Focusing Review

Highly Selective Molecular Recognition of Biologically Active Substances Using Liquid Phase Separation

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Abstract

The development of new chiral stationary phases has been very important in the accurate analysis of drug enantiomers and their metabolites in biological samples during drug discovery and development. New chiral stationary phases have been developed using conalbumin and flavoprotein from chicken egg whites, which have been applied to a broad range of drug enantiomers. The application and characterization of these two chiral columns for high−performance liquid chromatography have been documented. Both specific and non−specific interactions, based on the silica gel surface and linker moiety, influenced retention and chiral separation of solutes. Interactions between drug enantiomers and proteins, as a pseudo chiral stationary phase, were investigated with affinity capillary electrophoresis, in order to avoid the effects of non−specific interactions. The chiral discrimination region for ketoprofen on the flavoprotein surface was concluded to consist of an α−helix structure. Studies with chemically modified flavoprotein indicated that two types of interactions at the chiral discrimination region were required for chiral separation: a π−π interaction between a tryptophan residue and the aromatic ring of ketoprofen, and an ionic interaction between the carboxyl group of ketoprofen and an amino and carboxyl group of the protein.

In the body, drugs and biologically active substances having a carboxyl group have been known to transform various metabolites such as acyl glucuronide. The acyl adenylate has also been noted as a chemically active intermediate of coenzyme A ligation. Both the acyl adenylate and the acyl glucuronide produced protein adducts by reacting with nucleophilic groups such as amino groups on protein molecules. To characterize both active intermediates and protein adducts, analytical techniques conferring highly selective molecular recognition, such as high−performance liquid chromatography and mass spectrometry, were required.

Keywords: protein−conjugated chiral stationary phase, affinity capillary electrophoresis, chiral discrimination, acyl glucuornide, acyl adenylate, bile acid, protein−bound adduct

1. Introduction

Molecular recognition by biomolecules such as proteins is complicated because many intermolecular interactions, including ionic interactions, hydrogen bonding, hydrophobic interactions, and coordinate bonding, function between binding sites on the protein surface and the ligand. Biopolymers possess the ability to clearly discriminate between different binding molecules at the binding site. The affinity of binding depends on the sum total of these interactions, and proteins such as enzymes, antibodies, and receptors can therefore distinguish a specific ligand from others.

In liquid chromatography, four basic mechanisms underlie retention and separation of solutes: adsorption chromatography, partition chromatography, ion−exchange chromatography, and size exclusion chromatography. Except for size exclusion chromatography, each of these depends on hydrogen bonding, hydrophobic interactions, van der Waal's forces and ionic interactions, respectively. For example, the retention of solutes to the stationary phase can mainly be explained by hydrophobic interactions in the re-

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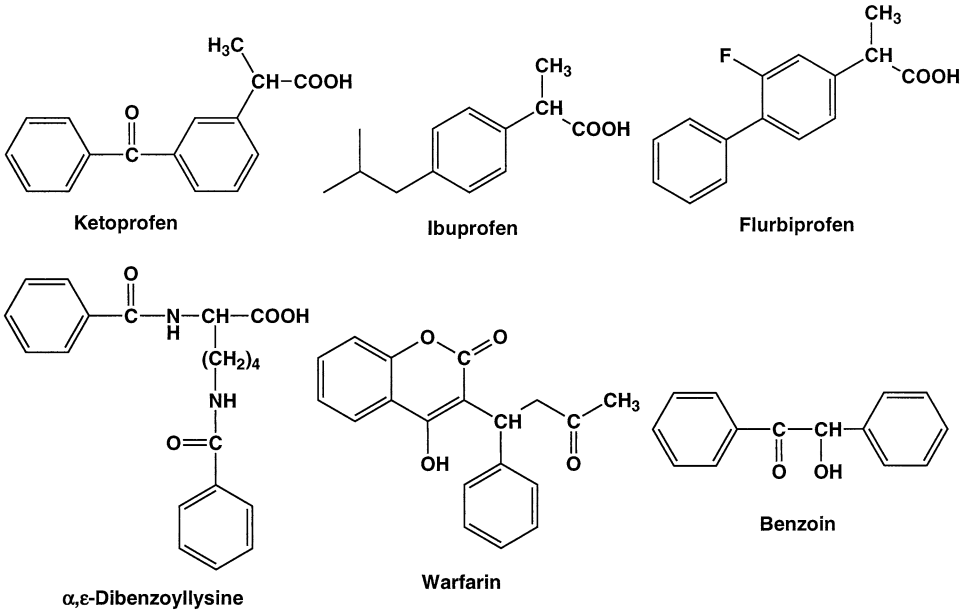
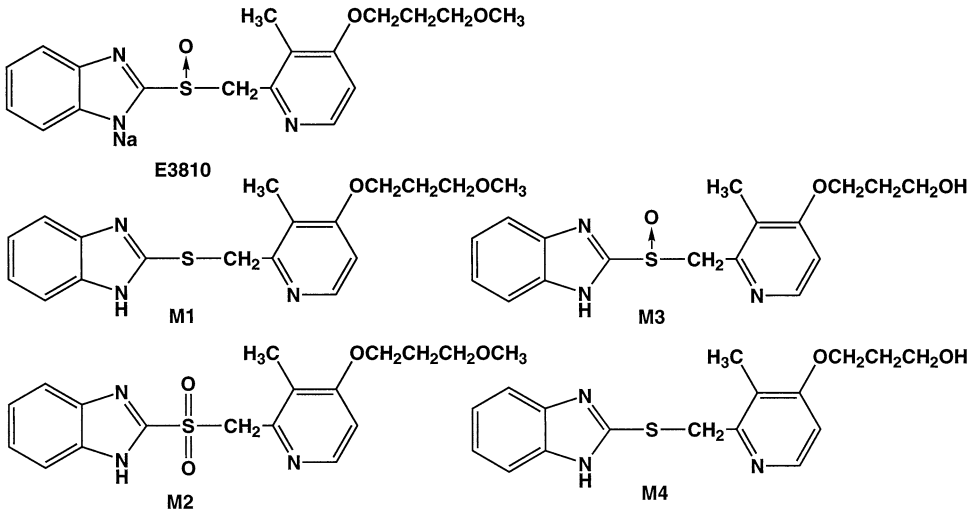
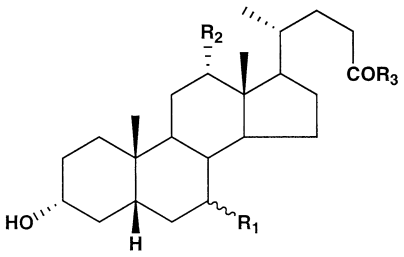






	R_1	R_2
Cholic acid (CA)	α-OH	OH
Chenodeoxycholic acid (CDCA)	α-OH	H
Deoxycholic acid (DCA)	H	OH
Ursodeoxycholic acid (UDCA)	β-OH	H
Lithocholic acid (LCA)	H	H

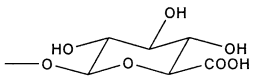
	R_3
Unconjugate	OH
Glycine conjugate	$NHCH_2COOH$
Taurine conjugate	$NHCH_2CH_2SO_3H$
Acyl glucuronide	

Figure 1. Structures of drugs and bile acids used in this review.

versed−phase partition liquid chromatography. Such hydrophobic interactions play a key role in anchoring molecules to binding sites on biopolymers. Chromatographic analysis of retention and separation mechanisms of solutes can be performed, and understanding these mechanisms may lead to a more complete understanding of intermolecular interactions between biologically active substances and biopolymers.

In this review, the mechanism for molecular recognition is analyzed for the interaction between drug enantiomers and proteins as chiral selectors. Affinity capillary electrophoresis (CE) is effective for this purpose. The combination of chemical modifications of the protein surface with spectroscopic analysis is also effective for analyzing the mechanism of interaction. On the other hand, highly specific molecular recognition and capturing of target molecules are available for analyzing post−translational modifications of proteins. Accurate chromatographic separation is also important for reliable analysis of unstable metabolites in biological fluids.

2. Separation of drug enantiomers by protein−conjugated chiral stationary phases

Chiral discrimination has been an issue in the development and use of pharmaceutical drugs, because drug enantiomers can have different pharmacokinetic properties and produce different physiological responses. For this reason, many studies have been conducted on optical resolution by high−performance liquid chromatography (HPLC), and the direct resolution of racemic compounds has been achieved by use of many chiral stationary phases (CSPs). The usefulness of protein−conjugated columns was first demonstrated by Allenmark *et al.* [1−3] and Hermansson [4]. Allenmark *et al.* successfully resolved acidic compounds using a bovine serum albumin−conjugated CSP, and Hermansson resolved racemic amines using α_1−acid glycoprotein−conjugated CSP. Miwa *et al.* have developed a highly effective CSP using ovomucoid, an acid glycoprotein found in chicken egg whites [5]. An ovomucoid column can achieve chiral resolution within a broad range, and is quite resistant to variations in pH, heat, and organic solvents [6,7]. An ovomucoid column alone, used as a protein−conjugated CSP, however, is not sufficient for separation of a great variety of drug enantiomers, and thus, the development of new CSPs is necessary.

