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Rapid LC-TOFMS method for identification of binding sites of covalent acylglucuronide–albumin complexes

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Abstract

A method for rapid identification of binding sites of covalent adducts was developed using delta bilirubin as a model compound. Delta bilirubin, containing intact human serum albumin (HSA), was digested with trypsin and the peptide fragments were monitored at 436 nm, but no predominant peaks were detected indicating the instability of the digested peptides containing bilirubin-related compounds. Therefore, the high-performance liquid chromatography time-of-flight mass spectrometer (LC-TOFMS) data of digested fragments of delta bilirubin were compared with those of control digests of HSA, revealing a characteristic peptide in the digest mixture of delta bilirubin. This peptide was sequenced by high-performance liquid chromatography time-of-flight tandem mass spectrometry (LC-TOFMS/MS) and identified as LDELRDEGKASSAK (Leu182 to Lys195) with a modification of a 178 Da increase at Lys190. This indicated the Lys190 to be a predominant covalent binding site of BGs on HSA via the imine mechanism and the binding between the bilirubin moiety and the glucuronic acid moiety to be unstable to digestion with trypsin. The method of comparing LC-TOFMS data requires no specific detection such as fluorescence or radioactivity for every compound. This should accelerate the structure elucidation of covalent adducts and be helpful for studying the relationship between the structure of ligands and specific binding sites.

Keywords: LC-TOFMS; Delta bilirubin; Covalent binding site; Acylglucuronide

1. Introduction

Acyl glucuronidation is a major metabolic pathway of many acidic compounds containing a carboxylic acid function such as non-steroidal anti-inflammatory drugs (NSAIDs) and bilirubin. Acylglucuronides are generally unstable and can undergo hydrolysis as well as spontaneous isomerization at physiologic pH [1], being capable of forming covalent adducts either directly or after isomerization and tautomerism [2,3]. As recently reviewed [4], covalent binding in vitro and in vivo has been demonstrated for bilirubin and some NSAIDs, and may play an important role in toxic side effects such as anaphylactic reactions or organ

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toxicity [5,6]. As yet, no definitive relationship has been established between the irreversible binding of acidic drugs to proteins and the incidence of immunologic reactions observed in human. Two covalent binding mechanisms of acylglucuronide have been proposed. One involves nucleophilic displacement of the glucuronosyl group by $-NH_2$ [7,8], –SH [9], or –OH [10,11] groups of the protein, to form covalent drug-protein adducts devoid of the glucuronic acid group. The other mechanism [12–14] 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 epsilon-amino on the protein to form an imine. The product in this case would be an adduct in which the drug is linked covalently to the protein via a glucuronic acid.

Such adducts could potentially undergo slow spontaneous Amadori rearrangement to form a more stable 1-amino-1-deoxyketose structure [15]. However, the reversible imine can be converted to a secondary amine in the presence of $NaCNBH_3$, increasing the chemical stability and the yield for structural analysis [2,12,14].

Direct bilirubins, bilirubin monoglucuronide (BMG) and bilirubin diglucuronide (BDG), are known to bind covalently to human serum albumin (HSA) to form adducts called delta bilirubin, whose clinical roles and detailed structure such as binding sites and binding mechanisms remain unclarified.

The objective of this study was to establish a high throughput method for the identification of covalent binding sites on albumin using delta bilirubin as a model adduct, which can be applicable to various compounds.

2. Experimental

2.1. Chemicals and reagents

HSA (fraction V), bilirubin, monoiodoacetic acid (sodium salt), sodium cyanoborohydride and dithiothreitol were purchased from Sigma Chemical Co. (MO, USA). Potassium dihydrogenphosphate, di-sodium hydrogenphosphate, glycine, ammonium hydrogen carbonate, acetic acid, tris(hydroxymethyl)aminomethane (Tris) and di-sodium dihydrogen ethylenediamine (EDTA) were obtained from Nacalai Tesque (Kyoto, Japan). Trypsin, ascorbic acid, hydrochloric acid, sodium chloride, sodium glucuronate and guanidine hydrochloride were purchased from Wako Pure Chemicals (Osaka, Japan). Chloroform, trifluoroacetic acid and ethanol were obtained from Katayama Chemical (Osaka, Japan), Pierce (IL, USA) and Sigma-Aldrich Japan (Tokyo, Japan), respectively.

