ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 1–9

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Enantioselective immunoaffinity extraction for simultaneous determination of optically active bufuralol and its metabolites in human plasma by HPLC

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Received 21 January 1997; accepted 12 May 1997

Abstract

A combined method of immunoaffinity extraction with high-performance liquid chromatography has been developed for the enantioselective determination of bufuralol and its metabolites in human plasma. The antibodies having high affinity toward the asymmetric center at the C-1 position of bufuralol and its 1′-oxidized metabolites and low affinity to their antipodes were elicited by immunization of rabbits with immunogens, (1*R*)- and (1*S*)-1′-oxobufuralol *O*-carboxymethyloxime-bovine serum albumin conjugates, respectively. 0.5 ml Of the immunoaffinity adsorbent (7.6 $mg \cdot ml^{-1}$ for anti-(1*S*)-antibody and 28.8 $mg \cdot ml^{-1}$ for anti-(1*R*)-antibody) prepared by immobilization of an antibody was capable of retaining up to 1 μg of (*R*)- and (*S*)-bufuralol and up to 500 ng of other metabolites. The adsorbates were recovered quantitatively by elution with methanol–10 mM ammonium acetate buffer (pH 5) (95:5, v/v) without any interfering peaks on the high-performance liquid chromatogram. The proposed method was evaluated to be useful for the simultaneous determination of optically active bufuralol and its metabolite in plasma with acceptable recovery and precision.

Keywords: Bufuralol; Immobilized antibody; Chiral separation; Immunoaffinity extraction; High-performance liquid chromatography

1. Introduction

The chromatographic determination of enantiomeric drugs in biological fluids is one of the most important subjects in biomedical analysis, because of their frequent use as a racemic mixture. Bufuralol, 1-(7-ethylbezofuran-2-yl)-2-*tert*-butylamino-1-hydroxyethane (**1**), is a potent non-selective β-adrenoseptor antagonist [1] with a β_2 partial agonist properties [2,3] and administered as a racemic mixture. The β-blocking po-

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0731-7085/98/$19.00
PII S0731-7085(97)00147-7

tency of (*S*)-bufuralol is approximately 100 times greater than that of the (*R*)-enantiomer [4] (Fig. 1). It is well known that bufuralol is transformed into many metabolites in man and animals [5] and differential metabolism of the two enantiomers occur due to genetic polymorphism [6,7]. Nevertheless, the diastereomeric carbinols (**3**) and the enantiomeric ketones (**2**) have the pharmacological activity comparable to the parent drug [6,8] for sufficient duration to contribute the activity, and are present to a large extent in biological fluid. Therefore, a reliable method for the separation and determination of bufuralol along with its metabolites in biological matrices is essential for pharmacokinetic and pharmacodynamic studies.

Among various methods, high-performance liquid chromatography (HPLC) is well recognized as a powerful tool for the separation and determination of racemates. However, HPLC alone is not necessarily effective for the simultaneous determination of racemates together with their metabolites having plural asymmetric centers.

Recently, immunoaffinity extraction, based on specific interaction between a matrix-bound antibody and soluble biochemicals, has been proposed for the purification of analytes in biological fluids with high selectivity and simplicity [9–12]. In our previous studies a novel method for simultaneous extraction of biologically active substances along with their metabolites has been developed with an immobilized antibody having a broad affinity spectra toward both a parent drug and its metabolites [13–15]. The present paper deals with an enantioselective extraction using an immunoadsorbent prepared by immobilization of an antibody with high recognition toward an essential asymmetric center combined with HPLC separation on a chiral stationary phase for the simultaneous determination of optically active bufuralol and its metabolites.

