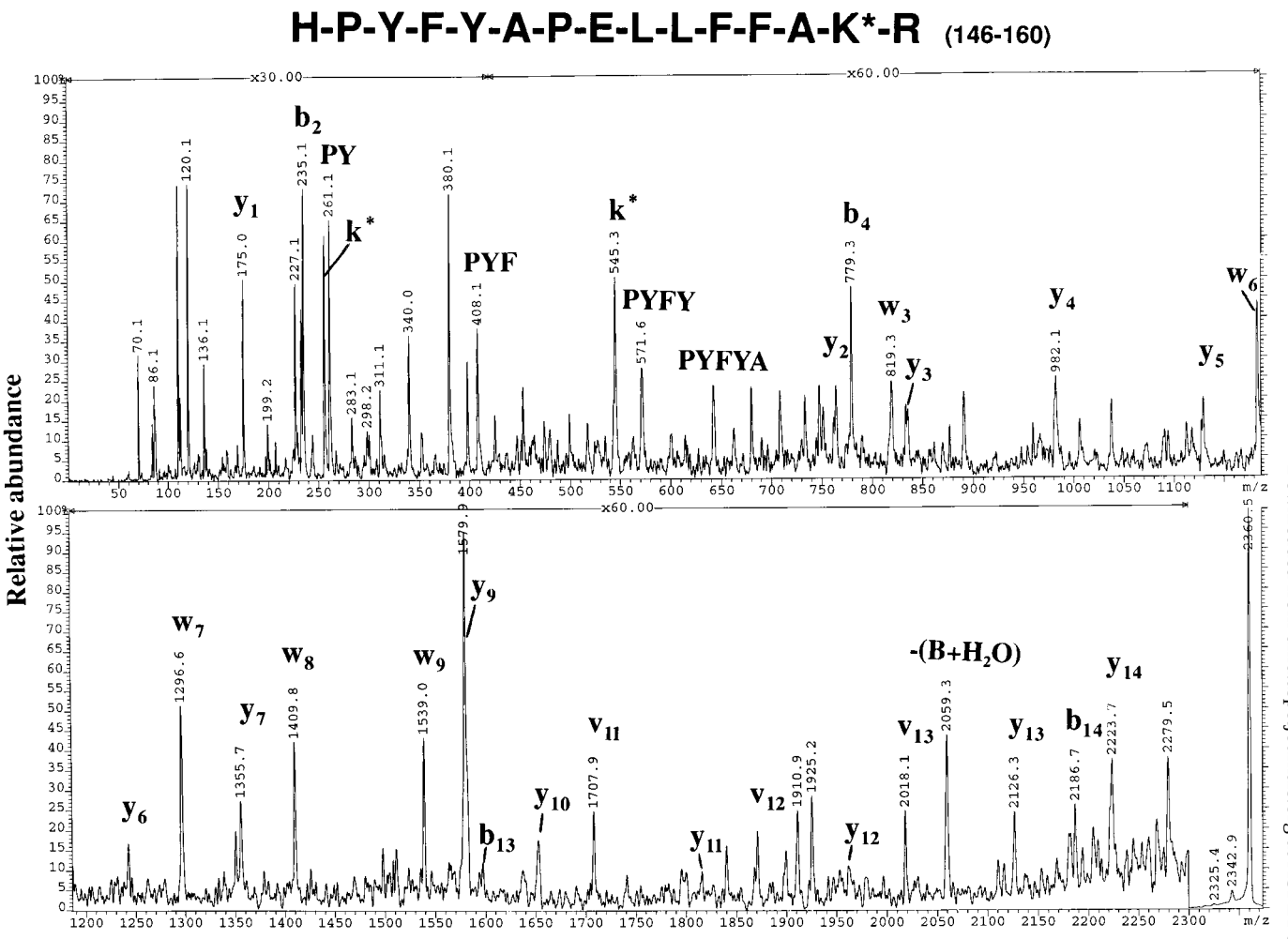

FIG. 5. *MALDI-high-energy CID spectrum of a peptide (residues 146–160) of HSA in which Lys-159 was modified (+461 mass units).*

*K**, BG-modified lysine residue; *k**, immonium and related ions of BG-modified lysine (reduced).

from hydrolyzed unbound benoxaprofen. As can be seen by comparing the chromatograms in fig. 1, the extensive suite of fluorescent peaks (fig. 1*a*) were not observed in the control experiment using the parent drug (fig. 1*b*). As expected, a very similar chromatographic pattern (fig. 2) was obtained in SIM studies when the benoxaprofen moiety was monitored at *m/z* 256 (fig. 3). Seventeen fractions corresponding to the numbered peaks (fig. 1) were collected and analyzed by MS.

Subsequent MS analysis of the modified peptides in these fractions revealed the type of BG adduct present at each protein binding site. This information is summarized in table 1, together with the molecular weight of each modified peptide and the type of MS sequence information used to establish the binding site. Three of the HSA modification sites identified in this study had been found in our previous studies with TG (Ding *et al.*, 1993). At least one modified peptide was found for every fraction collected. In the presence of $NaCNBH_3$, the modifications occurred exclusively on lysine residues, retaining the glucuronic acid moiety in the adduct. The percentage of total binding occurring at each site was not calculated, because of the presence of overlapping peaks and many incomplete cleavages at known tryptic sites. Despite the lack of semiquantitative knowledge at this level of detail, Lys-159 could be easily established as the major binding site in this experiment. For determination of adduct binding sites, the information from PSD spectra was sufficient in most cases, because the primary sequences of all peptides were known. However, the MALDI-CID experiments yielded much more explicit and complete information, allowing *de novo* interpretation of those spectra (Medzihradszky *et al.*, 1996, 1997). Water-loss ions were observed in the spectra of most modified peptides, which is typical for hydroxyl group-rich molecules under MALDI conditions.