2.2. Animals

Jcl:Sprague–Dawley male rats were used to obtain glucuronic acid conjugates of bilirubin. Bilirubin of 2.5 mg was dissolved in 0.2 ml of 1 N NaOH, followed by the addition of 1 ml of control plasma obtained from other rats. This was administered to a rat via the cervical vein cannula over about 5 min. Bile was collected from rats into brown glass tubes containing 0.1 ml of 50% ascorbic acid (aqueous solution) and 0.1 ml of acetic acid via bile duct cannulas for 5 h after administration of bilirubin. The collected bile was stored at -20 °C until the extraction of BGs. Administration of bilirubin and collection of bile were carried out in the dark.

2.3. Instrumentation

The HPLC system used was an Alliance 2690 separation module with a 996 photodiodearray detector (Waters, MA, USA). The mass spectrometer used was a Q-T of Ultima API with ESI probe (Micromass, Manchester, UK). These instruments were controlled using MassLynx™ software (version 3.5). Mass spectra obtained were processed with MaxEnt1™ or MaxEnt3™ software (Micromass) for interpretation as necessary. Data comparison was carried out using Metabolynx™ software (Micromass) with the parameters optimized.

2.4. Purification of bilirubin glucuronides from rat bile

A 4 ml of 0.4 M glycine buffer (adjusted to pH 1.8 with 1 M HCl), NaCl (1 g) and 10% ascorbic acid aqueous solution (1 ml) were added to a 0.5 ml of rat bile and it was extracted twice by 4 ml of $CHCl_3$/EtOH (1/1, v/v). The organic layer was transferred into another brown glass tube and washed twice with 1.2 ml of water. The washed organic layer was divided into brown glass tubes and dried under a stream of nitrogen gas at room temperature. The tubes were stored at −20 °C until use.

Structure confirmation of extracted glucuronides was conducted using liquid chromatography time-of-flight mass spectrometer (LC-TOFMS) and liquid chromatography time-of-flight tandem mass spectrometry (LC-TOFMS/MS). The column used was a CAPCELL PAK C18 UG300 (2.0 mm i.d. × 150 mm, Shiseido, Tokyo, Japan) at a flow rate of 0.2 ml/min. The gradient was 60% solvent A (0.1% trifluoroacetic acid in water)/40% solvent B (0.1% trifluoroacetic acid in acetonitrile) to 100% solvent B over 15 min, followed by 100% solvent B for 10 min. The eluent was monitored both with a photodiodearray detector at the wavelength of 436 nm and by TOFMS in ESI mode. The desolvation temperature, source temperature, flow rate of desolvation gas and cone gas were set at 350 °C, 150 °C, 500 l/h and 100 l/h, respectively. In the product ion scan, the collision energy was set at 40 eV.

2.5. Formation of delta bilirubin

HSA (fraction V, 3.25%) was incubated with purified BGs at 37 °C in the presence of 10 mM $NaCNBH_4$ at a total volume of 1 ml. The concentration of BGs was about 170 μM (bilirubin equivalent). The incubation buffer used was 0.1 M phosphate buffer (adjusted to pH 7.4 by mixing 0.1 M potassium dihydrogenphosphate and 0.1 M di-sodium hydrogenphosphate). After incubation, 1 ml of 0.5% acetic acid in water was added to the incubation mixture to acidify it.