2. Experimental

2.1. Materials

(1*R*)- (**1a**) and (1*S*)-Bufuralol (**1b**), (1*R*)- (**2a**) and (1*S*)-1′-oxobufuralols (**2b**), (1*R*,1′*R*)- (**3a**), (1*R*,1′*S*)- (**3b**), (1*S*,1′*R*)- (**3c**) and (1*S*,1′*S*)-1′-hydroxybufuralols (**3d**) were synthesized according to methods reported previously [8,16,17] and their optical purities were determined by HPLC to be more than 99%. An activated agarose, Affi-gel 10, and a dye binding assay reagent were obtained from Bio Rad (Hercules, CA). A Sep-Pak C_{18} cartridge was from Millipore-Waters (Milford, MA). Bovine serum albumin (BSA) and horseradish peroxidase (EC 1.11.1.7) (HRP) from *E. Coli* (150–200 U mg^{-1} protein) were supplied by Sigma (St Louis, MO). 3,3′,5,5′-Tetramethylbenzidine was purchased from Nacalai Tesque (Tokyo, Japan). Complete Freund's adjuvant was supplied by Iatron (Tokyo, Japan). Goat anti-rabbit immunoglobulin G (IgG) antiserum and normal rabbit serum were obtained from Daiichi Radioisotope (Tokyo, Japan). Rivanol (2-ethoxy-6,9-diaminoacridine lactate) was obtained from Wako (Osaka, Japan). All solvents were purified by distillation in glassware prior to use.

2.2. Apparatus

Melting points were measured on an electric micro hot-stage apparatus and uncorrected. Ultraviolet (UV) spectra were measured with a Hitachi Perkin-Ermer 139 UV-VIS spectrometer and

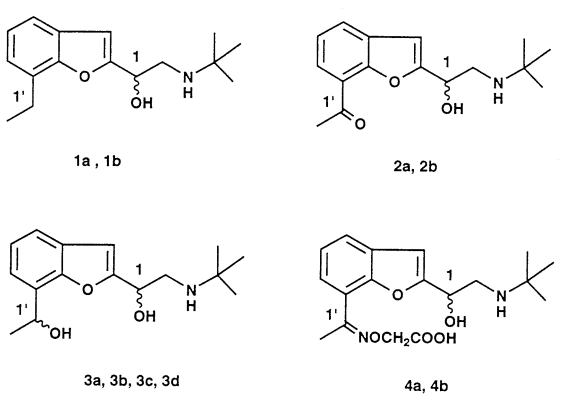

Fig. 1. Structures of bufuralol and related compounds. The a and b, and c and d series represent the compounds with the *R* and *S* configuration at the C-1 and C-1′ position, respectively: 1, bufuralol; 2, 1′-oxobufuralol; 3, 1′-hydroxybufuralol; 4, 1′-oxobufuralol *O*-carboxymethyloxime.

a Hitachi Sample Semiautomatic 100-20 spectrometer. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a Hitachi Model R-300 spectrometer at 300 MHz using tetramethylsilane as an internal standard (s, singlet; d, doublet; m, multiplet). HPLC was carried out on a JASCO Model PU-980 solvent delivery system equipped with a UV-970 spectrophotometer (at 248 nm). An Ultron ES-OVM (5 μm) column (150 × 4.6 mm i.d.) (Shinwa, Osaka, Japan) was used under ambient temperature at a flow rate of 1.0 ml min^{-1}.