Identification of Peptides Modified by a Reduced BG Group. A protonated species (MH^+) of molecular weight 2516.8 (*m/z* 2517.8 average mass) was detected in the HPLC fraction that eluted at 86 min (peak 16 in fig. 1 and table 1). With consideration of the theoretical molecular weight values for all possible tryptic and chymotryptic peptides that could be formed from the HSA protein sequence and the possible mass value for the borohydride-reduced benoxaprofen aldol tautomer (461 mass units), this measured value could correspond to the mass of residues 145–160 of HSA (Arg-His-Pro-Tyr-Phe-Tyr-Ala-Pro-Glu-Leu-Leu-Phe-Phe-Ala-Lys*-Arg, MW = 2055.1) plus that of covalently bound BG. This spectrum also showed direct evidence of the incorporation of both the benoxaprofen and reduced glucuronic acid moieties, *i.e.* the presence of fragment ions at *m/z* 244, 256, and 545, which reflect portions of the anticipated structure of a BG-modified lysine residue (fig. 3). This sequence assignment was established by interpretation of the fragmentation pattern observed in the PSD mass spectrum (fig. 4) associated with gating on this molecular ion. Furthermore, within an almost-complete amino-terminal **a**

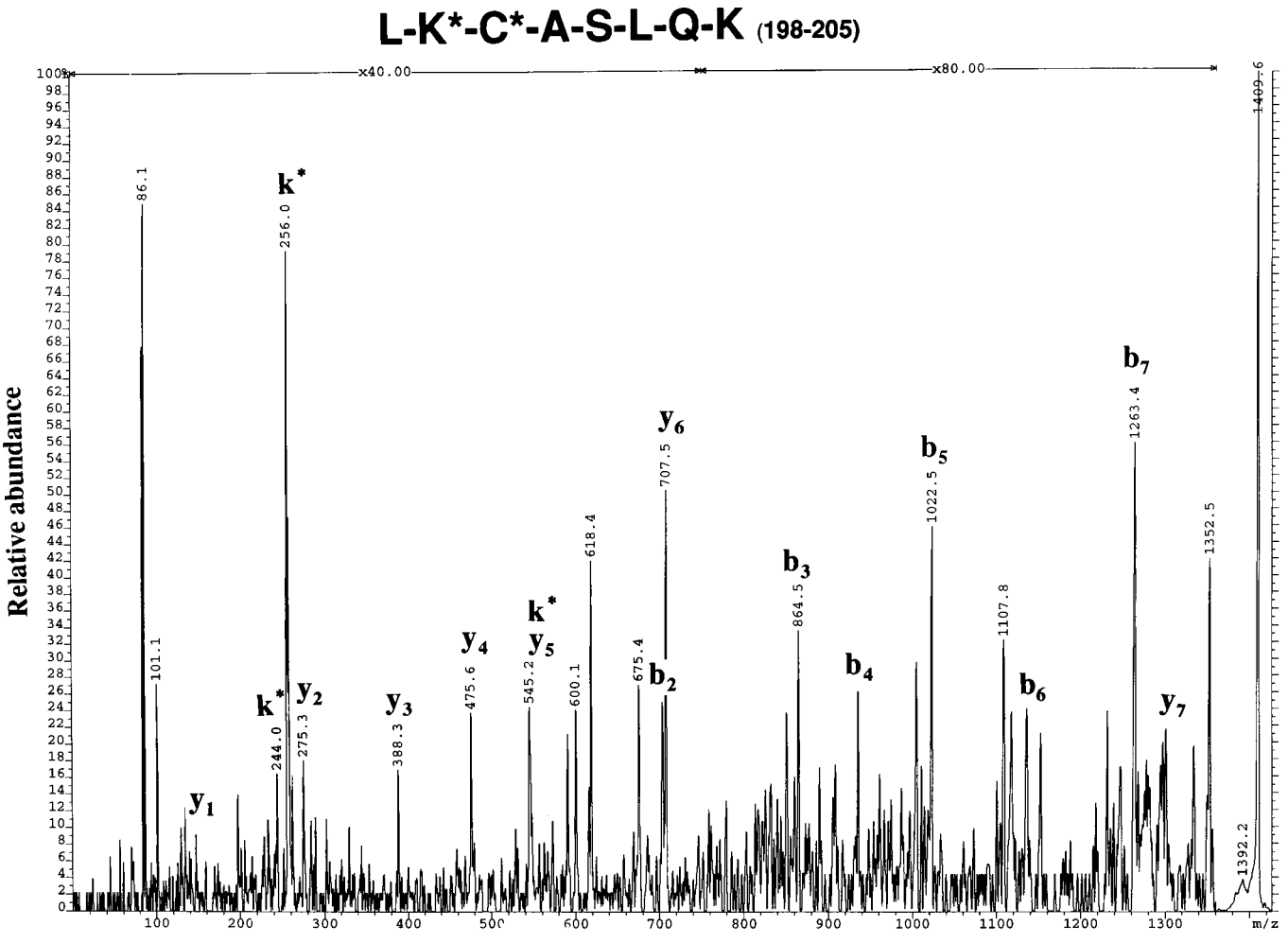

FIG. 6. *MALDI-high-energy CID spectrum of a tryptic peptide (residues 198–205) of HSA with Lys-159 modified by BG (+461 mass units).*

*K**, BG-modified lysine residue; *C**, carboxymethyl-cysteine; *k**, immonium and related ions of BG-modified lysine (reduced).

ion series (Medzihradszky and Burlingame, 1994) in the mass spectrum, only $\mathbf{a_{15}}$ was shifted up in mass by 461 units. This finding establishes unambiguously that Lys-159 is BG-modified. In addition, this particular site of modification was confirmed by the presence of and appropriate shifts in the redundant carboxyl-terminal **y** ion series ($\mathbf{y_4}$, $\mathbf{y_9}$, $\mathbf{y_{11}}$, $\mathbf{y_{12}}$, $\mathbf{y_{14}}$, and $\mathbf{y_{15}}$ were all shifted by 461 mass units).