2.6. Estimation of the formation of delta bilirubin

The formation of delta bilirubin was estimated by HPLC. The column used was MCI®GEL CHP2MG (4.6 mm i.d. × 250 mm, Chemco, Osaka, Japan). The linear gradient used was 70% solvent A/30% solvent B to 100% solvent B over 20 min, followed by 100% solvent B for 10 min, at a flow rate of 1 ml/min. Solvents A and B were as described in Section 2.4. The eluent was monitored with a photodiodearray detector at the wavelength of 436 nm to characteristically detect bilirubin-related compounds. In estimating the extent of formation of delta bilirubin, the eluent was not introduced into the mass spectrometer.

2.7. Trypsin digestion

Two-fold volume of reduction buffer (containing 6 M guanidine–HCl, 100 mM Tris and 1 mM EDTA, adjusted to pH 8.3 with 1 M HCl) was added to the incubation mixture, in which proteins were reduced with dithiothreitol (221 μmol) for 1 h at 60 °C in the dark. The reduced mixture was continuously alkylated with monoiodoacetic acid (410 μmol) at room temperature in the dark for 30 min. The reduced and alkylated protein mixture was dialyzed overnight against 100 mM ammonium hydrogen carbonate buffer (pH not adjusted) at room temperature in the dark, using a dialysis apparatus equipped with a membrane with a molecular cut-off of 13 000. The dialyzed proteins were then digested with 1% (mol/mol) trypsin for 24 h at 37 °C.

2.8. Analysis of tryptic digests

A portion of the tryptic digests was subjected to LC-TOFMS. The tryptic peptides were separated on a Vydac C18 column (2.1 mm i.d. × 250 mm, Grace Vydac, CA, USA) at a flow rate of 0.2 ml/min at 30 °C. The gradient elution employed was 95% solvent A/5% solvent B to 55% solvent A/45% solvent B over 90 min, to 100% solvent B over the next 10 min followed by 100% solvent B for 20 min. Solvents A and B were as mentioned in Section 2.4. The eluent was monitored both with a photodiodearray detector using a wavelength of

436 nm and by TOFMS. The conditions of TOFMS were as detailed in Section 2.4.

2.9. Data comparison method

The acquired LC-TOFMS data were compared using MetaboLynx software. This software automatically generates reconstructed ion chromatograms (RICs) and compares them between the analyte (digests of the adducts) and the control.

3. Results

Extracted BGs were reconstituted in 1 ml of 0.1% acetic acid (170 μM) and subjected to LC-TOFMS and LC-TOFMS/MS. Three main peaks, A, B and C, were observed in the HPLC chromatogram at 436 nm (Fig. 1, top of the left column). In the mass spectrum of peak A, $[M+H]^+$ was observed at m/z 937.4, corresponding to BDG. Both the mass spectra of peaks B and C showed the same $[M+H]^+$ ion at m/z 761.4, indicating that both peaks corresponded to BMG. All product ion spectra showed fragment ions at m/z 475 and 299 (Fig. 1, right column), which were assigned as indicated in the structures in Fig. 1. Thus, the peaks A, B and C were identified as BDG and BMGs, respectively. Identification of the isomers was not conducted in this study.

The time course of delta bilirubin formation is presented in Fig. 2. The formation of delta bilirubin showed a tendency to increase slightly until 48 h at all concentrations. This indicated that a high concentration of BGs and long-time incubation are required to maximize the formation of delta bilirubin. Therefore, 170 μM of BGs and 48 h incubation were employed to form delta bilirubin. The ratio of BGs linked covalently to HSA was calculated to be about 30% of the total incubated with HSA in the incubation of 170 μM BGs.

Delta bilirubin, which was a mixture of delta bilirubin and intact HSA, was reduced and alkylated as described in Section 2. A portion of the tryptic digests was analyzed using LC-TOFMS, but no prominent peaks were observed in the chromatogram monitored at the wavelength of 436 nm, which could characteristically detect bilirubin-related compounds. Digestion of delta bilirubin was confirmed to be adequate because it completely disappeared and many peptide fragments were observed in the chromatogram extracted at 210 nm. This result indicated that peptide fragments containing bilirubin or BGs were very unstable under the digestion conditions and spontaneously decomposed to become undetectable at 436 nm.