2.1. Development of protein−conjugated chiral stationary phases

There are two methods for immobilization of protein onto a support, immobilization by non−covalent conjugation and by covalent conjugation. Covalent conjugation of protein as a CSP onto a support provides superior reproducibility of immobilization, producing reproducible retention and high stability. Haginaka *et al.*

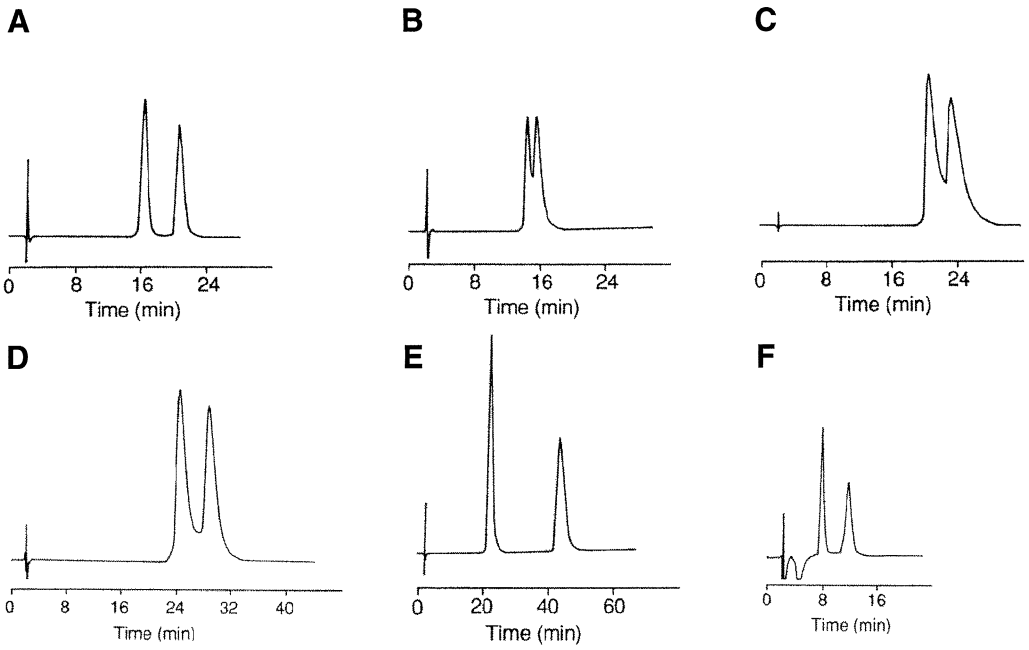


Figure 2. Typical Chromatograms of each compound on a flavoprotein−conjugated column: (A) ketoprofen; (B) ibuprofen; (C) flurbiprofen; (D) α, ε−dibenzoyllysine; (E) warfarin; (F) benzoin.
Conditions: mobile phase, (A), (B), (C), (E) 50 mM KH_2PO_4 /ethanol (9:1), (D) 50 mM potassium phosphate buffer (pH 4.0) /ethanol (94:6), (F) 50 mM potassium phosphate buffer (pH 5.6) /tert.−butanol (96:4); detection, UV at 230 nm; flow rate, 1.0 mL/min; column temperature, room temperature.

compared the efficiency of immobilization of the epoxide and active ester method, and found that the latter is more efficient as an immobilization method [8].

Conalbumin is an egg−white protein that binds iron, copper, manganese, and zinc at pH 6 or above, and acts to block the growth of bacteria. Its molecular weight is approximately 70000−78000, and its pI value is 6.1−6.6. Conalbumin was immobilized onto an aminopropyl silica gel support activated with *N*, *N*−disuccinimidyl carbonate [9], and this new CSP achieved chiral separation for a basic compound, azelastine, and was also able to separate drug enantiomers in a plasma sample with high sensitivity, using column−switching liquid chromatography/frit−fast atom bombardment mass spectrometry [10].

Flavoprotein is a glycoprotein made up of 14% carbohydrate, consisting of mannose, galactose, and glucosaminide. Its pI value is 3.9−4.1, which is similar to that of ovomucoid (3.9−4.3), and its molecular mass is 32000−36000. Interestingly, it also has the ability to bind riboflavin at a 1:1 ratio at pH 4.0 or above. This protein plays an important role in the transfer of riboflavin from blood to egg whites. It is very stable to heat, retaining its riboflavin−binding capacity after heating to 100℃ at pH 7.0 for 15 min. The flavoprotein−conjugated CSP performed chiral separation of acidic, weakly acidic, and neutral compounds as shown in Fig. 2 [11]. Racemic 2−arylpropionates are extensively used in clinical medicine as anti−inflammatory drugs. These profens, ketoprofen, ibuprofen and flurbiprofen, were separated using 50 mM KH_2PO_4 (pH 4.6)/ethanol (9:1, v/v) as a mobile phase, whereas ibuprofen and flurbiprofen were not appreciably resolved, despite their similar structures. α, ε−Dibenzoyllysine, an amino acid derivative and another carboxylic acid used in this experiment, also did not give good resolution with 50 mM potassium phosphate buffer (pH 4.0) /ethanol (94:6, v/v) as a mobile phase. On the other hand, both warfarin and benzoin, which are weakly acidic and neutral compounds, respectively, gave good results for chiral separation using 50 mM KH_2PO_4 (pH 4.6) / ethanol (9:1, v/v) and 50 mM potassium phosphate buffer (pH 5.6) /*tert*.−butanol (96:4, v/v), respectively.

2.2. Affinity of drug−protein for chiral discrimination

HPLC, using a protein−conjugated CSP (protein−CSP) and an aqueous mobile phase, can separate a broad range of enantiomers, and is effective for pharmacokinetic and toxicokinetic studies during drug discovery and development. Many kinds of protein−CSPs have been reported [1−5, 9, 11, 12]. The mechanism for chiral discrimination and retention with protein−CSP, however, has not yet been clarified in detail, because proteins are complex biopolymers consisting of l−amino acid residues. They are capable of numerous interactions with small molecules, such as ionic interactions, hydrophobic interactions, hydrogen bonding, and π−π interactions. Wainer and co−workers reported that $k/(k+1)$ showed a linear relation to protein−binding capacity when human serum albumin was employed as a chiral selector and allosteric effects were demonstrated [13−15]. Therefore, protein binding is essential for retention and chiral separation on protein−CSPs [16−18].

The retention and chiral separation properties of protein−CSPs have been investigated in detail, and the results indicated that the hydrophobic and ionic interactions between enantiomers and a chiral recognition moiety were important for chiral separation with each CSP. In addition, protein binding of enantiomers of native proteins was examined, and enantiomers, which displayed significant protein binding ability, showed strong retention in chromatography [19]. Also, each CSP had a similar property in that enantiomers, which showed significant differences in protein binding ability, were well resolved by chromatography. The results, however, also suggested that retention of solutes on protein−CSPs depended on different contributions from non−specific interactions.

Enzymes can efficiently discriminate between drug enantiomers, contributing to differences in metabolism and biological activity between enantiomers. Since chiral discrimination by proteins is due to differences in the affinity of drug enantiomers for binding sites, chiral separation on protein CSPs can be displayed by a general equation including the association constants [20]. The interactions between solutes and protein CSPs require consideration of the fact that the solute interacts not only with specific binding sites on a protein molecule, but also with non−specific regions, such as the surface of the silica support, aminopropyl spacer, and linkage region (Fig. 3). The theoretical equations for calculating the retention factor (k) and enantioselectivity (α) of protein CSPs were created

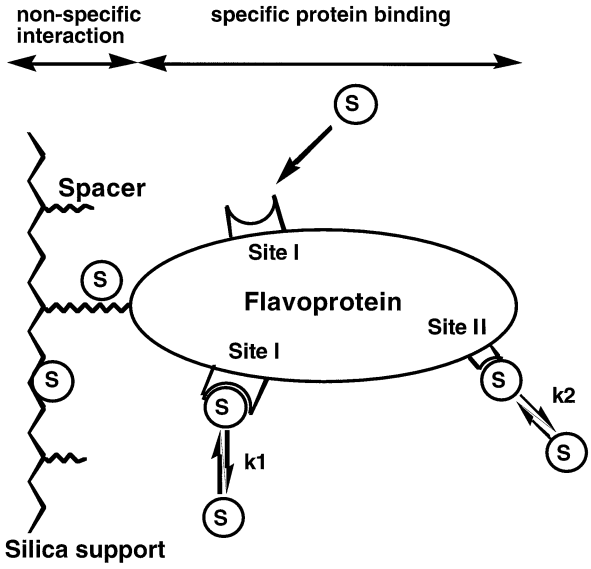


Figure 3. Schematic illustration of the interaction of solutes with a protein−CSP.

as follows:

$$k\mathrm{csp} = \frac{K\mathrm{p}\cdot[\mathrm{P}]\cdot V\mathrm{pro} + V\mathrm{nsp}}{V\mathrm{m}},$$

$$\alpha = \frac{K\mathrm{p}_2\cdot[\mathrm{P}]\cdot V\mathrm{pro} + K\mathrm{nsp}\cdot V\mathrm{nsp}}{K\mathrm{p}_1\cdot[\mathrm{P}]\cdot V\mathrm{pro} + K\mathrm{nsp}\cdot V\mathrm{nsp}},$$

where the association constant (*K*p) between solute and protein molecules is [PS] / ([S]•[P]), [P] is the molar concentration of protein molecules in the mobile phase, [S] is the molar concentration of solutes in the mobile phase, [PS] is the molar concentration of protein−solute complex, *V*pro and *V*nsp are the volume of the protein as a CSP and that of the non−specific regions in the column, *V*m is the mobile phase volume, and *K*nsp is the distribution constant of solute molecules in the non−specific phase. These equations show that the *k* and α values are influenced by the association constant, because [P], *V*pro, *V*nsp, *V*m, and *V*nsp are the eigenvalues for a column.