2.3. Preparation of (1R)- and (1S)-1′-oxobufuralol O-carboxymethyloxime (**4a** and **4b**)

A mixed solution of (1*R*)- (**2a**) (51 mg) or (1*S*)-1′-oxobufuralol (**2b**) (35 mg), *O*-carboxymethoxylamine HCl (51 mg for **2a** and 32 mg for **2b**) and sodium acetate (50 mg for **2a** and 35 mg for **2b**) in ethanol (3.5 ml) was stirred at room temperature for 2–4 h. After evaporation of the solvent, the residue was acidified with 5% AcOH and extracted with AcOEt. The H_2O layer was passed through a column (25 × 23 mm i.d.) of prep PAK-500 C_{18} (3 g, Millipore, Milford, MA). After washing with H_2O (15 ml), the resultant oxime derivative was eluted with methanol (5 ml). Recrystallization of the eluate from aqueous acetone gave **4a** (42 mg) and **4b** (37 mg) as colorless crystals, respectively. **4a**: mp 234–237°C (dec.). $[\alpha]_D + 51.9°$ (c = 0.10). Analysis calculated for $C_{18}H_{24}N_2O_5 \cdot 1/5H_2O$: C, 61.42; H, 6.99; N, 7.96. Found: C, 61.39; H, 6.83; N, 7.90. ^{1}H NMR ($CDCl_3$–CD_3OD) δ: 1.33 (9H, s, *tert*-C_4H_9), 2.41 (3H, s, CH_3), 4.61 (2H, s, $NOCH_2COO$), 5.22 (1H, m, 1′-H), 6.61 (1H, s, 3′-H), 7.25 (1H, d, $J = 6.6$ Hz, 5′-H), 7.38 (1H, d, $J = 6.6$ Hz, 4′-H), 7.49 (1H, d, $J = 6.6$ HZ, 6′-H). **4b**: mp 234-237°C. $[\alpha]_D$ −40.8° (c = 0.10). Analysis calculated for $C_{18}H_{24}N_2O_5 \cdot 1/8H_2O$: C, 61.65; H, 6.97; N, 7.99. Found: C, 61.58; H, 6.90; N, 7.79. ^{1}H NMR ($CDCl_3$–CD_3OD) δ: 1.27 (9H, s, *tert*-C_4H_9), 2.40 (3H, s, CH_3), 4.61 (2H, s, $NOCH_2COO$), 5.31 (1H, m, 1′-H), 6.56 (1H, s 3′-H), 7.25 (1H, d, $J = 6.6$ Hz, 5′-H), 7.36 (1H, d, $J = 6.6$ Hz, 4′-H), 7.47 (1H, d, $J = 6.6$ Hz, 6′-H).

2.4. Preparation of antiserum

To a solution of **4a** or **4b** (each 21 mg) in dry dimethylformamide (0.7 ml) was added tributylamine (120 μl) and isobutylchloroformate (60 ml) at 11°C, and the mixture was stirred for 5 h. Then BSA (100 mg) in H_2O (7.2 ml)–dimethylformamide (1.4 μl)–1M NaOH (80 μl) was added dropwise under ice cooling and the resulting solution was stirred overnight. The reaction mixture was dialyzed against cold running water at 4°C for 24 h. The protein was precipitated by addition of acetone followed by centrifugation at 3000 rpm for 10 min. This procedure was repeated until no unconjugated hapten could be detected by TLC. Lyophilization of the resulting solution afforded the BSA adduct (81 mg for **4a** and 96 mg for **4b**) as a fluffy powder. The number of hapten molecules incorporated into a BSA molecule was determined to be 6 for **4a** and 11 for **4b** by UV spectrophotometric analysis at 259 nm.

The hapten–BSA conjugate (1 mg) thus obtained was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into domestic female albino rabbits subcutaneously at multiple sites over the back. This procedure was repeated once every fortnight. The antisera prepared from blood by centrifugation at 3000 rpm for 10 min was stored at 4°C with 0.1% (w/v) NaN_3.

2.5. Preparation of HRP labeled bufuralol

A solution of the hapten (500 μg for **4a** and 570 μg for **4b**) in dry dimethylformamide (250 μl) was stirred with isobutylchloroformate (10 μl) and tributylamine (12 μl) for 35 min at 4°C. Then HRP (1 mg) in water (1.5 ml)–dimethylformamide (0.2 ml) was added under ice cooling and the mixture was stirred at 4°C for 3 h. During this time, the solution was kept to pH 8.0–8.5 by addition of 1M NaOH. The resulting solution was dialyzed against 0.05M phosphate buffer (pH 7.3) (PB) at 4°C overnight and diluted to 400 μg ml^{-1} with PB (pH 7.3) and stored at 4°C until use.