LC-TOFMS data obtained from delta bilirubin digests were compared with those from control HSA digests to find the characteristic peptide fragments of delta bilirubin. The control HSA used in the data comparison was prepared by incubating intact HSA and glucuronic acid to reject the binding of free glucuronic acid formed by the hydrolysis of BGs in the formation of delta bilirubin.

Data comparison was conducted using MetaboLynx software as mentioned in Section 2.9. Three candidates of the characteristic peptide of delta bilirubin were found, as listed in Table 1.

The product ion scan of m/z 849 was carried out and it was identified as the peptide from Leu182 to Lys195 (LDELRDEGKASSAK) with glucuronic acid bound at Lys190. The mass spectrum and the product ion mass spectrum of m/z 849 are shown in Fig. 3. Fragment ions containing Lys190 were assigned as indicated in Fig. 4.

The peptide observed at m/z 1019.7 was revealed to be an intact peptide whose sequence was AFKAWAVAR (Ala210–Arg218). It is inferred that this peptide was observed only in the digest of delta bilirubin because of the structural modification at Lys190 by bilirubin glucuronides.

The product ion scan of m/z 1419 was carried out, but no structural information was obtained because of its low ion intensity (about 1/5 of m/z 849).

No peptide fragments containing bilirubin itself were observed in this experiment. Therefore, Lys190 was tentatively identified as the major binding site of BGs via the imine mechanism.

In a separate experiment, it was confirmed that the peptide observed at m/z 849 was not formed by the digestion of HSA incubated with the control rat bile obtained from the rat not administered bilirubin.