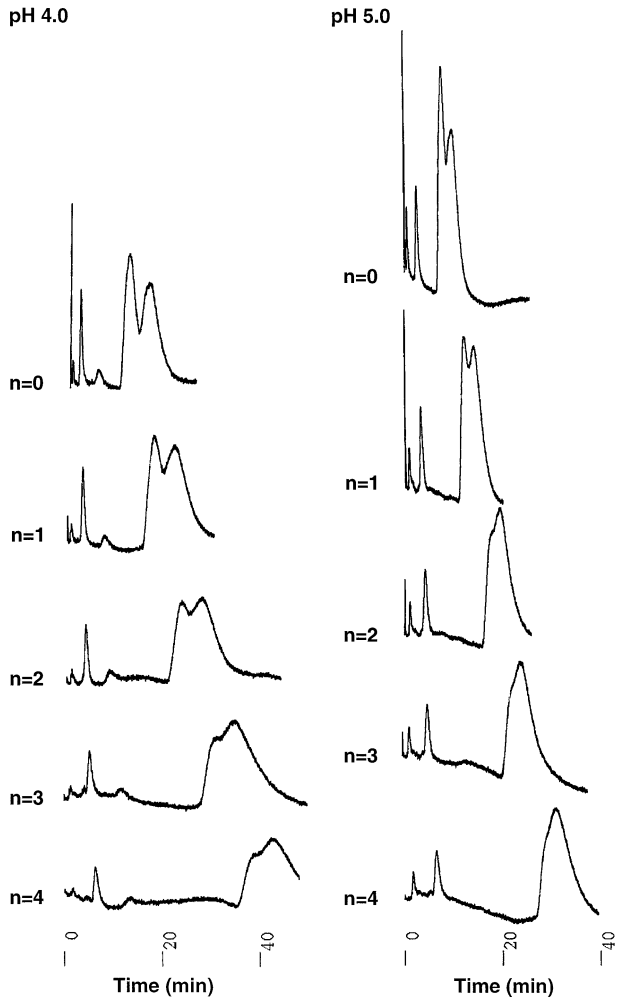


Figure 4. Chromatograms obtained in minicolumn experiments.
A flavoprotein−CSP minicolumn (4.0 mm I.D. x 10 mm) was used in combination with various numbers (n = 0−4) of non−specific stationary phase minicolumns.
Conditions: mobile phase, 20 mM potassium phosphate buffer (pH 4.0 or 5.0); flow rate, 0.5 mL/min; detection, UV at 254 nm; column temperature, 25℃; injection amount, 40 ng/5 μL as racemate.

The validity of the above theory was verified by experiments using a flavoprotein−CSP, in combination with variable numbers of non−specific stationary phases in series. The results indicated that specific and non−specific interactions contribute differently to the retention of ketoprofen enantiomers at pH 4.0 and 5.0 as shown in Fig. 4 and Table 1. At pH 4.0, the specific interaction with the protein has a greater effect than the non−specific interaction in the retention of both enantiomers. On the other hand, the ratio of the non−specific interaction at pH 5.0 is greater than that at pH 4.0. The ionic interaction between the carboxyl group in the ketoprofen molecule may be the predominant factor in the non−specific interaction. Overall, the results support the validity of the complex interactions. Thus, to understand chiral separation on protein−CSP, all interactions, including non−specific interactions must be taken into consideration. That is to say, chiral discrimination observed with native proteins is not necessarily reflected in chromatographic separation, because of effects of non−specific interactions.

Table 1. Effect of non−specific interaction on *k* and α values.
1. Experimental values

NSP	pH 4.0			pH 5.0		
	k_1	k_2	α	k_1	k_2	α
0	24.08	33.00	1.37	15.23	19.47	1.28
1	24.10	29.85	1.24	16.21	19.03	1.17
2	24.30	28.85	1.19	17.92	19.63	1.10
3	25.32	28.19	1.11	18.33	19.77	1.08
4	25.22	27.90	1.11	19.42	20.60	1.06

Chromatographic conditions: mobile phase, 20 mM potassium phosphate buffer; flow rate, 0.5 mL/min; detection, UV 254 nm; injection amount, 40 ng/5 μL.

2. Caluculated values

NSP	pH 4.0			pH 5.0		
	k_1	k_2	α	k_1	k_2	α
0	24.06	32.95	1.37	15.20	19.23	1.26
1	24.07	30.29	1.26	16.33	19.42	1.19
2	24.36	28.96	1.19	17.33	19.79	1.14
3	24.74	28.27	1.14	18.23	20.22	1.11
4	25.13	27.93	1.11	19.01	20.67	1.09

NSP: number (n) of non-specific columns connected with flavoprotein-CSP column.
k_1: *k* value of first-eluted enantiomer of ketoprofen.
k_2: *k* value of second-eluted enantiomer of ketoprofen.
α: enantioselectivity.

2.3. Analysis of the molecular recognition mechanism using affinity capillary electrophoresis

Kaliszan *et al.* investigated the relationship between chiral separation and physicochemical properties of solutes such as hydrophobicity, molecular size, and excess electron charge on the nitrogen atom [21−23]. They concluded that solutes bind to the chiral discrimination region through hydrophobic interactions and that a nearby ionic region is also related to the chiral separation. Pinkerton *et al.* found that only the third domain of turkey ovomucoid has chiral discrimination capacity, and they considered that ionic interactions, hydrophobic interactions, and hydrogen bonding all contribute to chiral discrimination, on the basis of NMR and computational studies [24]. Protein−CSPs not only show specific binding, but also non−specific interactions, e.g., at the silica gel surface, at unreacted aminopropyl groups, and in the linkage region. It is very difficult to investigate only the specific interaction involved in chiral discrimination by using HPLC [20]. Capillary electrophoresis (CE) has very high separation efficiency and many drug enantiomers have been separated by using various chiral selectors. Chiral separation by CE, using protein as a pseudo stationary phase, has been reported [25,26]. In CE, the protein used as a chiral selector is in a nearly native conformation in solution, so retention of the solute may reflect quite well the specific interactions between protein and solute in solution. Lloyd *et al.* compared retention on HPLC and CE with human serum albumin as a chiral selector, and found that the *k* value of benzoin on HPLC and CE showed a linear relationship in running buffer, containing various amounts of 1−propanol [27].

The content of organic modifiers greatly affected retention and chiral separation in chiral protein HPLC, implying that hydrophobic interactions between solute and protein binding sites are very important [28]. Table 2 shows the effect of the methanol content of the running buffer on binding and chiral separation in affinity CE. The *k* values increased slightly up to 20% methanol content, and then decreased at more than 25% methanol. Furthermore, they decreased with increasing ethanol content, in the range of 4−12%, in HPLC. On the other hand, the α values decreased with increasing of methanol content, and the chiral separation of ketoprofen was not achieved at 25% or more methanol. Fig. 5 shows the change in molecular ellipticity of flavoprotein, in relation to the methanol content of the running buffer. Increases in methanol content up to 20% caused a marked change of molecular ellipticity at 208 nm, reflecting conformational change of the secondary structure (α−helix) of flavoprotein, which coincides with a change of α value in affinity CE. The decrease in chiral separation of ketoprofen with increasing methanol content seems to be closely related to a conformational change in flavoprotein. The behavior of the *k* values indicated that electrostatic repulsion between negative charges of the carboxyl group in ketoprofen and a component of the specific binding site was reduced by the methanol−induced conformational change in flavoprotein, whereas hydrophobic interactions were unaffected. In addition, the decreased binding capacity at more than 25% methanol may be due to weakening of the interaction between the carboxyl group of ketoprofen and an amino group in the binding site.

In order to investigate the nature of the amino acid residue in the chiral discrimination region that interacts with ketoprofen, the molecular ellipticity of flavoprotein was measured with various concentrations of ketoprofen solution. Flavoprotein has a weakly negative Cotton effect at 245 nm in methanol−acetic acid buffer (pH 5.0, I=0.05) mixed solution (5:95) as shown in Fig. 6 A. When

Table 2. Effect of methanol content in running buffer on *k* and α values in affinity CE.

Methanol(%)	k_1	k_2	α
0	2.62	2.93	1.12
5	2.67	2.94	1.10
10	2.77	2.91	1.05
15	2.81	2.90	1.03
20	3.01	3.09	1.03
25	2.56	2.56	1.00
30	2.45	2.45	1.00

k_1, smaller *k* value; k_2, larger *k* value; α, enantioselectivity.
Conditions: capillary, μSIL (polyethylene glycol coated capillary, 375 μm O.D., 50 μm I.D.); total length, 40 cm; effective length, 25 cm; electrophoretic buffer, acetate buffer (pH 5.0, I=0.05) containing 200 μM flavoprotein; applied voltage, 10 kV; detection, UV 254 nm; sample amount, 1 mM.

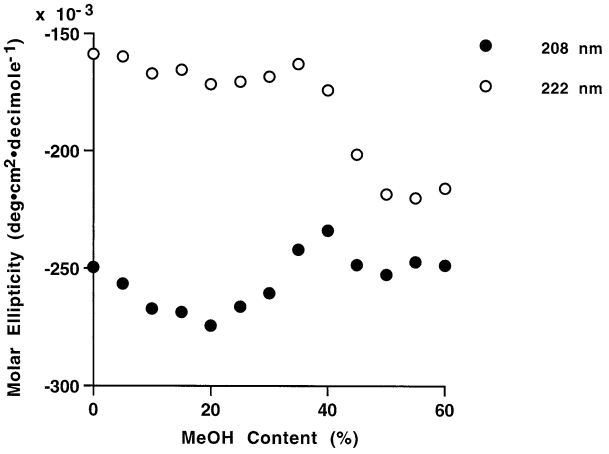


Figure 5. Effect of methanol content on the molar ellipticity at 245 nm of flavoprotein.
Conditions: sample concentration, 5 μM; solvent, acetate buffer (pH 5.0, I = 0.05) /methanol mixture; cell length, 1 mm; scan range, 200−350 nm; scan speed, 50 mm/min; resolution, 0.1 nm; band width, 1.0 nm; accumulation, 5.