Additional data regarding this modification at Lys-159 were obtained after analysis by MALDI-CID MS of the fraction eluting at 87.5 min (peak 17 in fig. 1 and table 1). In this case the $^{12}C^{35}Cl$ monoisotopic mass value (*m/z* 2360.5) corresponds to a tryptic peptide of HSA (His-Pro-Tyr-Phe-Tyr-Ala-Pro-Glu-Leu-Leu-Phe-Phe-Ala-Lys*-Arg, residues 146–160) modified by a BG group (fig. 5). In this CID spectrum, the abundant ion $MH^+ - 301$ corresponds to the loss of a benoxaprofen molecule from the modified molecular ion. In addition, **v** and **w** ions were observed, as expected, in this high-energy CID spectrum; they were not observed in PSD spectra. Also, a complete **y** ion series permitted determination of the complete sequence and structure of this peptide. Similarly, the major binding site, Lys-199, for TG (Ding *et al.*, 1993, 1995) was found to be modified by BG in fraction 8, eluting between 63 and 64 min, although the observed signal for this mass value was fairly weak. The parent-ion MH^+ at *m/z* 1409.6 corresponds to a BG-modified tryptic peptide of carboxymethylated HSA [Leu-Lys*-Cys(CH_2COOH)-Ala-Ser-Leu-Gln-Lys] (fig. 6).

Many other modifications could be confirmed by analysis of the PSD spectra recorded for the water-loss ions generated during the laser desorption process (table 1). For example, the MH^+ ion at *m/z* 1335.9 represents the water-loss species from a BG-modified peptide of HSA (Asp-Glu-Gly-Lys*-Ala-Ser-Ser-Ala-Lys). Peptide fragment ions ($\mathbf{a_4}$, $\mathbf{b_4}$, $\mathbf{a_5}$, $\mathbf{b_5}$, $\mathbf{a_6}$, $\mathbf{b_6}$, $\mathbf{b_7}$, $\mathbf{b_8}$, $\mathbf{y_6}$, $\mathbf{y_7}$, and $\mathbf{y_8}$) were all shifted by 443 mass units, which corresponds to the mass of a $BG-H_2O$ group (fig. 7). These shifts in mass values of sequence ion series indicate that the modification is at Lys-190 and that the facile loss of a water molecule occurs within the glucuronic acid moiety. Similarly, all other modifications to HSA have been characterized unequivocally (see summary in table 1).

Incubation Studies without $NaCNBH_3$. *Identification of Types of Adducts.* HPLC chromatograms of the tryptic digest of the reaction product from the incubation of BG with HSA at a BG/HSA molar ratio of 1:50 are presented in fig. 8. Again, the largest peaks (eluting at about 92 min) in both UV and fluorescence chromatograms represent hydrolyzed unbound benoxaprofen. In this case, 10 prominent fluorescent peaks that were not found in the control experiment were observed. All fractions corresponding to the numbered peaks were collected and subjected to MS analysis as described above. These MS analyses permitted the identification of the type(s) of adduct present at each binding site. This information is summarized in table 2, with the molecular weights and the type of sequence information used to establish the structures of all modified peptides. In this case, each of