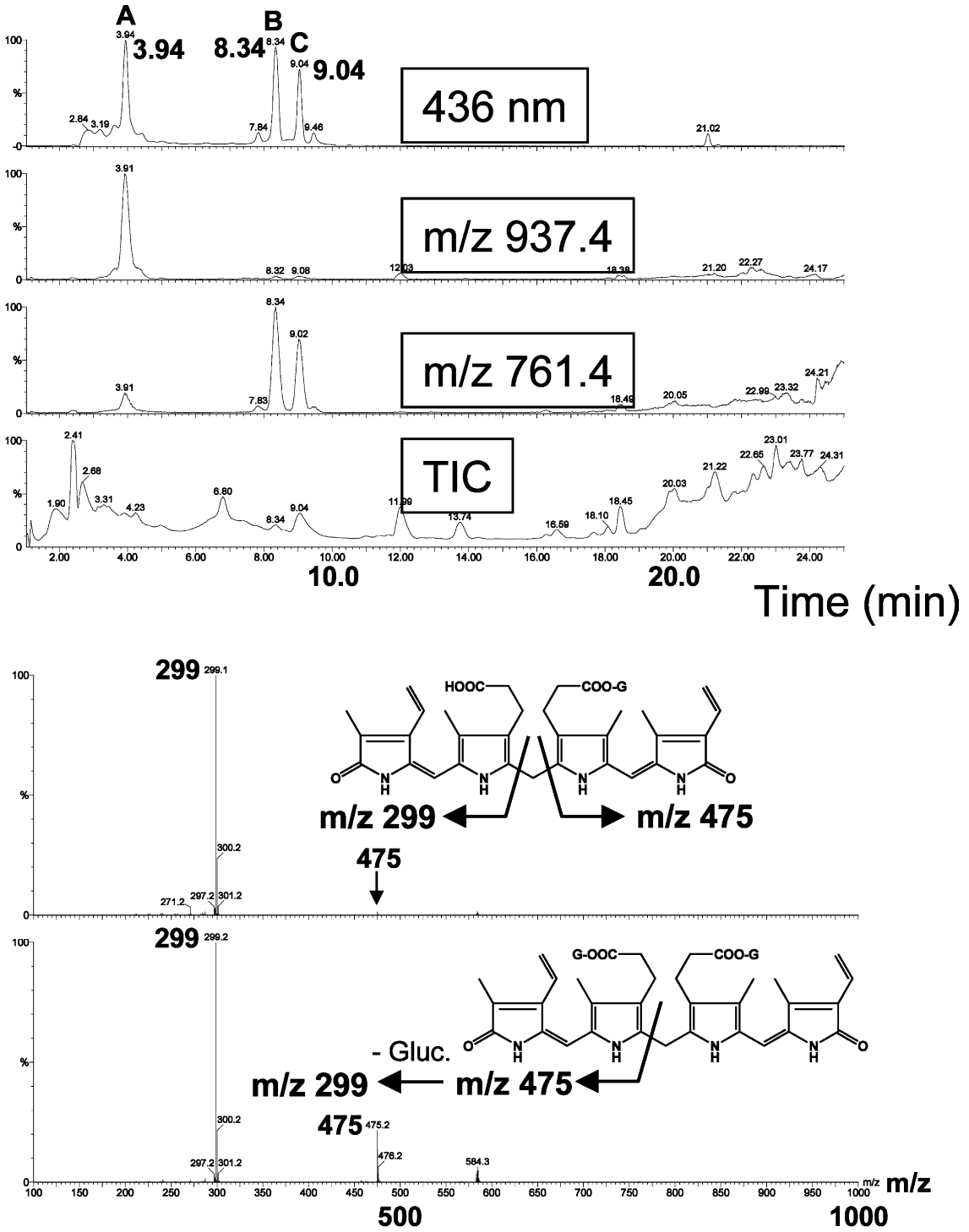


Fig. 1. HPLC, mass chromatograms and LC-TOFMS/MS spectra of bilirubin glucuronides purified from rat bile.

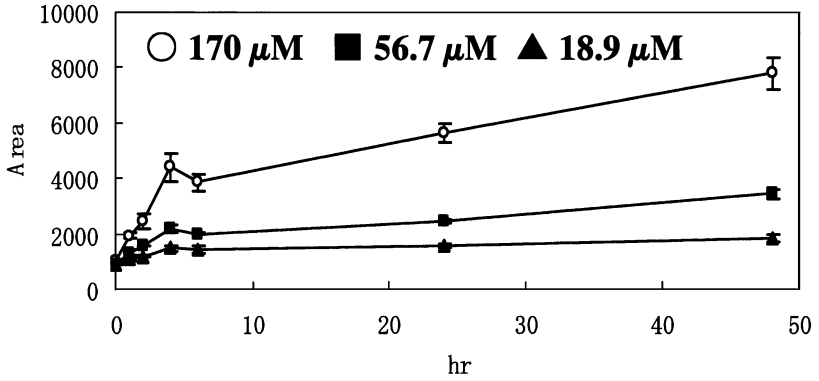


Fig. 2. Time course of formation of delta bilirubin at three concentration levels.