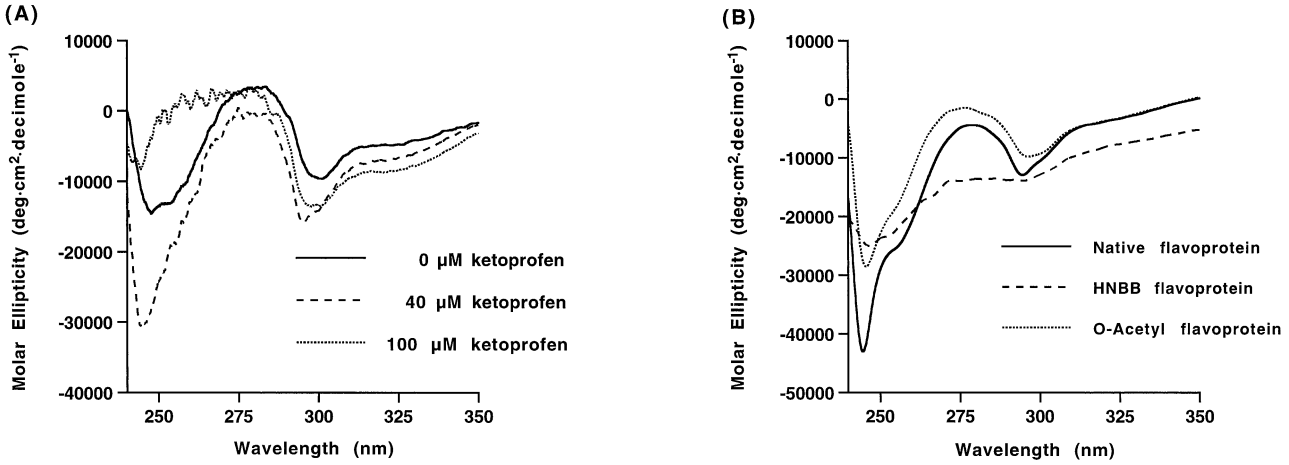


Figure 6. CD spectra of (A) flavoprotein induced by binding of ketoprofen and (B) modified flavoproteins.
Conditions: sample concentration, 50 μM; solvent, acetate buffer (pH 5.0, I = 0.05) /methanol (95:5); cell length, 10 mm; scan range, 240−350 nm; scan speed, 50 mm/min; resolution, 0.1 nm; band width, 1.0 nm; accumulation, 5.

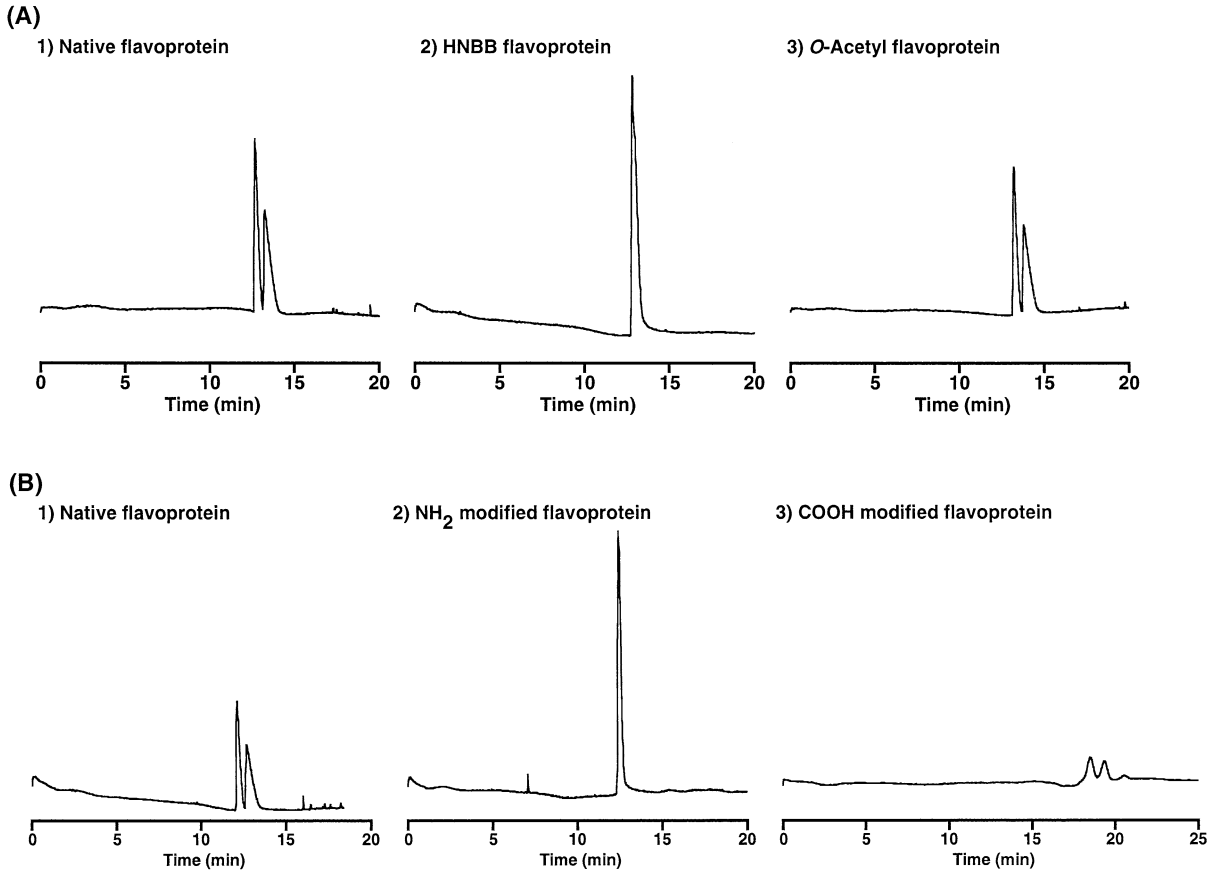


Figure 7. Electropherograms of ketoprofen by affinity CE using (A) native, tryptophan− and tyrosine−modified flavoproteins and (B) native, carboxyl and amino group modified flavoproteins as chiral selectors.
(A−1, B−1) native flavoprotein, (A−2) HNBB flavoprotein, (A−3) *O* −acetyl flavoprotein, (B−2) amino−modified flavoprotein, (B−3) carboxy−modified flavoprotein.
Conditions: capillary, μSIL (linear polyacrylamide coated capillary, 375 μm O.D., 50 μm I.D.); total length, 40 cm; effective length, 25 cm; electrophoretic buffer, acetate buffer (pH 5.0, I =0.05) /methanol (95:5) containing 200 μM flavoprotein or modified flavoprotein; applied voltage, 10 kV; detection, UV at 254 nm; sample amount, 1 mM.

the molar ratio of ketoprofen and flavoprotein was 1:1, the Cotton effect at 245 nm was enhanced, while it was decreased at a molar ratio of more than 2. Moreover, the addition of 100 μM of ketoprofen removed this Cotton effect. These results suggest a strong interaction between ketoprofen and the amino acid side chain, which contributed to the Cotton effect at 245 nm.

Tryptophan, tyrosine, phenylalanine, and cysteine residues influence the long−wavelength CD of proteins. Among them, tryptophan can be easily and specifically modified with 2−hydroxy−5−nitrobenzylbromide (HNBB) under aqueous conditions and tyrosine can be specifically *O*−acetylated by *N*−acetylimidazole. Fig. 6 B shows the CD spectra of native, HNBB−modified and *O*−acetyl−modified flavoprotein. The CD spectrum of *O*−acetyl−modified flavoprotein was similar in shape to that of native flavoprotein. In contrast, HNBB−modified flavoprotein decreased both the native Cotton effect at 245 nm and the positive Cotton effect at around 280 nm. These results suggested that a tryptophan residue on the flavoprotein surface is related to the flavoprotein−ketoprofen interaction. Fig. 7 A shows electropherograms of ketoprofen obtained with native, HNBB−modified, and *O*−acetyl−modified flavoprotein as chiral selectors. With *O*−acetyl−modified flavoprotein, the migration and chiral separation of ketoprofen were almost the same as with the native flavoprotein. The HNBB−modified flavoprotein, however, showed diminished chiral discrimination, though the migration time was almost unchanged. These results indicated that a tryptophan residue is involved in chiral discrimination of ketoprofen by flavoprotein, and a π−π interaction between the aromatic ring of ketoprofen and the indole side chain of the tryptophan residue presumably plays an important part in chiral separation of ketoprofen.

Chiral discrimination by flavoprotein also appears to be influenced by ionic interactions between the carboxyl group in ketoprofen and ionic group(s) at the binding site. Therefore, carboxyl groups of glutamic acid and aspartic acid residues on the flavoprotein, whose pI values are 3.9−4.1, were modified by coupling with glycine ethyl ester in the presence of 1−ethyl−3 (3−dimethylaminopropyl) carbodiimide, and amino groups of lysine and arginine residues were modified by acylation using sulfosuccinimidyl acetate. Fig. 7 B shows electropherograms of ketoprofen obtained with native, amino−modified, and carboxy−modified flavoprotein as chiral selectors. The modification of amino groups quenched chiral separation capacity, and the peak shape was sharper than that with native flavoprotein, though the migration time was almost the same. This phenomenon is considered to be due to loss of ionic interactions between the carboxyl group in ketoprofen and an amino group in the chiral discrimination region. On the other hand, the carboxyl−modified flavoprotein achieved chiral separation of ketoprofen, although the migration time was longer due to reduced electrophoretic mobility of the protein based on the change in native charge. These results indicated that ionic interactions between a carboxyl group in ketoprofen and an amino group in the chiral discrimination region are important for chiral separation. A repulsive interaction between the carboxyl group of ketoprofen and a carboxyl group of the protein, however, destabilizes the interaction.