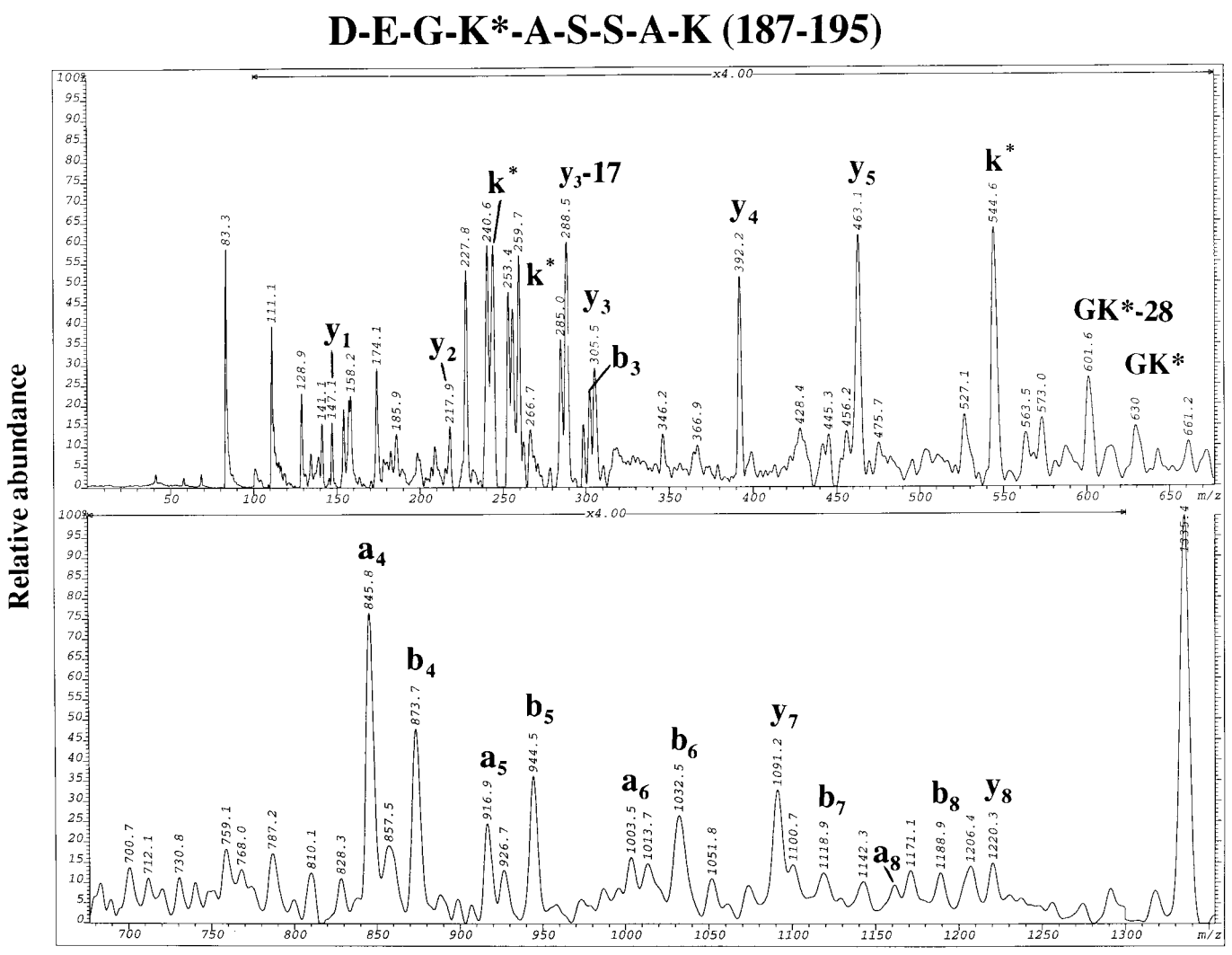

FIG. 7. *MALDI-PSD spectrum of a BG-modified peptide (residues 187–195) of HSA in which there was water loss from the glucuronic acid moiety attached to the Lys-190 residue.*

*K**, BG-modified lysine with loss of one water molecule (+443 mass units); *k**, its immonium and related ions.

the fractions collected contained one or two modified peptides. These results show that Lys-159 was the predominant binding site in this experiment also and that only three of the previously identified TG binding sites (Lys-199, Ser-480, and Arg-222) were modified by BG (Ding *et al.*, 1995).

Identification of Peptides Modified by a BG Group (Imine-Based Mechanism). Three of the major modified peptides (peaks 7, 8, and 12; table 1) that were detected in the presence of $NaCNBH_3$ were also found to be modified in the absence of $NaCNBH_3$ (eluting as peaks 1, 2, and 4; table 2). Of course, the mass of the modifying group was 2 mass units less than that discussed above. The component that eluted at 65.5 min (peak 1 in fig. 8) yielded a moderately abundant molecular ion (MH^+) at 1407.8 Da (monoisotopic) in MALDI spectra. This value corresponds to a tryptic peptide from a carboxymethylated region of HSA [Leu-Lys-Cys(CH_2COOH)-Ala-Ser-Leu-Gln-Lys, MH^+ at *m/z* 948.5] plus a covalently bound, unreduced, BG moiety (459 Da) (fig. 9). The presence of immonium ions formed from the BG-modified lysine (*m/z* 560 and 543) and the most characteristic fragment ion of benoxaprofen at *m/z* 256 indicate the incorporation of an unreduced BG moiety in this peptide. The shift in the mass values of several sequence ions ($\mathbf{a_2}$, $\mathbf{b_2}$, $\mathbf{b_3}$, $\mathbf{b_4}$, $\mathbf{b_5}$, $\mathbf{b_6}$, and $\mathbf{y_7}$) by 459 Da further established that the modification was at Lys-199. Similarly, modification of the major binding site Lys-159 could be shown easily by the PSD and CID spectra of two BG-modified peptides (Phe-Phe–Ala-Lys*-Arg, MH^+ at *m/z* 1127.4; Phe–Ala-Lys*-Arg, MH^+ at *m/z* 980.4) formed from nonspecific cleavages occurring during the rather long tryptic digestion. In these three examples of peptide modification, the glucuronic moiety group was retained within the protein-drug adducts.

Identification of Peptides Modified Only by a Benoxaprofen Group. Investigation by MALDI MS of the fraction eluting at 69.5 min (peak 5 in fig. 8) revealed a molecular ion (MH^+) at 1231.6 Da. This mass value matches that of a peptide with the same sequence as a peptide eluting at 65.5 min [Leu-Lys*-Cys(CH_2COOH)-Ala-Ser-Leu-Gln-Lys, MH^+ at *m/z* 948.5] but in this case modified by a benoxaprofen group only (283 mass units) (fig. 10). This high-energy CID spectrum displays a complete series of **b** and **y** ions. The mass difference observed either between $\mathbf{b_1}$ and $\mathbf{b_2}$ or between $\mathbf{y_6}$ and $\mathbf{y_7}$ equals the mass value corresponding to a lysine residue modified by benoxaprofen only (283 + 128 = 411 mass units). This modification was confirmed by the presence of the most stable fragment ion of benoxaprofen at *m/z* 256, together with the immonium ions expected for benoxaprofen-modified lysine, *i. e.*, *m/z* 384 and 367. Similarly, the presence in this fraction of a peptide with modification of Lys-159 by

