Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 3, pp. 389–395, 1994

0731–7085/94 $6.00 + 0.00

Novel immunoaffinity extraction for liquid chromatographic determination of major metabolites of 4-acetoxy-2-(4-methylphenyl)benzothiazole in plasma

SHINICHI MIYAIRI,† HITOSHI SHIMADA,‡ NORIO AWATA,‡ JUNICHI GOTO*† and TOSHIO NAMBARA†

† *Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan*
‡ *Product Research and Development Laboratories, Pharmaceuticals Research Center, Kanebo Ltd, 1-5-90, Tomobuchi-cho, Miyakojima-ku, Osaka 534, Japan*

Abstract: Group extraction of the metabolites of 4-acetoxy-2-(4-methylphenyl)benzothiazole has been achieved through the use of an immunoaffinity adsorbent. The antisera elicited from an immunogen, 4-hydroxy-2-(4-formylphenyl)benzothiazole O-carboxymethyloxime-bovine serum albumin conjugate, were characterized to have a broad affinity spectrum for major metabolites oxidized at the 4-methyl group of the benzene moiety. One millilitre of the immunoaffinity adsorbent prepared by immobilization of antibodies (12.5 mg ml^{-1}) was capable of retaining up to 4 μg of benzothiazoles. The adsorbates were recovered quantitatively by elution with 90% (v/v) methanol without any interfering peaks on the high-performance liquid chromatogram. The peak-height ratio of each metabolite to an internal standard was plotted against the concentration of the former substance; good linearity was observed in the range of 10–500 ng ml^{-1}.

Keywords: *Immobilized antibody; immunoaffinity extraction; immunoadsorption column; high-performance liquid chromatography; 4-acetoxy-2-(4-methylphenyl)benzothiazole.*

Introduction

A simple, sensitive and reliable method for the analysis of a drug and its metabolites in biological matrices is essential for pharmacokinetic and pharmacodynamic studies. Currently, various useful tools and techniques are available for this purpose. However, a tedious clean-up procedure is usually a prerequisite to remove interfering endogenous substances. It is, therefore, necessary to establish an efficient method for the separation and purification of trace target compounds prior to the analysis.

Recently a selective extraction method with immobilized antibody columns through the immunoaffinity has been developed for determination of oestradiol and 11-dehydrothromboxane B_2 in plasma [1–4]. Since the immunoaffinity extraction procedure is applied to the simultaneous determination of biologically active substances and related compounds, plural antibodies corresponding to each analyte should be prepared [5, 6]. The use of an antibody having a broad affinity spectrum for both a drug and its metabolites would be favourable for the xenobiotic metabolism study.

The present paper deals with the immunoaffinity extraction of 4-hydroxy-2-(4-methylphenyl)benzothiazole, an active metabolite of 4-acetate (KB-2683) which is a promising anti-rheumatoid arthritis agent [7], together with its further metabolites. The application of this method as a clean-up step for high-performance liquid chromatographic determination of benzothiazoles in plasma is also described.

Materials and Methods

Materials

4-Hydroxy-2-(4-methylphenyl)benzothiazole (**1**), its acetate (**2**) and related compounds were prepared by New Drug Research Laboratories, Pharmaceuticals Research Center of Kanebo Ltd [7]. 2-(4-Methylphenyl)-benzothiazole-4,5-quinone, 4,5-diacetoxy-2-(4-

*Author to whom correspondence should be addressed.

methylphenyl)benzothiazole and 4,5-dihydroxy-2-(4-methylphenyl)benzothiazole were prepared from **1** as described previously [8].

An activated agarose, Affigel-10, and a dye-binding assay reagent were obtained from Bio-Rad Ltd (Hercules, CA, USA). β-Galactosidase (EC 3.2.1.23) from *Escherichia coli* (900 units mg^{-1} protein), bovine serum albumin (BSA) and γ-globulin were supplied by Sigma Chemical Co. (St Louis, MO, USA). *o*-Nitrophenyl-β-D-galactopyranoside was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Goat anti-rabbit immunoglobulin G (IgG) antiserum and normal rabbit serum were obtained from Daiichi Radioisotope Laboratories, Ltd (Tokyo, Japan). Rivanol (2-ethoxy-6,9-diaminoacridine lactate) was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Apparatus

^{1}H-Nuclear magnetic resonance (NMR) spectra were recorded on a JNM-FX-100 spectrometer (JEOL, Tokyo, Japan) at 100 MHz using tetramethylsilane as an internal standard. Abbreviations used are s = singlet, d = doublet and dd = doublet of doublets. Apparatus used for HPLC was a 6000A solvent delivery system equipped with a 441-UV spectrophotometer (at 313 nm) (Millipore–Waters, Milford, MA, USA). A 150 × 4.6 mm i.d. column packed with 5-μm Inertsil ODS-2 (GL Science Inc., Osaka, Japan) was used at ambient temperature. Melting points (mp) were measured on an electric micro hot-stage apparatus and are uncorrected.