2.6. Procedure for EIA

The HRP-labeled bufuralol (40 ng, 100 μl), standard (*R*)- or (*S*)-bufuralol (0, 20, 50, 100, 300, 600, 1000 and 2000 pg) in PB (pH 7.3) containing 0.9% (w/v) NaCl and 0.1% (w/v) gelatin (buffer A, 0.1 ml) or buffer A (0.1 ml), and 0.5% normal rabbit serum diluted with buffer A (0.1 ml) were added to diluted antiserum (0.1 ml), and the whole was allowed to stand at 4°C for 4 h. After addition of goat anti-rabbit IgG antiserum (0.1 ml) diluted to 1:30 with buffer A containing 0.1% EDTA, the solution was further allowed to stand at 4°C for 15 h. The resulting mixture was diluted with 0.05 M PB (pH 7.3) (1.5 ml) and centrifuged at 3000 rpm for 10 min. The immune precipitate was collected by aspirating off the supernatant and then washed with PB (pH 7.3) (1.5 ml) by repeating the procedure. The precipitate was suspended in 1.8 ml 0.05 M sodium acetate–citrate buffer (pH 4.2) containing 0.42 mM 3,3′,5,5′-tetramethylbenzidine and 3% dimethylsulfoxide and, following preincubation at 37°C for 3 min, 0.2 ml 0.02% H_2O_2 was then added. After the mixture was incubated at 37°C for 45 min, 0.05 M H_2SO_4 (2 ml) was added to terminate the reaction. The absorbances at 450 nm were measured.

2.7. Determination of affinity of antibody

An affinity spectrum of the antibody was assessed by EIA using seven kinds of related compounds. The relative magnitude of affinity was expressed as the percentage of the amount of bufuralol which reduced the enzymic activity in the immune precipitate by half to the amount of each the compound listed in Table 1.

2.8. Immobilization of antibody

A mixture of antiserum (32 ml for anti-(1*R*)- and 33 ml for anti-(1*S*)-bufuralol antibody, respectively) and a 0.4% (w/v) rivanol solution (110 ml) was vortex-mixed gently on ice for 30 min and then centrifuged at 3000 rpm for 10 min at 4°C. To the supernatant was added activated charcoal (3.6 g), and the whole was stirred for 15 min on ice. After centrifugation at 3000 rpm for 10 min, the supernatant was passed through a membrane filter (pore size 0.45 μm) and subjected to lyophilization. After dissolution in 0.1 M Hepes (pH 8.0) (3.5 ml), the content of protein in the dried residue was determined by the dye-binding assay using γ-globulin as a standard [9]. The IgG fraction (126 mg for anti-(1*R*)-bufuralol antibody and 33 mg for anti-(1*S*)-bufuralol antibody, respectively) was added to Affi-gel 10 (4.1 ml) prerinsed with isopropanol (10 ml), 10 mM acetate buffer (20 ml) and 0.1M Hepes (pH 8.0) (10 ml), and the suspension was then gently stirred at 4°C for 24 h. After addition of 1 M ethanolamine (pH 8.0, 0.6 ml), the adsorbent was gently stirred at room temperature for 1 h and then washed successively with 0.1 M Hepes (pH 8.0, 20 ml) and PB (pH 7.3, 20 ml) until the absorbance at 280 nm of the eluate had disappeared. These were then stored at 4°C in PB (pH 7.3) containing 0.2% (w/v) NaN_3. The immunosorbent (0.5 ml) thus obtained was packed into a disposable pipette (290 × 6 mm i.d.) silanized with trimethylchlorosilane and used after successive washing with water (10 ml) and PB (pH 7.3, 10 ml)

Table 1
Relative affinity of antibody as determined by EIA

Compound	Relative affinity (%)	
	1*S*	1*R*
(1*R*)-bufuralol	0.83	100
(1*R*)-1′-oxobufuralol	0.56	196
(1*R*, 1′*R*)-1′-hydroxybufuralol	5.98	27.1
(1*R*, 1′*S*)-1′-hydroxybufuralol	7.31	53
(1*S*)-bufuralol	100	3.52
(1*S*)-1′-oxobufuralol	121	2.10
(1*S*, 1′*S*)-1′-hydroxybufuralol	96.1	0.42
(1*S*, 1′*R*)-1′-hydroxybufuralol	101	0.22

1*S*, anti-(1*S*)-bufuralol antiserum; 1*R*, anti-(1*R*)-bufuralol antiserum.

2.9. Determination of the capacity of the immunoaffinity adsorbent

The immobilized antibody (0.5 ml) in a glass column (6 mm i.d.) equilibrated with PB (pH 7.3) was charged with bufuralol and its related compounds in PB (pH 7.3), and then washed with 1

M NaCl (5 ml) and water (10 ml). The non-adsorbed analytes present in the washings were extracted on a Sep-Pak C18 cartridge, followed by elution with methanol (5 ml). The adsorbed analytes were then eluted with 10 mM ammonium acetate buffer (pH 5.0)—methanol (5:95 v/v) (5 ml) and bufuralol and its related compounds present in these fractions were determined by HPLC. The evaluation of the immunoaffinity adsorbent using human plasma (1 ml) spiked with bufuralol and its metabolites was also performed in the same manner.