These studies have established that the chiral recognition region of flavoprotein for ketoprofen in affinity CE consists of an α−helix structure, and the critical groups involved are a tryptophan residue, an amino group, and a carboxyl group of the protein. At more than 25% methanol, the putative π−π interaction between the aromatic rings of ketoprofen and the tryptophan residue is retained, so that binding capacity is largely maintained, although the α−helical structure is considered to be substantially denatured, thereby abrogating the ionic interaction, and consequently, chiral discrimination.

2.4. Quantitative determination of drug enantiomers and their metabolites

E 3810 is an antiulcer agent which inhibits gastric acid secretion as a consequence of blocking an H^+, K^+−ATPase. The region around the sulfur atom and the side chain on the pyridine ring of E 3810 is mainly metabolized. To confirm the utility of the flavoprotein column, E 3810 and its metabolites were applied with a column−switching technique using an avidin−conjugated column for deproteinization, as a pretreatment column made it possible to perform automatic in−line analysis [29]. Typical chromatograms of a blank plasma sample and a sample spiked with E 3810 and its metabolites are shown in Fig. 8, A and B. A chromatogram of a plasma sample obtained following the intravenous administration of racemic E 3810 is shown in Fig. 8 C. (S)−(−)−E 3810 and (R)−(+)−E 3810 were separated from one another on the flavoprotein column, in that order of elution, and M 3 enantiomers were also separated. All peaks of interest were clearly separated with chiral separation by this column−switching system using the flavoprotein column, and there was no interference at the retention time of any of the eluted compounds of interest.

A column−switching high−performance liquid chromatographic method using a flavoprotein column for simultaneous determination of drug enantiomers and their metabolites has been demonstrated. This method employs an avidin column as a pretreatment column for in−line deproteinization and concentration, allowing the direct injection of a large volume of plasma. This type of on−line automatic system using a protein−CSP allows simple, rapid, accurate, and precise determination of drug enantiomers and their metabolites in biological samples, and should be applicable to enantioselective pharmacokinetic studies of various drugs.

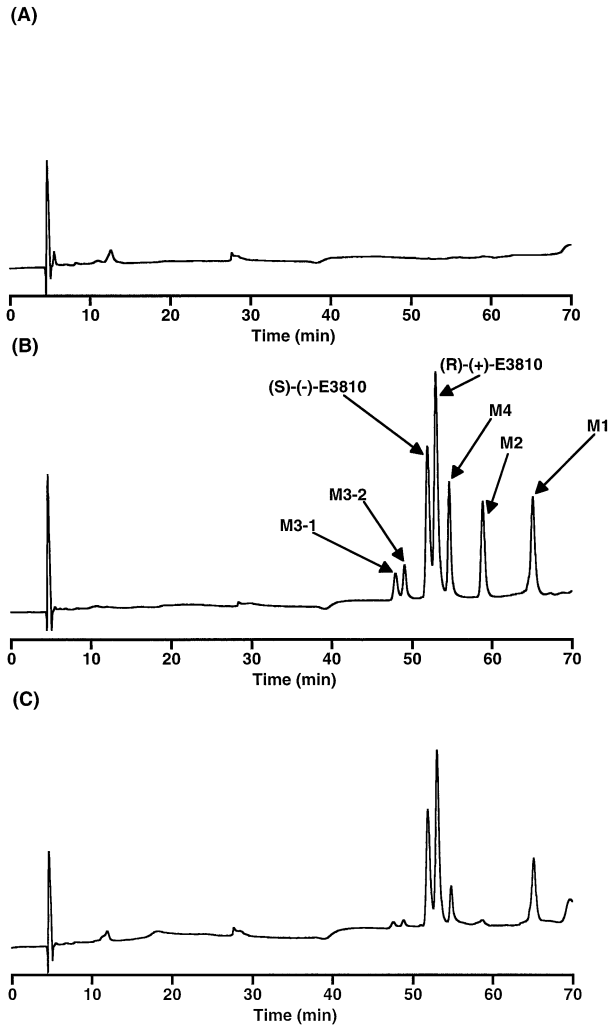


Figure 8. Typical chromatograms of (A) blank beagle dog plasma, (B) plasma spiked with 10 μg/mL of E 3810 enantiomers and 1 μg/mL of their metabolites and (C) plasma from a beagle dog 30 min after intravenous administration of racemic E 3810 (3 mg/kg). Conditions: analytical column, flavoprotein column (4.6 mm I.D. x 250 mm); trapping column, avidin column (4.0 mm I.D. x 10 mm); mobile phase for trapping, 0.1 M potassium phosphate buffer (pH 7.5) at a flow rate of 1.0 mL/min; mobile phase for analysis, A) 20 mM potassium phosphate buffer (pH 5.5)/acetonitrile (99:1), B) 20 mM potassium phosphate buffer (pH 5.5)/acetonitrile (1:1); gradient program, 0% of solvent B for 30 min and 0 to 40% of solvent B over 35 min at a flow rate of 1.0 mL/min; detection, UV at 290 nm.

3. Analysis of low molecular weight compounds−protein adducts

Glucuronidation, which converts lipophilic substances into water−soluble forms, plays a significant role in the metabolism of drugs and endogenous compounds. For a long time, this conjugation process, which is catalyzed by the hepatic glucuronosyltransferase, has been considered an important detoxification mechanism. Recent observations indicate, however, that compounds which act as substrates for the transferase become more toxic upon glucuronidation. Acyl glucuronides of carboxylic acid derivatives are chemically active and irreversibly bind to proteins to produce protein−bound adducts [30−32], which may result in hypersensitivity reactions to acidic compounds [33,34]. Therefore, the formation of acyl glucuronides by the hepatic glucuronosyltransferase requires further characterization.

In contrast, it has been proposed that during the activation of the carboxyl group of biological interest to the acyl coenzyme A (CoA) thioester, which is known to be the intermediate for the formation of amino acid conjugates, the acyl adenylate is first formed and acyl CoA synthetase may be responsible for its formation [35−37]. This acyl adenylate, which is a mixed anhydride consisting of a carboxylic acid derivative and an adenosine monophosphate, shows high reactivity towards nucleophiles such as amino groups on proteins. Most bile acids are metabolized to amino acid conjugates in the hepatocyte through the carboxyl group at position C−24. Therefore, acyl adenylates can be produced as active intermediates during amino acid conjugations of bile acids, and both the biosynthesis of bile acid acyl adenylates and their reactivity to amino groups on protein molecules requires further investigation.

3.1. Acyl glucuronide in the human body

Nonsteroidal anti−inflammatory drugs (NSAIDs), α−arylpropionic acid derivatives having an asymmetric center at the α−position of the carboxyl group, are commonly used in racemate form. Almost all NSAIDs are subjected to a chiral inversion of the *R*−enantiomer into its counterpart, the *S*−enantiomer, through the acyl CoA thioester. Only the *R*−enantiomer is a substrate for hepatic acyl CoA thioester ligases. On the other hand, the enantiomer of flurbiprofen does not act as a substrate for acyl CoA thioester ligases [38], and therefore, there is no chiral inversion of flurbiprofen in humans [39], which suggests that producing amino acid conjugates of flurbiprofen by phase II metabolic reactions would be difficult.

Glucuronidation is one of the major phase II metabolic pathways for endogenous compounds, drugs, and other xenobiotics. The stereoselectivity of acyl glucuronosyltransferases towards NSAIDs has been investigated. *R*−Flurbiprofen metabolism into its acyl glucuronide was found to occur at a rate 2−fold higher than that of the *S*−enantiomer [40]. Furthermore, based on a kinetic study with rat liver microsomes, Magdalou *et al.* found a 5−fold higher rate of formation of flurbiprofen acyl glucuronide than

ibuprofen acyl glucuronide [41]. Therefore, acyl glucuronidation, where a stereoselective reaction may take place, is regarded to be the most important phase II reaction for flurbiprofen.

The elimination of coexisting substances, such as protein and inorganic salts in biological fluids, is a common prerequisite for the separation and determination of trace compounds. Because acyl glucuronides are chemically unstable due to the active ester bond, biological samples must be rapidly stabilized or quenched by exposure to acidic conditions to prevent degradation of biosynthetic acyl glucuronides during sample handling and storage. Therefore, urine specimens were immediately acidified with 10% trichloroacetic acid upon their collection. An LC/ESI−MS system with a simple column−switching technique was employed for an on−line pretreatment procedure, enabling the direct injection analysis of target compounds [42]. This LC/ESI−MS method was effective for the simultaneous resolution of *S* − and *R* −flurbiprofen glucuronides and an internal standard (IS), possessing a fine base−line separation with a detection limit of 7.4 pg (17.6 fmol) /injection of the *S* −flurbiprofen glucuronide, at a signal−to−noise ratio of 10 under a selected ion monitoring mode.