Preparation of benzothiazole-related compounds

4-Acetoxy-2-(4-diacetoxymethylphenyl)-benzothiazole (3). To a solution of **2** (3.36 g) in acetic acid (42 ml), acetic anhydride (42 ml) and concentrated H_2SO_4 (3.16 ml) was added CrO_3 (8.4 g) in portions at <-5°C. The whole was stirred at <-8°C for 3 h and then poured into chilled water (800 ml). The precipitate formed was collected by filtration, washed with water, and dissolved in ethyl acetate (EtOAc). The organic layer was washed with 5% (w/v) Na_2CO_3 and water, successively, dried over anhydrous Na_2SO_4 and evaporated. The crude product was purified by column chromatography on silica gel (100 g). Elution with chloroform ($CHCl_3$)–EtOAc (20:1, v/v) and recrystallization of the eluate from methylene chloride–methanol (MeOH) gave **3** (1.28 g) as colourless needles. mp 135–137°C. Anal. Calcd for $C_{20}H_{17}NO_6S$: C, 60.14; H, 4.29; N, 3.51. Found: C, 60.14; H, 4.29; N, 3.51. NMR($CDCl_3$)δ: 2.16 (6H, s, 4'-$CH(OCOC\underline{H}_3)_2$), 2.48 (3H, s, 4-$OCOCH_3$), 7.18 (1H, dd, J = 1.4 and 7.9 Hz, 5-H), 7.38 (1H, dd, J = 7.9 and 7.9 Hz, 6-H), 7.59 (2H, d, J = 8.6 Hz, 3'-H and 5'-H), 7.69 (1H, s, 4'-$C\underline{H}(OCOCH_3)_2$), 7.75 (1H, dd, J = 1.4 and 7.9 Hz, 7-H), 8.08 (2H, d, J = 8.6 Hz, 2'-H and 6'-H).

4-Hydroxy-2-(4-formylphenyl)benzothiazole (4). A solution of **3** (2.66 g) in 45% (v/v) MeOH (121 ml) and concentrated HCl (5.4 ml) was refluxed under an N_2 atmosphere for 90 min. After dilution with water, the precipitate formed was collected by filtration, washed with water, and recrystallized from EtOAc to give **4** (1.66 g) as pale yellow needles. mp 185–187°C. Anal. Calcd for $C_{14}H_9NO_2S$: C, 65.86; H, 3.55; N, 5.49. Found: C, 66.02; H, 3.64; N, 5.47. NMR(dimethyl sulphoxide-d_6)δ: 6.94 (1H, dd, J = 1.3 and 7.9 Hz, 5-H), 7.30 (1H, dd, J = 7.9 and 7.9 Hz, 6-H), 7.54 (1H, dd, J = 1.3 and 7.9 Hz, 7-H), 8.06 (2H, d, J = 8.4 Hz, 3'-H and 5'-H), 8.28 (2H, d, J = 8.4 Hz, 2'-H and 6'-H), 10.10 (1H, s, 4'-CHO).

4-Hydroxy-2-(4-formylphenyl)benzothiazole O-carboxymethyloxime (5). A mixture of **4** (797 mg), O-carboxymethoxylamine HCl (822 mg) and anhydrous sodium acetate (925 mg) in ethanol (34 ml) was stirred at room temperature for 90 min. After dilution with water, the resulting solution was adjusted pH >10 with 5%(w/v) NaOH and washed with ether. The pH of the aqueous layer was then readjusted to pH <2 with concentrated HCl and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated. The residue was recrystallized from aqueous dioxane to give **5** (740 mg) as colourless needles. mp 214–216°C. Anal. Calcd for $C_{16}H_{12}N_2O_4S$: C, 58.53; H, 3.68; N, 8.53. Found: C, 58.41; H, 3.55; N, 8.43. NMR($CDCl_3$–CD_3OD)δ: 4.75 (2H, s, N-OCH_2COO), 6.95 (1H, dd, J = 1.8 and 8.0 Hz, 5-H), 7.24 (1H, dd, J = 8.0 and 8.0 Hz, 6-H), 7.38 (1H, dd, J = 1.8 and 8.0 Hz, 7-H), 7.68 (2H, d, J = 8.4 Hz, 3'-H and 5'-H), 8.04

(2H, d, J = 8.4 Hz, 2′-H and 6′-H), 8.23 (1H, s, 4′-CH=N).