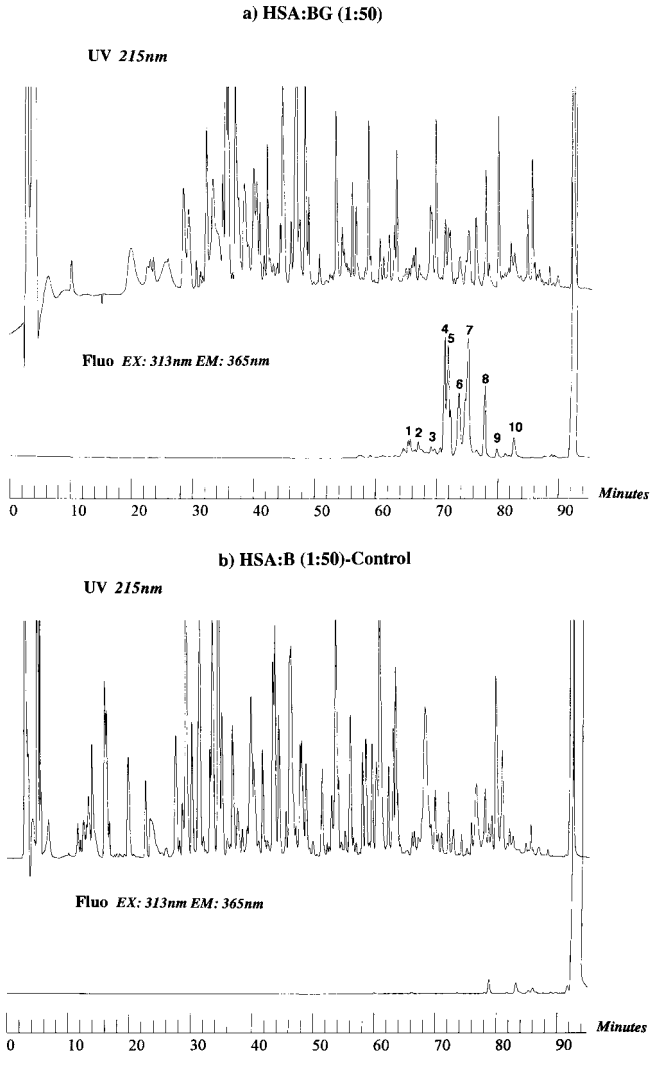

FIG. 8. *HPLC chromatograms of tryptic digests obtained after incubation of HSA with BG (at a HSA/BG molar ratio of 1:50) for 96 hr* (a) *and HPLC chromatograms of tryptic digests from a control experiment in which incubation conditions were the same as described in a but using benoxaprofen instead of BG (*b*).]*

a, *Upper*, profile of UV absorbance at 215 nm; *lower*, profile of fluorescence emission at 365 nm with excitation at 313 nm.

direct nucleophilic displacement of the glucuronic acid moiety was established by interpretation of the high-energy CID mass spectrum (peak 5 in table 2).

In addition, serine residues (table 2) were found to be major binding sites in this experiment. For example, the fraction eluting at 73.5 min (peak 6 in fig. 10) displayed a strong signal with MH^+ at 1423.6 Da using MALDI. This mass value is equal to that of a tryptic peptide of carboxymethylated HSA [Cys(CH_2COOH)-Cys(CH_2COOH)-Thr-Glu-Ser*-Leu-Val-Asn-Arg, MH^+ at *m/z* 948.6] plus the mass anticipated for a covalently bound benoxaprofen moiety (283 mass units) (fig. 11). The presence of an abundant ion at *m/z* 256 also implies the presence of a covalently bound benoxaprofen group. In addition, the modification of a serine residue in this sequence is required by the shift in mass of several sequence ions ($\mathbf{b_5}$, $\mathbf{y_5}$, $\mathbf{y_6}$, $\mathbf{y_7}$, and $\mathbf{y_8}$) by 283 units. This nucleophilic displacement by serine is further supported by the presence of two immonium ions (*m/z* 343 and 326), indicating serine with a benoxaprofen molecule covalently attached to its α-hydroxyl group. From this analysis and comparison with the known amino acid sequence of HSA, modification of Ser-480 was unequivocally identified. As can be seen by inspection of table 2, additional direct modifications occur on other serine residues, as well as on Arg-222 (peak 8).

TABLE 2

Identified binding sites for covalent binding of BG to HSA

HPLC Peak[a]	HSA Residues	Molecular Weights of Peptides	Sequence Information	Modifications[b]
1	198–205	1407.6	PSD, CID	BG-K-199[c]
2	157–160	980.4	PSD, CID	BG-K-159
3	467–472	957.4	PSD, CID	B-S-470
4	156–160	1127.4	PSD, CID	BG-K-159
5	198–205	1231.6	PSD, CID	B-K-199
	157–160	804.4	PSD, CID	B-K-159
6	476–484	1423.5	PSD, CID	B-S-480[c]
7	309–313	892.3	PSD, CID	B-S-312
8	219–225	1158.5	PSD, CID	B-R-222[c]
9	484–490	1177.4	CID	B-S-489
10	485–490	1021.3	CID	B-S-489

[a] HPLC peak numbers correspond to the labeled peaks in fig. 8.
[b] B, benoxaprofen; K, lysine; S, serine; R, arginine.
[c] Modification sites also found for TG.

In most of these cases, the interpretation of PSD spectra provided sufficient information to identify the peptide involved as well as to establish the exact location of this adduct within the peptide sequence. However, elucidation of the peptide sequence and adduct site arising from direct modification of Ser-489 (peaks 9 and 10 in table 2) could be performed only with MALDI-high-energy CID experiments.