Table 1
Candidates of characteristic peptide fragments of delta bilirubin

Rt (min)	*m/z*	Charge
23.6	849.0	+2
40.9	1019.7	+1
48.8	1419.0	+1

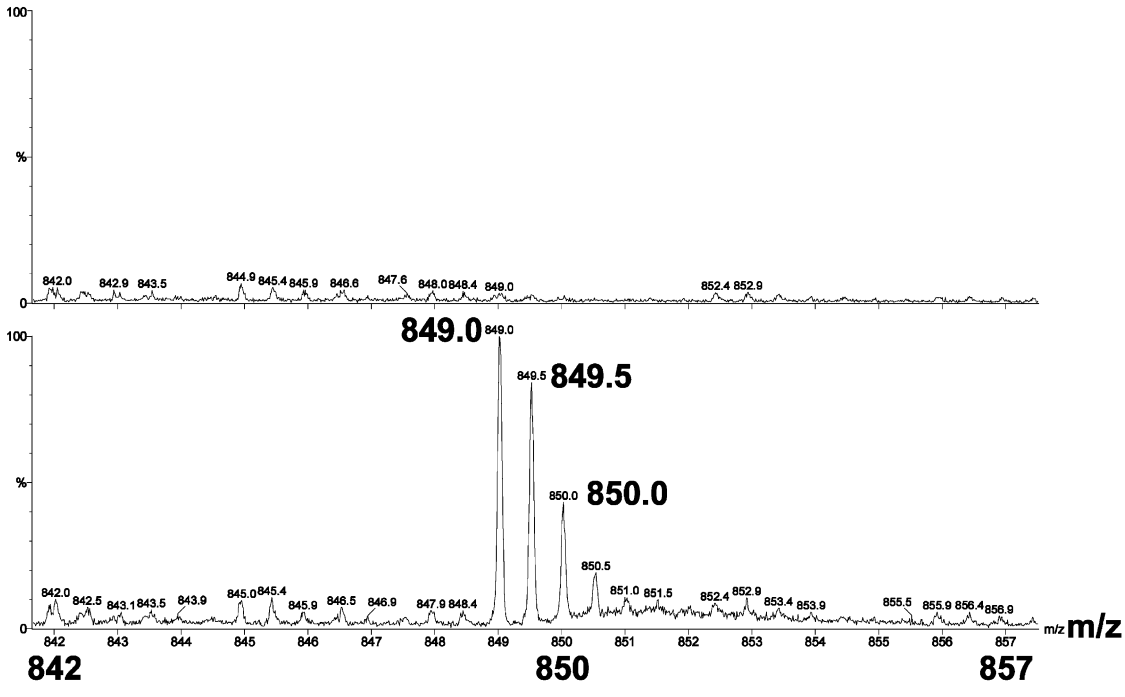
4. Discussion

Though the BGs binding sites and mechanisms of delta bilirubin have not been identified, some speculations have been made. van Breemen et al. [7] argued that the covalent binding of BGs to HSA proceeded with the displacement by one or more nucleophilic functional groups on the HSA molecule of glucuronic acid from its carboxylic acyl linkage with albumin based on evidence of reactivity with diazotized sulfanilic acid such as bilirubin. Yoshida et al. [16] analyzed azo-pigments obtained from delta bilirubin, and the results indicated that covalent binding of bilirubin to HSA occurred exclusively on the conjugated dipyrrolic half of the bilirubin conjugate. Neither study offered firm evidence of displacement of the glucuronic acid moiety or the formation of imine.

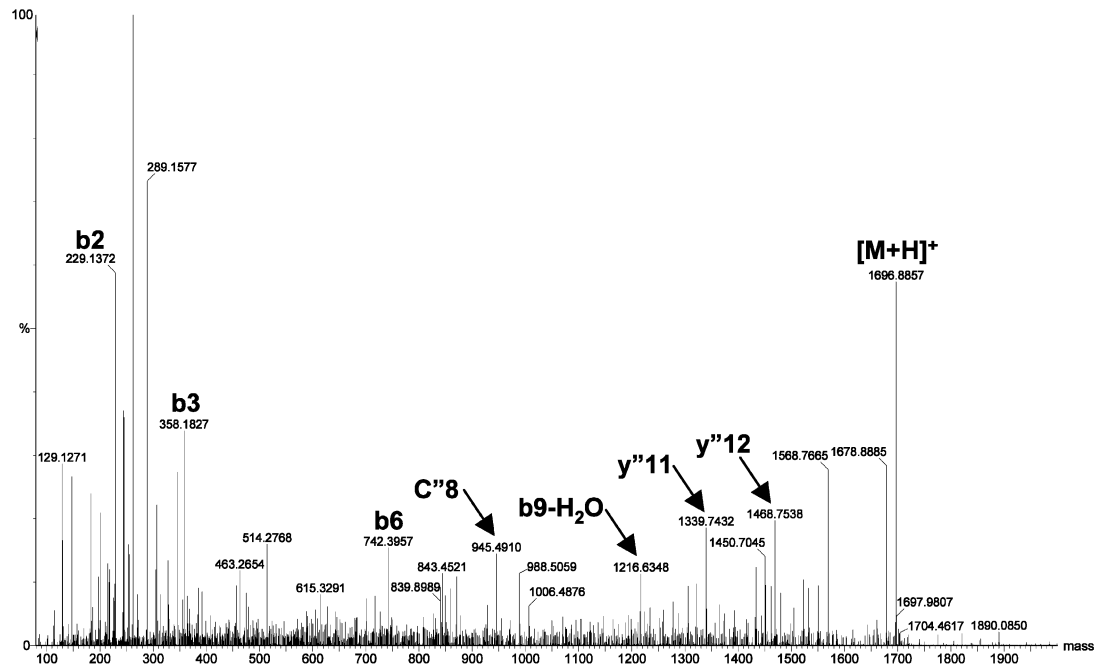
In this study, the method of comparing LC-TOFMS data also revealed the glycation site by glucuronic acid to be Lys525 by the comparison between HSA incubated with glucuronic acid and intact HSA. This result was quite reasonable because this residue, Lys525, is known to be the principal glycation site by glucose in vivo (data not shown) [17,18], indicating the ability of this method to identify the predominant binding site.