4-Hydroxy-2-(4-formylphenyl)benzothiazole O-(p-nitrophenyloxycarbonyl)methyloxime (6). A mixture of **5** (200 mg), *p*-nitrophenol (85 mg) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (140 mg) in 10% (v/v) aqueous dioxane (6 ml) was stirred at room temperature for 1 h. The resulting solution was diluted with EtOAc, washed with 5% (w/v) $NaHCO_3$ and water, successively, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified by column chromatography on silica gel (18 g). Elution with $CHCl_3$–EtOAc (5:1, v/v) and recrystallization of the eluate from acetone–hexane gave **6** (28 mg) as colourless needles. mp 146–159°C (decomposition). Anal. Calcd for $C_{22}H_{15}N_3O_6S$: C, 58.79; H, 3.36; N, 9.35. Found: C, 58.89; H, 3.52; N, 9.20. NMR($CDCl_3$–CD_3OD)δ: 5.04 (2H, s, N-OCH_2COO), 6.93 (1H, dd, J = 1.6 and 8.0 Hz, 5-H), 7.25 (1H, dd, J = 8.0 and 8.0 Hz, 6-H), 7.38 (2H, d, J = 9.1 Hz, 2-H and 6-H of *p*-nitrophenyl moiety), 7.40 (1H, dd, J = 1.6 and 8.0 Hz, 7-H), 7.71 (2H, d, J = 8.4 Hz, 3′-H and 5′-H), 8.09 (2H, d, J = 8.4 Hz, 2′-H and 6′-H), 8.27 (2H, d, J = 9.1 Hz, 3-H and 5-H of *p*-nitrophenyl moiety), 8.29 (1H, s, 4′-CH=N).

Preparation of antiserum

A mixture of the activated ester (**6**) (38 mg) and BSA (100 mg) in pyridine–50 mM sodium phosphate buffer (PB) (pH 7.5) (2:1, v/v) (1.5 ml) was stirred at 4°C for 2 days. 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 thin-layer chromatography. The precipitate was then dissolved in 50% (v/v) aqueous pyridine and dialysed against cold running water overnight. Lyophilization of the resulting solution gave the hapten–BSA conjugate as a fluffy powder. The number of hapten molecules incorporated into a BSA molecule was determined to be 11 by UV spectrophotometric analysis at 323 nm.

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

EIA

Preparation of β-galactosidase-labelled antigen. The activated ester (**6**) (33.3 μg) in dioxane (0.1 ml) was added to PB (pH 7.3) (0.2 ml) containing β-galactosidase (500 μg). The mixture was allowed to stand at 4°C for 3 days with occasional shaking. The resulting solution was dialysed against the buffer (3 × 1 l) for 2 days, diluted with the buffer to the concentration of 500 μg ml^{-1} and, after addition of BSA (5 mg), stored at 4°C. For EIA, this stock solution was diluted with PB (pH 7.3) containing 0.9% (w/v) NaCl, 0.1% (w/v) gelatin, and 0.5% (v/v) normal rabbit serum.

Procedure for EIA. The hapten-enzyme conjugate (0.1 μg, 0.1 ml) was mixed with **1** (or each benzothiazole-related compound) (0 and 20–700 pg) in PB (pH 7.3) containing 0.9% (w/v) NaCl and 0.1% (w/v) gelatin (0.1 ml) and diluted antiserum (0.1 ml) sequentially, and the mixture was allowed to stand at 4°C for 4 h. To the mixture was added goat anti-rabbit IgG antiserum (0.1 ml) diluted to 1:30 (v/v) with PB (pH 7.3) containing 0.9% (w/v) NaCl, 0.1% (w/v) gelatin, and 0.3% (w/v) EDTA, and the whole was allowed to stand at 4°C for 16 h. The resulting mixture was diluted with 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 suspended in 1 ml PB (pH 7.3) containing 0.12% (w/v) *o*-nitrophenyl-β-D-galactopyranoside, 0.2% (w/v) $MgCl_2$, and 0.7% (v/v) 2-mercaptoethanol was incubated at 37°C for 1 h. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml) and the absorbance at 420 nm was measured.

Determination of affinity of antibody for benzothiazole analogues

An affinity spectrum of the antibody was assessed by EIA using 13 benzothiazole-related compounds. The relative magnitude of affinity was expressed as the ratio of the amount of each compound which reduced the enzyme activity in the precipitate by half to the

amount of 4-hydroxy-2-(4-methylphenyl)-benzothiazole (**1**) (Table 1).