This method was applied to assay 11 human urine samples from healthy volunteers, who received a 40−mg tablet of flurbiprofen orally. Urine specimens were collected 3 hours after drug administration and subjected to the LC/ESI−MS analysis, with a selected ion−monitoring mode utilizing corresponding deprotonated molecules (*m/z* 419 for glucuronides and *m/z* 314 for IS). The typical mass spectrum and chromatogram of urinary flurbiprofen glucuronides are illustrated in Fig. 9 A and Fig. 9 B, respectively. The

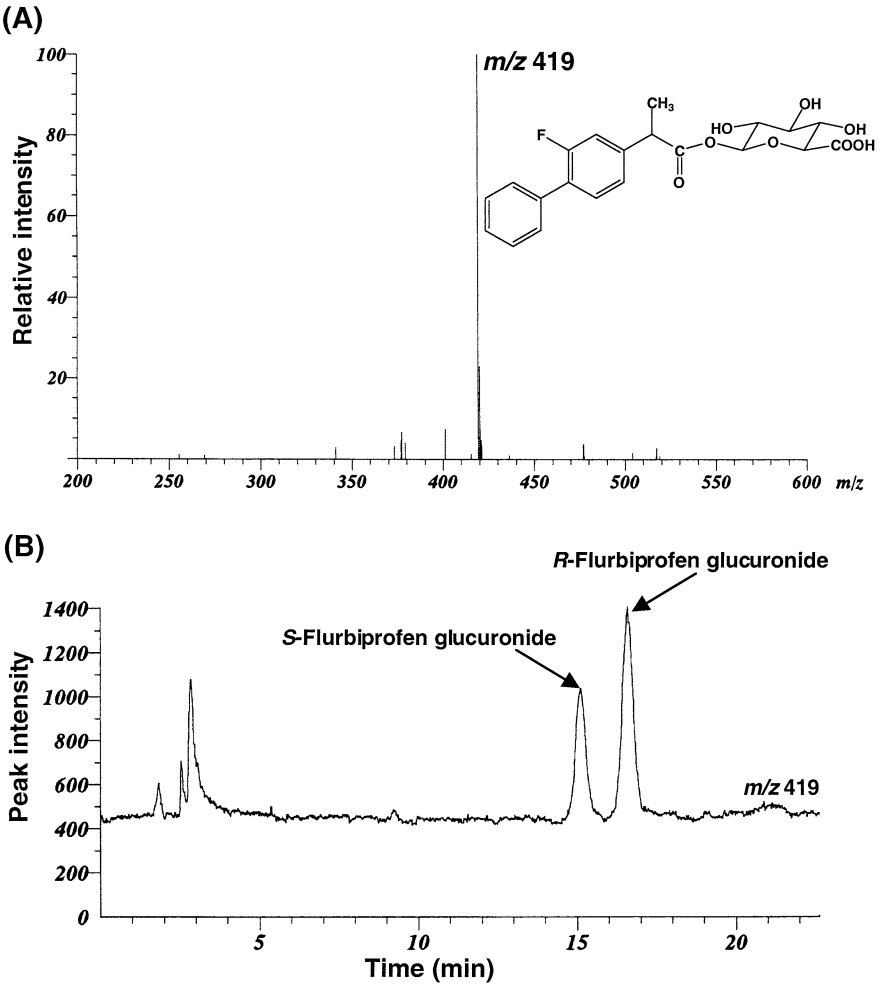


Figure 9. (A) Electrospray negative ion mass spectrum of urinary (R)−flurbiprofen glucuronide and (B) typical selected ion recording of a urine sample.
Conditions: electrospray voltage, −2.5 kV; orifice voltage, 0 V; ring lens voltage, −60 V; orifice temperature, 150℃; desolvating plate temperature, 250℃; analytical column, TSKgel ODS−80 Ts (2.0 mm I.D. x 150 mm); trapping column, Inertsil ODS−3 (4.0 mm I.D. x 10 mm); mobile phase for trapping, 100 mM ammonium acetate buffer (pH 4.0) at a flow rate of 1.0 mL/min; mobile phase for analysis, 20 mM ammonium acetate buffer (pH 5.6)/acetonitrile/ethanol (20:7:2) at a flow rate of 200 μL/min.

Table 3. Concentration of the flurbiprofen glucuronides in human urine 3 hours after oral administration of 40 mg flurbiprofen tablet.

No.	Sex	Age	R-Form (μg/mL)	S-Form (μg/mL)	R/S ratio
1	M	50	23.2±0.61	18.0±0.46	1.29
2	M	43	10.7±0.12	6.0±0.29	1.77
3	M	35	9.9±0.23	5.3±0.11	1.88
4	M	26	6.8±0.08	4.1±0.05	1.66
5	M	24	21.0±0.47	13.0±0.39	1.62
6	M	24	16.4±1.16	14.2±0.85	1.16
7	M	24	30.5±0.46	18.9±0.28	1.62
8	M	23	9.7±0.89	5.9±0.15	1.64
9	M	23	27.0±0.36	14.7±0.16	1.84
10	M	21	6.8±0.32	3.9±0.18	1.75
11	F	23	22.0±0.42	14.2±0.37	1.55
Average			16.7±8.4	10.7±5.7	1.62±0.22

high−resolution mass values corresponding to deprotonated molecules were 419.1130 and 419.1171 for the peaks corresponding to the *S*− and *R*−flurbiprofen glucuronides (theoretical exact mass value: 419.1142) on the chromatogram, with mass errors of only 1.2 and 2.9 mMU, respectively. As shown in Table 3, the concentrations of *R*− and *S*−flurbiprofen glucuronides in human urine from healthy subjects were 6.8~29.4 μg/mL and 3.9~18.0 μg/mL, respectively, suggesting that a slightly higher value of the acyl glucuronide exists for the *R*−enantiomer.

3.2. Production of protein bound adduct through acyl glucuronides

Bile acids are synthesized from cholesterol in hepatocytes and excreted as glycine or taurine conjugates into the intestine via the bile duct. In the intestinal lumen, bile acids undergo deconjugation and dehydroxylation at the 7 α−hydroxy group by intestinal bacteria. These bile acids are then re−adsorbed from the ileum−proximal colon and returned to the liver via the portal vein. Conjugation with sulfuric acid and glucuronic acid also takes place through the 3 α−hydroxy group of bile acids. The levels of sulfates in urine significantly increase in patients with hepatobiliary disease. Although the conjugation of bile acids with glucuronic acid involves the hydroxyl group at the C−3 position on the steroid nucleus, the levels of bile acid 3−glucuronides in human urine are very low [43]. Acyl glucuronides of bile acids, conjugated through the carboxyl group at C−24, have also been demonstrated to be present in human urine. Bile acid acyl glucuronides were also detected at low levels in human urine obtained from both healthy subjects and from patients with hepatobiliary disease [44]. These acyl glucuronides were also preferentially biosynthesized following incubation with rat hepatic microsomal fractions [45]. It has been reported that the acyl glucuronide of a drug having a carboxyl group is capable of reacting with protein, such as human serum albumin, to form a covalent drug−protein adduct [32].

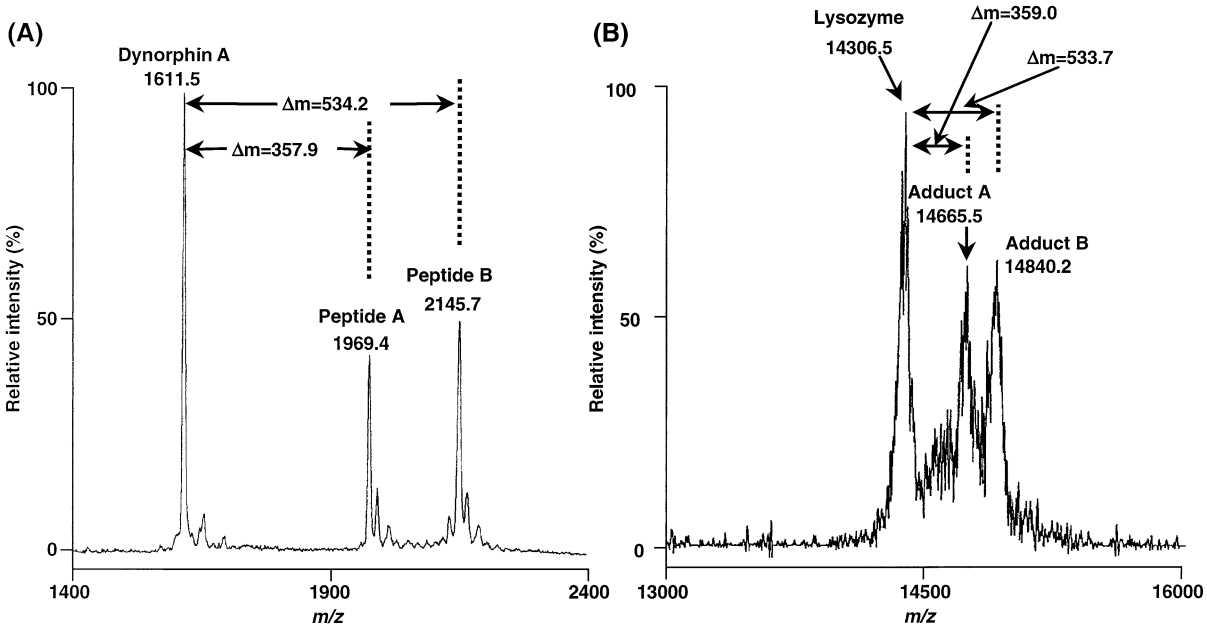


Figure 10. MALDI−TOF mass spectra of (A) the incubation mixture of LCA 24−G with dynorphin A and (B) the incubation mixture of LCA 24−G and lysozyme.
Conditions: mass spectrometer, Voyager RP with a 337 nm pulsed nitrogen laser (Perseptive Biosystems); matrix, (A) α−cyano−4−hydroxycinnamic acid, (B) 3,5−dimethoxy−4−hydroxycinnamic acid; accelerating voltage, 25.0 kV; grid voltage, 14.0 kV; guide wire voltage, 25 V.