Covalent adducts of some NSAIDs have been investigated in detail. Ding et al. [2] identified the covalent binding sites of tolmetin glucuronide (TG) using secondary ion mass spectrometry (SIMS) and MALDI-TOFMS. TG became linked to Lys199 and to a lesser extent Lys195 and Lys525 via the imine mechanism, which retains the glucuronic acid moiety. On the other hand, TG linked directly to lysines (Lys199 and Lys541), serines (Ser220, Ser232 and Ser480) and arginine (Arg222). The relationship between the concentration of TG and the profile of the binding sites was also investigated, and indicated that the imine mechanism was favored at lower concentrations of TG, which were closer to the physiological concentration. Lys159 of HSA was identified as the major binding site of benoxaprofen glucuronide by Qui et al. [3]. This binding pattern at Lys159 was both the imine mechanism and the nucleophilic displacement. Some other binding sites were also identified, leading to the conclusion that the specific covalent binding profile appeared to be drug-dependent.

Lys190 belongs to subdomain IIA, which is known to be a non-covalent protein binding site of quercetin, thyroxine, tolbutamide, aspirin, warfarin, camptothecin and bilirubin, thus suggesting that this subdomain possesses affinity for bilirubin. The residue of Lys190 is also a covalent



(A)



(B)

Fig. 3. Mass spectra of the specific peptide: (A) LC-TOFMS spectra of control digests (upper) and delta bilirubin digests (lower); (B) LC-TOFMS/MS spectrum of m/z 849; (C) magnified spectrum of B (m/z 80–500).