Discussion

Glucuronides of propionic acid NSAIDs generally have a much longer half-life *in vitro* at physiological pH than do those of acetic acid NSAIDs (Spahn-Langguth and Benet, 1992), and they are generally considered to be much less reactive because of the presence of an α-methyl group. This work confirms that BG (benoxaprofen is a propionic acid NSAID) can react with HSA *in vitro* to form covalent adducts *via* the same two mechanisms found in studies with TG (tolmetin is an acetic acid NSAID) (Ding *et al.*, 1993, 1995). The nucleophilic displacement mechanism generates an adduct in which the glucuronic acid moiety is not retained, whereas the imine mechanism yields an adduct containing the glucuronic acid moiety. We also showed that the extent of covalent binding was much higher in the presence of $NaCNBH_3$ (an imine-trapping reagent), possibly resulting from the much lower initial BG/HSA ratio of 3 needed to allow characterization of the modified peptides, which is consistent with our previous findings with other NSAIDs (Smith *et al.*, 1990; Munafo *et al.*, 1990). Only lysine ϵ-amino groups were covalently modified when imine-trapping reagents were used, because the nucleophilic displacement mechanism was completely overshadowed by the imine-based mechanism. Lys-159 was the major binding site in HSA under both incubation conditions. In the absence of $NaCNBH_3$, Lys-159 reacted by both the imine-based mechanism and the nucleophilic displacement mechanism, as did Lys-199 to a lesser extent. Ser-312, Ser-480, and Arg-222 were the other three major binding sites, which reacted only *via* nucleophilic displacement. Minor binding sites included Ser-470 and Ser-489.

Although the mechanisms for covalent binding remain the same, the binding patterns are very different for different drugs. Of the binding sites on HSA for TG, only a limited number were also found for BG (tables 1 and 2). Lys-159 was not found to be modified by TG in our previous studies. However, it was the major binding site for BG, compared with Lys-199 for TG. With the imine-trapping reagent, only 3 of 13 BG modification sites were also found for TG (table 2).

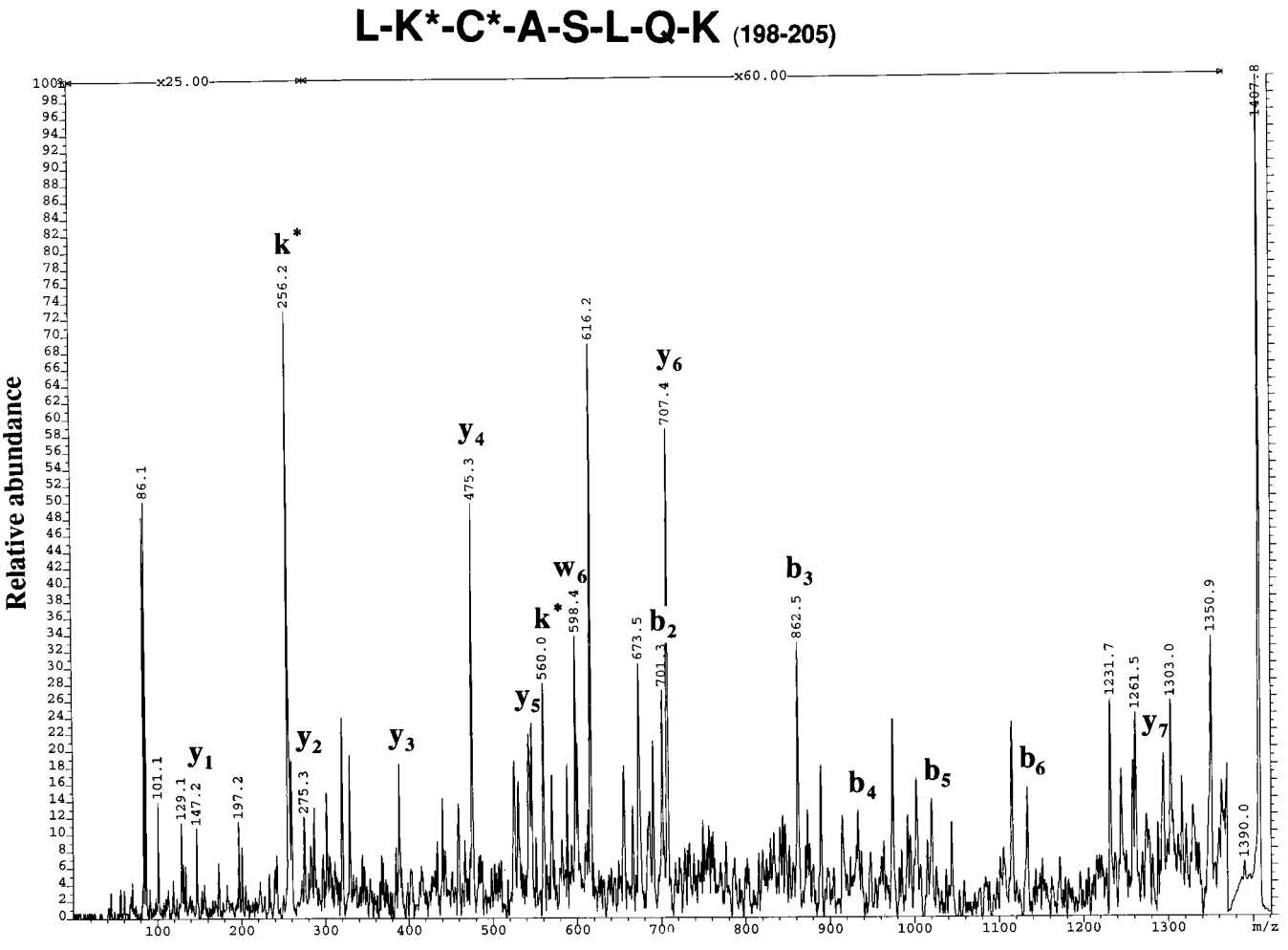

FIG. 9. *MALDI-high-energy CID spectrum of a tryptic peptide (residues 198–205) of HSA with Lys-199 modified by BG* via *an imine-based mechanism in the absence of* $NaCNBH_3$.

*K**, BG-modified lysine with retention of the glucuronic acid moiety (+459 mass units); *C**, carboxymethyl-cysteine; *k**, immonium and related ions of BG-modified lysine (unreduced).