3. Results and discussion

3.1. Preparation of antiserum

Bufuralol having an asymmetric center at the C-1 position in the molecule mainly undergoes aliphatic oxidation at C-1′, resulting in making the new chiral center appearance to give 1′-hydroxylated bufuralols (carbinols) (**3a**–**d**), which are further transformed into the 1′-ketonic derivatives (**2a**–**b**). To prepare an immunoaffinity adsorbent used for enantioselective extraction of optically active bufuralol and its 1′-oxidative metabolites, the antibody should have discriminative abilities toward the C-1 asymmetric center with significant binding ability to metabolites as well as to the parent compound. It is well known that an antibody raised against a haptenic molecule usually shows significant cross-reactivity with compounds homologous around the bridge portion used for conjugation with a carrier protein. Therefore, an initial effort was directed to the preparation of (1*R*)- and (1*S*)-1′-oxobufuralol *O*-carboxymethyloxime derivatives (**4a** and **4b**) as pertinent haptens by condensation of (1*R*)- (**2a**) and (1*S*)-1′-oxobufuralol (**2b**) with carboxymethoxyl amine. The haptenic compounds obtained were then covalently coupled with BSA by a mixed anhydride method, respectively, to produce bufuralol–BSA conjugates. A satisfactory number of hapten molecules (6 for **4a** and 11 for **4b**) were incorporated in each conjugate. The immunogen thus obtained was administered subcutaneously to individual rabbits with complete Freund's adjuvant. The evaluation of the titer was carried out by incubation of variously diluted antiserum with a bufuralol–HRP conjugate. The appropriate antisera were obtained 6 months after initial immunization.

The EIA system with these antibodies provided the optimum dilution of both antisera to be 5000 (v/v) and feasible dose response curves for (*R*)- and (*S*)-bufuralol in the range of 0.25–10 ng per tube. The affinity spectra of antibodies were determined by ascertaining the ability of optically active bufuralol and its metabolites to compete with an enzyme-labeled antigen in binding to the antibody, and the results obtained are listed in Table 1. As expected, both antisera are characteristic of discriminating the corresponding antipodes with their cross-reactivity of a few per cent or less. The affinity spectrum of the antibody elicited from (1*R*)-bufuralol–BSA conjugate contrasted sharply with that of the antibody obtained from (1*S*)-bufuralol–BSA conjugate. The anti-(1*R*)-bufuralol antibody showed high affinity to (1*R*)-bufuralol and its 1′-oxygenated metabolites where the magnitudes of the cross-reactivities to carbinol derivatives was somewhat low (27 and 53%, respectively). On the other hand, the reactivities toward their corresponding (1*S*)-antipodes were significantly lower as expected. A similar phenomenon was observed for the contrasted anti-(1*S*)-bufuralol antibody, showing 96–121% cross-reactivity to (1*S*)-bufuralol and its 1′-oxygenated metabolites with low affinity toward the corresponding (1*R*)-antipodes. These results are ascribable to the site of conjugation, since (1*R*)- and (1*S*)-bufuralols were coupled with BSA through the bridge at the C-1′ position remote from the C-1 asymmetric carbon.

3.2. Immunoaffinity extraction

It was obvious from the results so far obtained that the group separation of optically active bufuralol and its 1′-oxidized metabolites based on the chiral recognition toward C-1 asymmetric center of these molecules was accomplished by the use of these antisera. Accordingly, the immobilized antibodies were then prepared. Immunoglobulin G, obtained as a γ-globulin

fraction from antisera by the method of Horejsi and Smentana [18] with a minor modification, were then covalently coupled on a cross-linked agarose having a 10 carbon-atom spacer with an *N*-succinimidyl ester moiety as a reacting group. The amount of IgG immobilized on an agarose matrix was estimated to be 28.8 and 7.6 $mg \cdot ml^{-1}$ of the gel for anti-(1*R*)- and (1*S*)-bufuralol antibody, respectively.