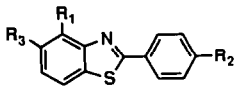
Immobilization of antibody

A mixture of antiserum (35.8 ml) and a 0.4% (w/v) rivanol solution (125 ml) was vortex-mixed gently on ice for 10 min and then centrifuged at 3000 rpm for 10 min at 4°C. To the supernatant (151 ml) was added activated charcoal (3.6 g), and the whole was stirred for 10 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 PB (pH 7.3) (15 ml), the content of protein in the dried residue was determined by the dye-binding assay using γ-globulin as a standard [9]. To the fraction (21 mg ml^{-1}) was added Affigel-10 (22 ml), and the suspension was stirred gently at 4°C for 52 h. The gel was collected on a sintered glass funnel, suspended in PB (pH 7.3) (20 ml), and treated with 1 M ethanolamine in PB (pH 7.3) (2.2 ml) at 4°C for 2 h. The gel was again collected on a sintered glass funnel, washed with PB (pH 7.3) (200 ml), and stored at 4°C in PB (pH 7.3) containing 0.02% (w/v) NaN_3.

Determination of the capacity of the immunoaffinity adsorbent

The immobilized antibody (1 ml) in a glass column (6 mm i.d.) equilibrated with PB (pH 7.3) containing 0.9% (w/v) NaCl was charged a benzothiazole derivative in PB (pH 7.3). The column was washed with PB (pH 7.3) containing 1 M NaCl (10 ml), and water (10 ml), successively. The non-adsorbed analytes present in the aqueous solution which passed through the immunoadsorption column were extracted with EtOAc. The adsorbed analytes were then eluted with 90% (v/v) MeOH (5 ml). The benzothiazole derivatives present in these fractions were determined by HPLC using 20 mM KH_2PO_4-acetonitrile in various ratios as the mobile phase.

Table 1
Affinity spectra of antibody raised against 4-hydroxy-2-(4-formylphenyl)benzothiazole O-carboxymethyloxime (**5**)–BSA conjugate to benzothiazole-related compounds, determined by EIA

Compound 			Relative affinity antiserum	
R_1	R_2	R_3	No. 1	No. 2
OH	CH_3	H	1.00	1.00
OH	CH_2OH	H	1.56	2.66
OH	CHO	H	1.29	1.89
OH	COOH	H	0.88	1.13
OH	H	H	1.68	1.41
$OCOCH_3$	CH_3	H	1.02	1.25
$OCOCH_3$	H	H	1.42	1.12
$OCOC_2H_5$	CH_3	H	0.90	0.84
H	CH_3	H	0.25	0.34
OCH_3	CH_3	H	0.10	0.001
OH	CH_3	OH	0.003	0.005
$OCOCH_3$	CH_3	$OCOCH_3$	0.003	0.003
o-Quinone (=O	CH_3	=O)	0.008	0.006

Evaluation of the immunoaffinity adsorbent using human plasma

Human plasma (1 ml) spiked with **1**, its metabolites and an I.S. (4-hydroxy-2-phenylbenzothiazole) were diluted with PB (pH 7.3) (9 ml) and then loaded on to the immunoadsorption column. The extracted benzothiazole derivatives were determined by HPLC using 100 mM ammonium acetate buffer (pH 3.0)–acetonitrile (1:1, v/v) as the mobile phase.

Results and Discussion

Preparation of hapten

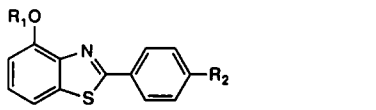
To prepare an immunoaffinity adsorbent used for the simultaneous extraction of a drug and its metabolites, the immobilized antibody should have significant binding abilities to the metabolites as well as to the parent compound. The structure of a hapten is crucial to obtain an antibody with a broad immunoaffinity spectrum. For this purpose, it is necessary to introduce a bridge at the major metabolic site of a drug, because antibody raised against a hapten molecule usually shows significant cross-reactivity with compound(s) homologous around the bridge portion [10].

The model compound, 4-hydroxy-2-(4-methylphenyl)benzothiazole (**1**), is an active metabolite of 4-acetoxy-2-(4-methylphenyl)-benzothiazole (**2**). In the general view of the metabolism of the xenobiotic, **1** undergoes oxidation predominantly at the 4-methyl group of the benzene moiety by hepatic cytochrome P-450. Therefore, the O-carboxymethyloxime of the 4'-formyl metabolite (**4**) appeared to be a pertinent hapten for obtaining an antibody with a broad affinity spectrum for major metabolites because the carboxyl group is available for coupling with the carrier protein.