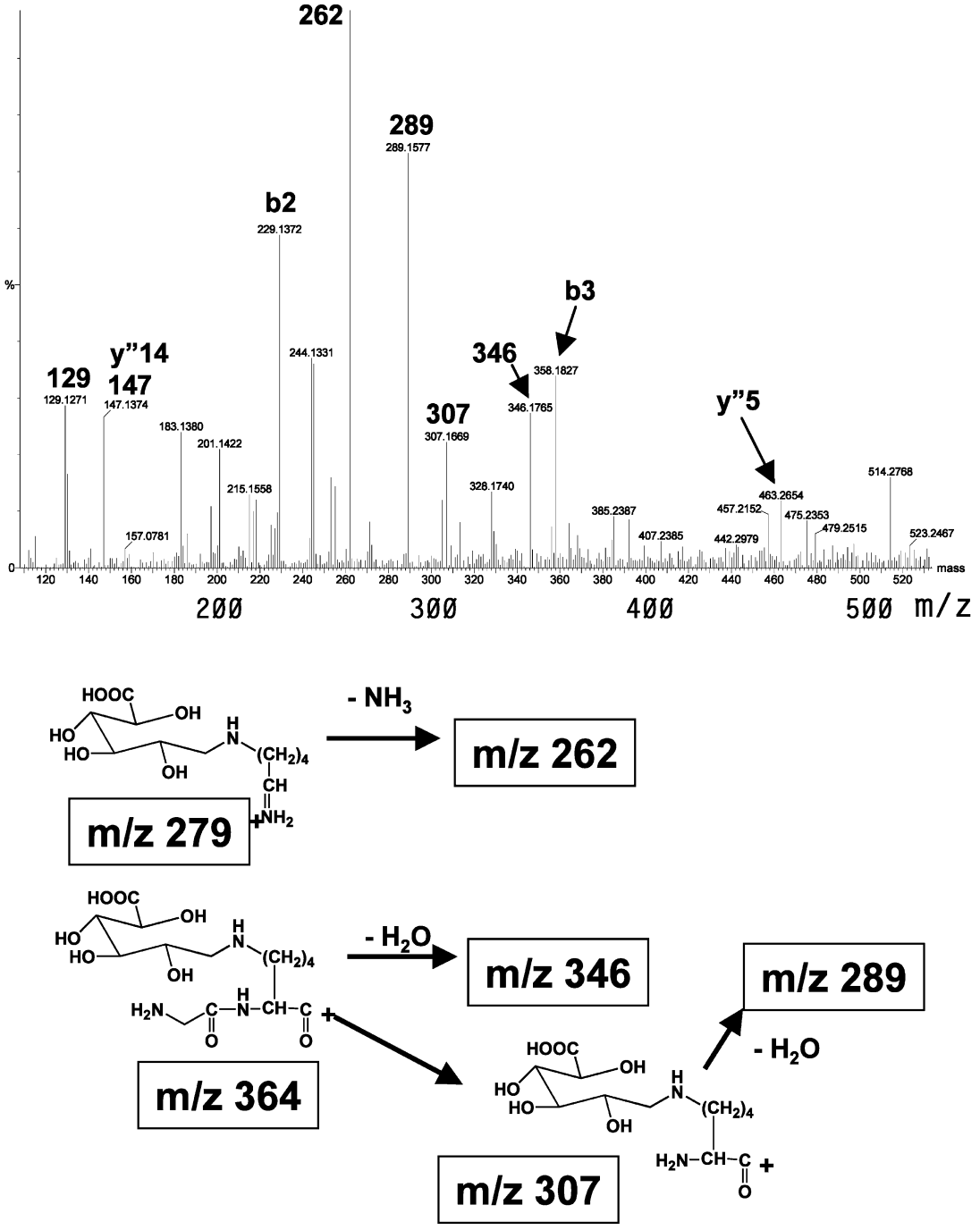


Fig. 4. Assignment of fragment ions containing Lys190.

binding site of vitamin B6, whose binding mechanism is Schiff-base formation, indicating that the amino functional group of this residue has high reactivity with aldehyde to form Schiff-base.

These findings offer support for the results of this study that the predominant binding site is Lys190 and its mechanism involves imine formation.

In both studies about adducts of NSAIDs, the binding sites were identified by sequencing peptide fragments digested with trypsin, and the target peptide fragments containing the drug were detected using a typical method such as UV or fluorescence detection. However, binding site identification could not be carried out in the same manner for the cases in which the drug moiety disappeared because of poor stability of the linkage or the lack of a characteristic detection method for the drug. In such cases, the LC/MS data comparison method demonstrated in this study can be very useful for identifying peptide fragments containing a covalently bound drug or its traces. The advantages of this method are that no characteristic detection method is required (not drug-dependent) and processes from the identification of peptide fragments derived from covalent adducts to their sequencing can be automated to accelerate binding site identification of a drug or its metabolites. This method is considered to be very useful particularly in the early stage of drug development, where a rapid method is required for the evaluation of many drug candidates.

5. Conclusion

Lys190 of HSA was identified as a predominant binding site of BGs using only LC-TOFMS data, indicating the usefulness of this method. This data comparison method is considered to be applicable to ligands that cannot be detected specifically such as UV detection at a characteristic wavelength or fluorescence detection or radiometric detection. This method should facilitate identification of binding sites at an early stage of drug development.

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