The condition for elution of bufuralol and 1′-oxobufuralol, which have a relatively high affinity to the antibody, from a column packed with a corresponding immunoadsorbent (0.5 ml) was assessed with various eluents where the recovery from the adsorbent was determined by HPLC. The independent use of an organic solvent, methanol or acetonitrile, which has been commonly applied to elute target compounds on an immunosorbent, was not suitable for the quantitative recovery of bufuralol and its derivatives. It is well known that the addition of an organic salt to an organic solvent has a good effect on the elution of the adsorbates on the gel, implying that electrostatic bonds that could mediate the specific interaction between the bound ligand and the immobilized antibody might be minimal. The combined use of an organic solvent with an organic salt was therefore undertaken. As a result, 5 ml methanol–10mM ammonium acetate buffer (pH 5) (95:5 v/v), which could be easily removed by evaporation to dryness without any interference for a subsequent HPLC analysis, was chosen as a suitable eluant for the quantitative recovery of these compounds (Table 2). The capacity of the adsorbents for target compounds was then evaluated individually by the determination of bufuralol and its metabolites in the adsorbate fraction after passing through the column packed with corresponding immobilized anti-(1*R*)- or (1*S*)-bufuralol antibody. As shown in Table 3, the amount of (1*R*)- and (1*S*)-bufuralol and their 1′-ketonic derivatives in the adsorbate fraction increased with an increasing loaded amount up to 0.5–1 μg. The capacity of these adsorbents for the corresponding 1′-carbinol was estimated to be 0.1–1 μg. We have previously shown that even compounds with a low reactivity to antibody can also be captured on an immunosorbent together with compounds having a high affinity because of the use of a great excess of an immobilized antibody. Actually when the corresponding antipode was loaded alone, a small part was adsorbed (Table 4). Fortunately these antipodes were not captured on the immunoadsorbent, being loaded with appropriate optical isomers, such as exist in biological materials, because of their low affinities (Table 5). These results indicate that immobilized antibodies are capable of retaining optically active bufuralol and its metabolites selectively in biological fluids in which analytes are present in low concentrations.

3.3. Chromatographic condition

The next effort was directed to the development of chromatographic conditions of HPLC on a chiral stationary phase in order to obtain the complete separation of four target compounds classified into two groups of 1*R* and 1*S* configurations which was attained by immunoaffinity extraction described above. From the results, the use of an Ultron ES-OVM column with a linear gradient elution from 14:1 to 2:1 0.3% ammonium

Table 2
Recovery of bufuralol and 1′-oxobufuralol from the corresponding immunoadsorption column

Compound	Recovery (%, mean ± S.D.)	
	Eluent (5ml)	
	$CH_3OH–H_2O$[a]	CH_3OH–10 mM CH_3COONH_4[a]
Anti-(1*R*)-bufuralol antibody		
(1*R*)-bufuralol	99.4 ± 5.5	98.9 ± 1.2
(1*R*)-1′-oxobufuralol	83.9 ± 6.0	100.4 ± 2.1
Anti-(1*S*)-bufuralol antibody		
(1*S*)-bufuralol	97.8 ± 1.5	99.3 ± 3.7
(1*S*)-1′-oxobufuralol	85.7 ± 6.1	102.5 ± 2.9

$n = 5$.
[a] (95:5 v/v).

Table 3
Extraction efficiency of bufuralol and its metabolites with the immunoadsorption column as determined by HPLC