Without the imine-trapping reagent, the two major binding sites for BG (Lys-159 and Ser-312, together with Ser-470 and Ser-489) (table 2) were not modified by TG in our previous studies (Ding *et al.*, 1995). Although many different binding sites have been identified, the main sites still seem to be clustered in the two principle binding regions (IIA and IIIA) on HSA (He and Carter, 1992), as we found previously for TG (Ding *et al.*, 1995). The most important of these is around Lys-199 and includes Lys-159, Arg-222, and possibly Ser-312. These amino acids are located in or near the hydrophobic binding pocket of subdomain IIA (He and Carter, 1992). The second binding area, including Ser-470, -480, and -489, is located in the IIIA binding pocket. The data indicate that covalent modification sites vary from drug to drug, although they are located in two principle binding regions on HSA.

The discovery that glucuronides of different NSAIDs display different binding site preferences was unexpected. Because retention of the common constituent, the glucuronic acid moiety, at the major binding sites (Lys-159 for BG and Lys-199 for TG) could potentially explain the immunological cross-reactivities observed with several NSAIDs (Spahn-Langguth and Benet, 1992), the glucuronic acid moiety might also be important in determining the binding sites on HSA for those acyl glucuronides and could result in similar binding patterns for glucuronides of different NSAIDs. Apparently this was not the case here. It is likely that noncovalent binding of the drug moiety of those acyl glucuronides plays a dominant role in determining their covalent binding sites on HSA. Serum albumin is known to have greater affinity for small, negatively charged, hydrophobic molecules (He and Carter, 1992). Most hydrophobic aromatic NSAIDs bind to subdomains IIA and IIIA of HSA both *in vitro* and *in vivo* (Honore and Brodersen, 1984), which is consistent with the fact that covalent binding sites are located in these two regions for both BG and TG. Different binding site preferences within these two regions could result from the different binding orientations of these drugs.

HPLC and SIM profiles, together with the identified modification sites, show a much more extensive binding pattern for BG than for TG. This is consistent with the considerably tighter reversible binding of benoxaprofen to HSA (Honore and Brodersen, 1984). However, given the much higher sensitivity of fluorescence detection, compared with UV detection, and the increased sensitivity of the instrumentation we currently use, this comparison may be inappropriate.

It is the continuous development of various MS technologies that makes this work possible. Mainly because of the much lower level of covalent binding, the molecular ions of many BG-modified peptides could barely be detected by liquid secondary ion mass spectrometry (LSIMS), which was exclusively used to characterize the structure of TG-modified peptides in our previous work (Ding *et al.*, 1993, 1995).

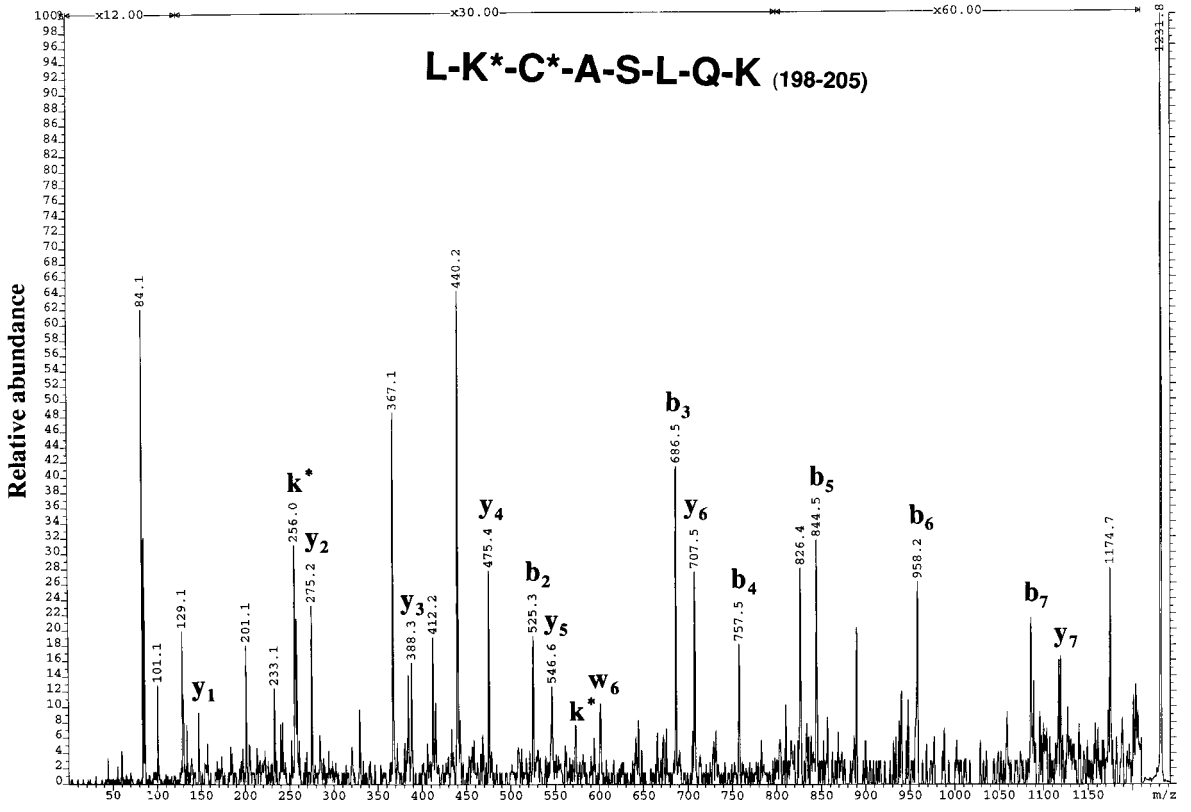

FIG. 10. *MALDI-high-energy CID spectrum of a tryptic peptide (residues 198–205) of HSA with Lys-199 modified by BG* via *a nucleophilic displacement mechanism. K**, modified lysine with benoxaprofen directly attached to its ϵ-amino group (+283 mass units); *k**, its immonium and related ions; *C**, carboxymethyl-cysteine.