Table 4. Observed and calculated peptide fragment of covalent adduct produced by incubation of LCA 24−G and lysozyme.

Amino acid residue		Sequence	*m/z* ([M+H]$^+$)		Binding site
from	to		calcd.	obsd.	
1	13	KVFGRCELAAAMK	1482.8	1482.7	
1	13	KVFGRCELAAAMK	1841.4	1841.1	K-1-LCA
1	13	KVFGRCELAAAMK	2017.5	2017.4	K-1-LCA 24-G
97	116	KIVSDGDGMNAWVAWRNRCK	2365.7	2365.8	
97	116	KIVSDGDGMNAWVAWRNRCK	2724.4	2724.1	K-97-LCA
97	116	KIVSDGDGMNAWVAWRNRCK	2900.4	2900.0	K-97-LCA 24-G

LCA 24−G, which was effectively biosynthesized by the action of hepatic glucuronosyltransferase, was incubated with dynorphin A, and the mass spectrum of the reaction mixture was obtained using MALDI−TOFMS. In addition to the protonated form of dynorphin A at *m/z* 1611.5, intestine ions were newly observed at *m/z* 1969.4 and 2145.7 along with the corresponding potassium adduct ions (Fig. 10 A). The modified peptide A was the covalent binding product of LCA with the peptide through the Arg−1 or the ε−amino function of Lys−8 of dynorphin A, and the modified peptide B was the peptide covalently bound to LCA 24−G.

The next lysozyme as a model protein was incubated with LCA 24−G, and MALDI−TOFMS analysis gave only three ions at *m/z* 14306.5, 14665.5, and 14840.2, corresponding to lysozyme, protein−LCA adduct, and protein−LCA 24−G adduct, respectively (Fig. 10 B). The lyophilized powder was then subjected to reduction and S−carboxymethylation. After protein digestion with lysylendopeptidase, the obtained peptide fragment mixture was subjected to HPLC separation. The peak of interest was collected and subjected to MALDI−TOFMS analysis. The mass values obtained for the lysylendopeptidase digests of the modified lysozyme were compared with those obtained for the authentic lysozyme. As a result, the covalent LCA− and LCA 24−G−lysozyme adducts bound through Lys−1 and Lys−97 of the protein were definitely confirmed (Table 4).

3.3. Inhibition of acyl glucuronidation by bile acids

Acyl glucuronides are hydrolyzed into aglycones under alkaline conditions and, in the presence of alcohol, easily generate the corresponding esters of aglycones under neutral conditions. To characterize the rat hepatic bile acid glucuronosyltransferase, a reliable method for determining such unstable 24−Gs was needed. LC/ESI−MS in the negative ion detection mode was therefore employed, due to its high specificity and selectivity. Various amounts of bile acids were also incubated with the enzyme preparation [47]. A saturation curve following the Michaelis theory was not observed for LCA as a substrate, and the initial velocity of LCA glucuronidation decreased with increasing concentrations of substrates (data not shown). This result strongly suggested that LCA had inhibitory activity towards bile acid acyl glucuronidation. In human body fluids, most bile acids exist as glycine and taurine conjugates,

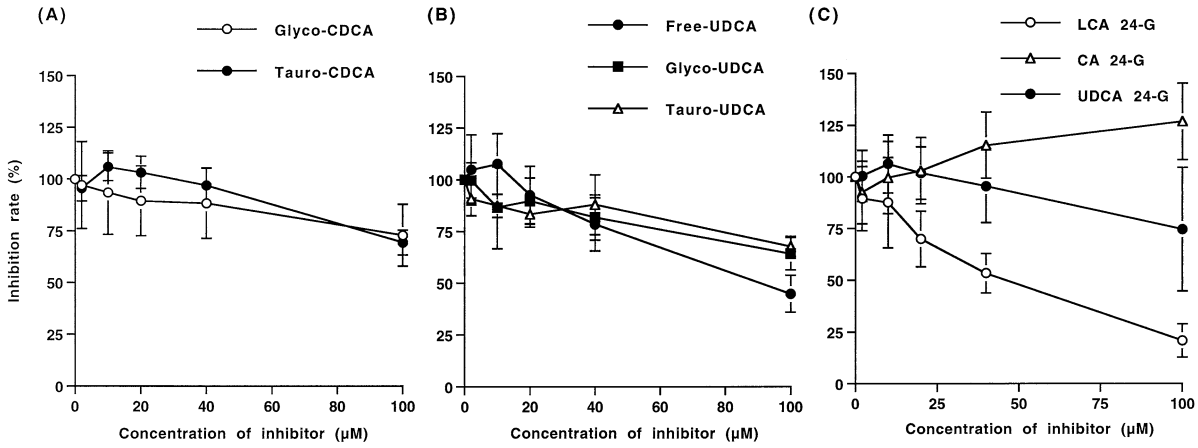


Figure 11. Effect of (A) amino acid conjugated CDCA, (B) unconjugated and amino acid conjugated UDCA and (C) bile acid acyl glucuronides on the formation of CDCA acyl glucuronide.
Twenty μM of CDCA was incubated with microsomal preparations (400 μg of protein/mL) in the presence of various amounts of bile acid derivatives as inhibitors at 37℃ for 10 min.

which are the analogues of substrates. The inhibitory effects of glycine and taurine conjugated CDCA on the formation of CDCA 24−G were therefore investigated. Both glycine and taurine conjugates inhibited the metabolism of CDCA into its 24−G by 20−25%, as illustrated in Fig. 11 A. Glycine and taurine conjugated UDCA also inhibited CDCA 24−G formation by 20−25%, and unconjugated UDCA inhibited the glucuronidation of CDCA 2−fold more potently than conjugated UDCAs (Fig. 11 B).

The enzyme reaction product, the bile acid acyl glucuronide, is a kind of derivatives of the substrate. LCA 24−G, as an inhibitor, was therefore added into the incubation mixture. Addition of an equal molar amount of LCA 24−G into the incubation mixture resulted in a 40% inhibition of acyl glucuronidation of CDCA (Fig. 11 C). In addition, the formation of CDCA 24−G was reduced by 25% in the presence of a 5−fold molar excess of LCA 24−G. UDCA 24−G also inhibited the acyl glucuronidation of CDCA and its degree of inhibition was almost identical to that of glycine and taurine conjugated UDCA. CA 24−G in the incubation mixture resulted in increased formation of the CDCA 24−G. Since the acyl glucuronides seem to be an activated form of glucuronic acid, the excess amounts of CA 24−G may act as a glucuronic acid moiety donor, such as UDPGA.

3.4. Enzymatic formation of acyl adenylate

Prior to conjugation with glycine and taurine, the carboxyl groups of bile acids must be activated by hepatic bile acid acyl−CoA synthetases, transforming them into the corresponding CoA thioester. This enzyme system, existing in a distinct compartment from fatty acid−CoA synthetases, is localized to hepatic microsomal fractions. The reaction mechanism governing the biosynthesis of fatty acyl−CoA proceeds via a ping−pong mechanism. The first step is the transfer of an adenyl group to form an acyl−adenylate (AMP) and pyrophosphate. The acyl−CoA is then formed by displacing the AMP moiety with CoA. The carboxylic acid derivatives may also be condensed by AMP during the biosynthesis of those acyl−CoAs.

To confirm the preferential formation of CA−AMP, preceding the production of the CA−CoA, LC/ESI−MS was applied to the separation and characterization of cholyl−adenylate in an incubation mixture with a rat liver microsomal fraction [48]. The potassium salt of cholic acid was incubated with a hepatic microsomal fraction from a male Wistar rat, and then the reaction mixture was subjected to solid−phase extraction and a portion of the extract was subjected to LC/MS analysis. Typical selected ion recordings are illustrated in Fig. 12 A. In the presence of ATP in the incubation mixture, the peak corresponding to cholyl−adenylate was detected, whereas no peak of cholyl−adenylate on the chromatogram was observed in the absence of ATP in the incubation medium.

Cholic acid was then incubated in the presence of ATP with CoA. As shown in Fig. 12 B, peaks of not only cholyl−adenylate but also of cholyl−CoA were detected. The amount of cholyl−adenylate was increased by the addition of excess cholic acid, in contrast to increasing relative amounts of cholyl−CoA by using low concentration of cholic acid as a substrate. These results may indicate that enzymatic formation of cholyl−adenylate is preferred to the biotransformation of cholyl−CoA.