Compound	Recovery (%)			
	Amount loaded (μg)			
	0.1	0.5	1	2
Anti-(1*R*)-bufuralol antibody				
(1*R*)-bufuralol	103.4 ± 1.4	99.4 ± 5.5	94.4 ± 2.6	52.4 ± 3.4
(1*R*)-1′-oxobufuralol	100.4 ± 2.1	100.2 ± 9.0	61.5 ± 1.5	30.9 ± 2.0
(1*R*, 1′*R*)-1′-hydroxybufuralol	107.2 ± 1.7	97.8 ± 6.8	33.1 ± 2.4	19.9 ± 3.7
(1*R*, 1′*S*)-1′-hydroxybufuralol	98.7 ± 4.7	66.1 ± 7.5	31.4 ± 2.7	14.4 ± 1.4
Anti-(1*S*)-bufuralol antibody				
(1*S*)-bufuralol	97.5 ± 4.0	97.8 ± 1.5	87.6 ± 2.7	47.1 ± 1.6
(1*S*)-1′-oxobufuralol	102.5 ± 2.9	100.4 ± 1.8	61.8 ± 8.7	33.4 ± 4.5
(1*S*, 1′*S*)-1′-hydroxybufuralol	102.1 ± 3.3	93.1 ± 2.2	96.2 ± 7.2	49.9 ± 6.1
(1*S*, 1′*R*)-1′-hydroxybufuralol	99.5 ± 1.7	97.1 ± 2.9	94.3 ± 7.6	44.8 ± 7.3

$n = 5$.

acetate buffer (pH 6.7) and acetonitrile at a rate of 1 ml min^{-1} was effective without any significant leading and/or tailing. All the four compounds were completely separated within 30 min and the elution order was carbinol, 1′-ketonic derivative and then bufuralol. Irrespective of the configuration at C-1, the carbinols having 1′S configuration were eluted earlier.

Table 4
Adsorption of optical antipodes on the immunoadsotption column

Compound (500 ng each)	Adsorption (ng, mean ± S.D.)
Anti-(1*R*)-bufuralol antibody	
(1*S*)-bufuralol	137.5 ± 4.0
(1*S*)-1′-oxobufuralol	348.5 ± 20.5
(1*S*, 1′*R*)-1′-hydroxybufuralol	37.0 ± 5.5
(1*S*, 1′*S*)-1′-hydroxybufuralol	54.0 ± 5.0
Anti-(1*S*)-bufuralol antibody	
(1*R*)-bufuralol	57.5 ± 1.5
(1*R*)-1′-oxobufuralol	21.5 ± 4.0
(1*R*, 1′*R*)-1′-hydroxybufuralol	5.0 ± 1.0
(1*R*, 1′*S*)-1′-hydroxybufuralol	13.0 ± 3.5

$n = 5$.

3.4. Application to a plasma sample

The proposed immunoaffinity extraction procedure combined with HPLC for the separation of bufuralol and its metabolites in biological fluids was then applied to human plasma spiked with bufuralol and its metabolites. After dilution with PB, 1 ml human plasma spiked with 50 or 100 ng

Table 5
Adsorption of optical antipodes on the immunoadsorption column in the presence of (1*R*)- and (1*S*)-1′-ethyloximinobufuralol (4 and 3 μg)

Compound (200 ng each)	Adsorption (ng, mean ± S.D.)
Anti-(1*R*)-bufuralol antibody	
(1*S*)-bufuralol	4.2 ± 0.8
(1*S*)-1′-oxobufuralol	N.D.
(1*S*, 1′*R*)-1′-hydroxybufuralol	N.D.
(1*S*, 1′*S*)-1′-hydroxybufuralol	N.D.
Anti-(1*S*)-bufuralol antibody	
(1*R*)-bufuralol	8.8 ± 1.7
(1*R*)-1′-oxobufuralol	N.D.
(1*R*, 1′*R*)-1′-hydroxybufuralol	N.D.
(1*R*, 1′*S*)-1′-Hydroxybufuralol	N.D.

$n = 5$.