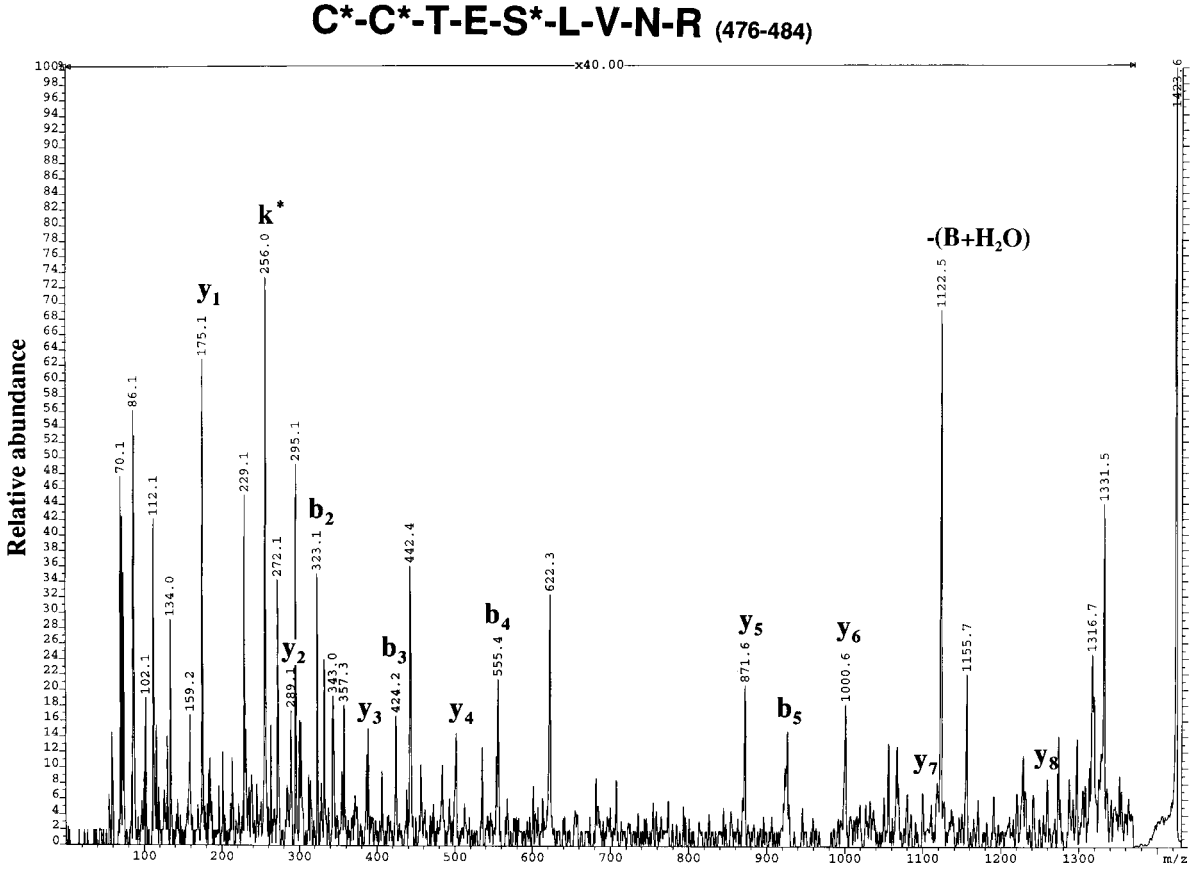

FIG. 11. *MALDI-high-energy CID spectrum of a tryptic peptide (residues 476–484) of HSA in which benoxaprofen was attached to the β-hydroxyl group of Ser-480* via *an ester linkage.*

*S**, modified serine (+283 mass units); *s**, its immonium and related ions; *C**, carboxymethyl-cysteine.

However, conclusive structural information was obtained for all modified peptides by MALDI, MALDI-PSD, and MALDI-CID MS. With these new technologies, structural studies of peptide samples in the mid- to high-femtomole range have become routine in our laboratory (Clauser *et al.*, 1995). Another crucial advantage of these new technologies is the ability to analyze peptides with higher molecular weights (Medzihradszky *et al.*, 1997). Complete PSD and CID spectra of modified peptides with molecular weights of >2500 (table 1) can be obtained in this greatly reduced range, which was not readily achievable in our previous work. In particular, high-energy MALDI-CID MS not only generates **d**, **v**, and **w** ions to distinguish leucine from isoleucine but also has the ability to select the ^{12}C isobar of the molecular ion in a mixture, to yield clean spectra with very high signal/chemical noise ratios (Medzihradszky *et al.*, 1997). This latter advantage is well illustrated by the fact that modification at Ser-489 cannot be verified with PSD spectra (data not shown), whereas the information content of MALDI-CID spectra allows *de novo* interpretation of the spectra of these two modified peptides (table 2); this capacity is very useful, especially when both of these peptides result from nonspecific enzymatic cleavages.

Clearly, the next challenge in this research would be determination of the covalent binding of NSAIDs to albumin *in vivo*. Such studies would involve detection and characterization of the binding of significantly lower concentrations of BG present in the circulation. It is not clear whether sufficient quantities of covalently modified circulating albumin could be obtained to permit analogous determination of binding sites by presently available MS methods. If technically feasible, such studies will be pursued in our laboratory.

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