Since acyl−adenylates correspond to the activated form of carboxylic acids, it seems that cholyl−adenylate reacts with compounds having an amino group. Therefore, the nonenzymatic condensation of cholyl−adenylate with taurine to produce taurocholate was performed. The cholyl−adenylate was incubated with or without taurine and a portion of the incubation mixture was subjected to an LC/MS analysis monitored with a deprotonated molecule having an *m/z* 736 for cholyl−adenylate and 514 for taurocholate. As depicted in Fig. 12 C, cholyl−adenylate was transformed into taurocholate in the presence of taurine, exhibiting the nonenzymatic

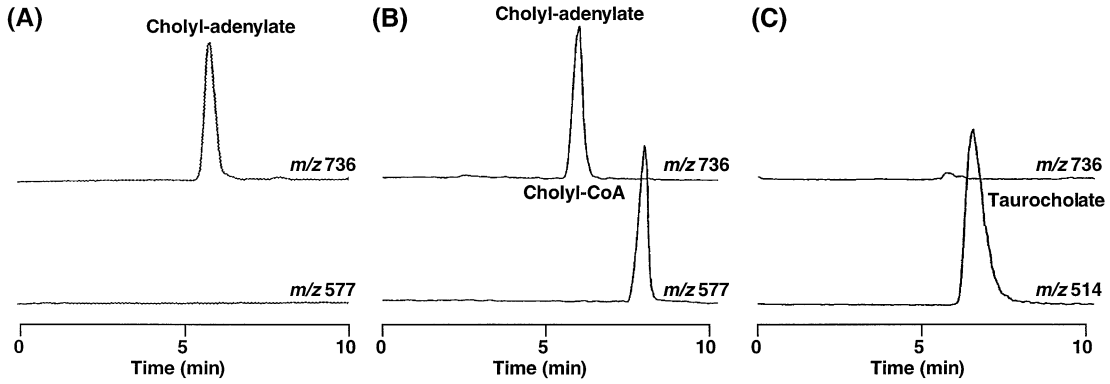


Figure 12. Selected ion recordings of (A) an incubation mixture of cholic acid in the presence of ATP without CoA or (B) with CoA using male Wistar rat liver microsomal preparations (250 μg of protein) and (C) an incubation mixture of cholyl adenylate in the presence of taurine without any enzymes.

amidation of bile acids.

3.5. Production of protein bound adducts through acyl adenylate

Transacylation mechanisms to produce drug−protein adducts operate via nucleophilic substitution through the ε−amino group of lysine in proteins. The structural analysis of such drug−protein adducts is needed, and mass spectrometry is a powerful tool for the structural characterization of proteins with low sample levels. This method can also characterize the binding sites of low molecular weight compounds on a covalently modified protein. Immunoaffinity extraction with a highly specific and selective immobilized antibody is effective for the group separation of modified and unmodified peptides as an efficient pretreatment procedure. Substance P (SP), a lysine−containing undecapeptide amide formed by the post-translational processing of preprotachykinin, was used as a model peptide. The extraction of the adduct chemically synthesized SP bound (S)−ibuprofen was performed using immobilized anti−(S)−ibuprofen antibody, and can clearly distinguish the S−form from the R−form [49]. This enrichment procedure is very useful for selection of targets of post−translationally modified peptides from complex peptide fragment mixtures.

The deoxycholyl adenylate was incubated with an equimolar amount of SP, and the reaction was monitored for 72 h by analyz-

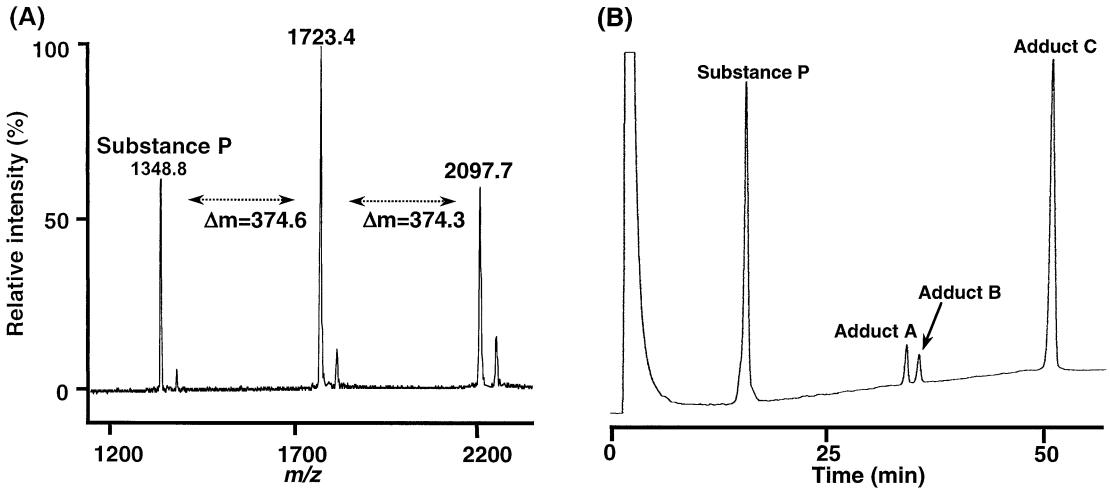


Figure 13. (A) MALDI−TOF mass spectrum and (B) HPLC chromatogram of the reaction mixture resulting from incubation of deoxycholyl adenylate with substance P.
Conditions: mass spectrometer, Voyager RP with a 337 nm pulsed nitrogen laser (Perseptive Biosystems); matrix, α−cyano−4−hydroxycinnamic acid; accelerating voltage, 25.0 kV; grid voltage, 14.0 kV; guide wire voltage, 25 V; column, PRODIGY 5 u C 8 (4.6 mm I.D. x 150 mm); mobile phase, A, water/acetonitrile (9:1) containing 0.08% TFA, B, acetonitrile containing 0.08% TFA; gradient program, 1 to 50% of solvent B over 60 min; detection, UV at 215 nm.

Table 5. Observed and calculated *m/z* values of Lys−C digests of covalent adducts produced by incubation of CA−AMP and lysozyme.

	Amino acid residue		Sequence	*m/z* [M+H]$^+$		Modification site
	from	to		Calcd.	Obsd.	
R1	1	13	KVFGRCELAAAMK	1873.4	1875.2	1K
R2	1	13	KVFGRCELAAAMK	2265.9	2264.0	1K*
R3	97	116	KIVSDGDGMNAWVAWRNRCK	2756.3	2757.3	97K
R4	98	129	IVSDGDGMNAWVAWRNRCKGTDVQAWIRGCRL	4142.9	4145.3	116K
R5	97	129	KIVSDGDGMNAWVAWRNRCKGTDVQAWIRGCRL	4271.0	4273.6	97K or 116K
R6	97	129	KIVSDGDGMNAWVAWRNRCKGTDVQAWIRGCRL	4661.5	4664.5	97K and 116K
R7	14	96	RHGLDNYRGYSLGNWVCAAKFESNFNTQATNRN TDGSTDYGILQINSRWWCNDGRTPGSRNLCNIPC SALLSSDITASVNCAK	9845.3	9853.8	33K

*: Both amino functions of N-terminal and side chain of Lys-1.

ing a portion of the mixture by MALDI−TOFMS [50]. As illustrated in Fig. 13 A, in addition to $[M+H]^+$ for SP at *m/z* 1348.8, additional abundant ions were detected at *m/z* 1723.4 and 2097.7. These values were shifted by 374 and 748 Da (ΔM) from that of the unmodified SP, indicating the addition of one and two molecules of DCA, respectively. Liquid chromatographic separation of the reaction mixture was carried out. The SP, deoxycholyl adenylate, and DCA−SP adducts, represented as adducts A, B, and C, were effectively resolved (Fig. 13 B). The fractions corresponding to peaks A, B, and C were collected, and the components in these fractions were confirmed as the modified peptides by observation of the $[M+H]^+$ ions at *m/z* 1722.3, 1722.7, and 2096.7, respectively, in the MALDI−TOF mass spectra. PSD analyses of these modified peptides were then carried out in order to establish the binding site of DCA. In the PSD mode, an amino acid sequence of a modified peptide can be established by comparison of the product ion pattern of a modified peptide with that of an unmodified peptide.

Next, the ten−fold molar amount of cholyl adenylate was incubated with lysozyme as a model protein. The mass spectrum indicated an abundant ion at *m/z* 15079.9, and a moderately−abundant ion at *m/z* 15428.0, along with the $[M+H]^+$ ion of unmodified lysozyme. These *m/z* values correspond to lysozyme modified with two and three molecules of CA, where the discrepancies between the theoretical and observed mass values were 5 (0.03% error) and 46 (0.3% error) Da, respectively. The whole reaction mixture was then subjected to reductive S−alkylation followed by proteolytic digestion with endoproteinase Lys−C and analysis by MALDI−TOFMS. For a comparison of the mass pattern of the reaction mixture with that of lysozyme alone, the 7 peptide residues modified with CA (R−1−R−7) were monitored as shown in Table 5. It is known that lysozyme has six lysine residues: 1, 13, 33, 96, 97, and 116, and the binding sites of CA obtained by mass analysis were Lys−1, −33, −97, and −116 residues, respectively, which exist at the protein surface.

4. Conclusion

This review has discussed the development, application, and characterization of protein−conjugated chiral stationary phases using conalbumin and flavoprotein from chicken egg whites for separation of drug enantiomers and their metabolites. Protein−conjugated chiral stationary phases not only display specific interactions but also non−specific interactions based on the silica gel surface and linker moieties. These non−specific interactions influenced both retention and chiral separation of solutes. Affinity capillary electrophoresis was very useful for mechanistic analysis of interactions mainly contributing to chiral discrimination by the protein molecule. Coupling with specific chemical modification of amino acid residues or surface functions was also useful for analyzing interactions between solutes and chiral discrimination regions. The interactions functioning on chromatography are the same as those interactions between solutes and their binding sites on the protein surface, and they contribute a main part of binding to the active site on enzymes. Acyl glucuronides and acyl adenylates produced by the action of enzymes are chemically active compounds, which produces protein adducts displaying immunogenicity in the body. The enzymatic formation of these active chemicals and their reactivity towards amino groups on proteins has been demonstrated. To characterize both active intermediates and protein adducts, analytical techniques including highly selective molecular recognition such as high−performance liquid chromatography and mass spectrometry are required.

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