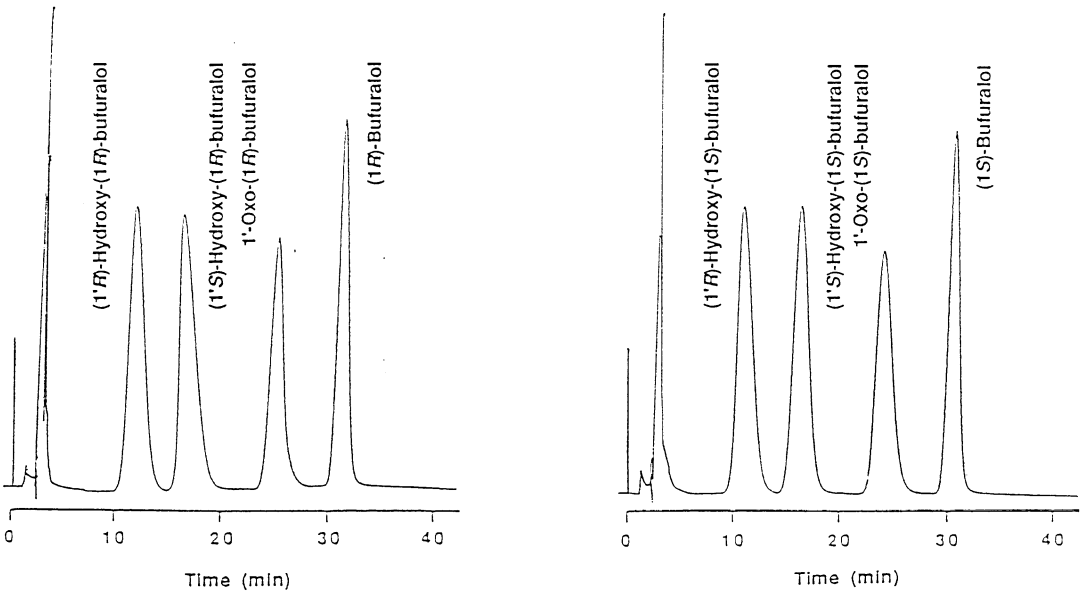

Fig. 2. HPLC chromatograms of eluates from immunoadsorption columns loaded with a plasma specimen spiked with a mixture of 50 ng each of optically isomereric bufuralol and its metabolites

each bufuralol and its metabolites was loaded onto an anti-(1*R*)-bufuralol antibody immunoadsorbent column, and the washes were then loaded onto an anti-(1*S*)-bufuralol antibody immunoadsorbent column. The adsorbates on the immunoadsorbent were eluted from the column and then subjected to HPLC analysis on an Ultron ES-OVM column with 0.3% ammonium acetate (pH 6.7)–acetonitrile as a mobile phase in a linear gradient elution from 14:1 (v/v) to 2:1 (v/v). The peaks of bufuralol and its metabolites were clearly observed on a chromatogram without any significant interference, as illustrated in Fig. 2. The recovery rates were estimated by determining the amount of representative compounds added to human plasma. As listed in Table 6, values for bufuralol and its metabolites were more than 96% with an acceptable S.D. It is to be noted that the immunoadsorption column prepared from antisera against bufuralol–1′-CMO–BSA conjugate will serve for an efficient clean-up of optically active bufuralol and its metabolites in plasma with high selectivity and specificity. There was no evidence of deterioration of the immunoadsorbent

Table 6
Recovery of bufuralol and its metabolites added to human plasma

Compound	Added (ng)	Recovery (%, mean ± S.D.)
(1*R*)-bufuralol	50	97.8 ± 6.7
	100	99.5 ± 7.4
(1*R*)-1′-oxobufuralol	50	100.4 ± 2.2
	100	102.1 ± 6.9
(1*R*, 1′*R*)-1′-hydroxybufuralol	50	98.7 ± 6.9
	100	96.7 ± 7.3
(1*R*, 1′*S*)-1′-hydroxybufuralol	50	96.7 ± 8.2
	100	101.0 ± 4.4
(1*S*)-bufuralol	50	101.9 ± 2.9
	100	101.5 ± 3.1
(1*S*)-1′-oxobufuralol	50	100.3 ± 5.3
	100	101.3 ± 4.1
(1*S*, 1′*R*)-1′-hydroxybufuralol	50	97.5 ± 8.7
	100	97.6 ± 7.2
(1*S*, 1′*S*)-1′-hydroxybufuralol	50	101.6 ± 7.5
	100	100.6 ± 8.3

$n = 5$.

stored at 4°C in PB. In studies of variation on the level of bufuralol in plasma, it has been confirmed that an immobilized antibody column was stable at room temperature for 12 months and after at least 100 times of repeated use. The use of immunoaffinity extraction combined with HPLC will be useful for pharmacokinetic and pharmacodynamic studies of optically active drugs and metabolites having plural chiral centers.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan and the Japan Health Sciences Foundation.

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