On selective oxidation of the 4'-methyl group with CrO_3 at $<-5°C$ **2** was converted into the acetal (**3**) [11]. The formyl derivative (**4**) obtained from **3** by hydrolysis was condensed with O-carboxymethoxylamine to yield 4-hydroxy-2-(4-formylphenyl)benzothiazole O-carboxymethyloxime (**5**) which, in turn, was converted into the activated ester with *p*-nitrophenol (**6**) (Fig. 1).

Characterization of antisera

The appropriate antisera were obtained 6 months after initial administration of immunogen prepared from the activated ester (**6**) and BSA. The optimum dilution of both antisera was determined to be 1:10,000 (v/v). The β-galactosidase-labelled antigen was prepared with 20 times excess of **6** to the enzyme where 50% of the immunoreactive enzyme was found in the precipitate formed by the second antibody (goat anti-rabbit IgG antibody). The EIA systems with antisera Nos 1 and 2 provided feasible dose–response curves for **1** in the ranges 30–1000 pg and 20–300 pg per tube, respectively. The affinity spectra of these antisera were determined by ascertaining the ability of **1** and its 12 related benzothiazoles to compete with the enzyme-labelled antigen in binding to the antibody. The relative affinity of antibodies to compounds related to **1** was expressed as the ratio of the amount of **1** which reduced the enzyme activity in the bound fraction by half to the amount of benzothiazoles (Table 1). The antibodies showed significant affinities to the 4-acetate (**2**) and its homologous propionate whereas magnitudes for the corresponding methyl ether derivative were less than 0.10. In contrast, antibodies showed excellent affinities (>0.88) to oxygenated metabolites at the 4'-methyl group, namely hydroxymethyl ($\mathbf{R_2 = CH_2OH}$), formyl ($\mathbf{R_2 = CHO}$) and carboxyl ($\mathbf{R_2 = COOH}$) derivatives as well as the demethylated compound ($\mathbf{R_2 = H}$) (>1.41) as anticipated. These results imply that antibodies have a broad affinity spectrum for both **1** and its metabolites oxidized at the 4-methyl group of the benzene moiety. Furthermore, the demethylated compound, 4-hydroxy-2-phenylbenzothiazole, could be used as an appropriate internal standard (I.S.) for the determination of **1** and metabolites.



1 : R_1 = H, R_2 = CH_3

2 : R_1 = CH_3CO, R_2 = CH_3

3 : R_1 = CH_3CO, R_2 = $CH(OCOCH_3)_2$

4 : R_1 = H, R_2 = CHO

5 : R_1 = H, R_2 = $CH{=}NOCH_2COOH$

6 : R_1 = H, R_2 = $CH{=}NOCH_2COO$-C$_6$H$_4$-NO_2

Figure 1
Structures of benzothiazole-related compounds including the hapten.

Preparation and characterization of immunoaffinity adsorbent

Immunoglobulin G was obtained as the γ-globulin fraction from antisera by the method of Horejsi and Smetana with minor modifications [12]. The immunoglobulin was coupled to agarose through the spacer having an N-succinimidyl ester. The amount of IgG immobilized on the agarose matrix was estimated to be 12.5 mg ml^{-1} of gel.

Elution of 4-hydroxy-2-(4-methylphenyl)-benzothiazole (**1**) from the immunoadsorption column was assessed with various eluents where the recovery from the adsorbent was determined by HPLC. As listed in Table 2, **1** was eluted from the adsorbent (1 ml) almost quantitatively with 5 ml of either 95% (v/v) or 90% (v/v) aqueous MeOH; in contrast, the use of 95% (v/v) acetone and 90% (v/v) acetonitrile resulted in low recoveries of 77% and 45%, respectively. Accordingly, 90% (v/v) MeOH was chosen as the eluent for further experiments. This result is compatible with that for 11-dehydrothromboxane B_2 [3].

The capacity of immunoaffinity adsorbent was then evaluated by determination of benzothiazole derivatives in the adsorbate and non-adsorbate fractions. As depicted in Fig. 2, the recovered amount of **1** in the adsorbate frac-

Table 2
Recovery of 4-hydroxy-2-(4-methylphenyl)benzothiazole in the adsorbate fraction from the immunoadsorption column with various eluents

Eluent*		Recovery (%)
Methanol–H_2O	(90:10, v/v)	98
	(95:5, v/v)	96
Acetone–H_2O	(90:10, v/v)	94
	(95:5, v/v)	77
Acetonitrile–H_2O	(90:10, v/v)	45

*5 ml.

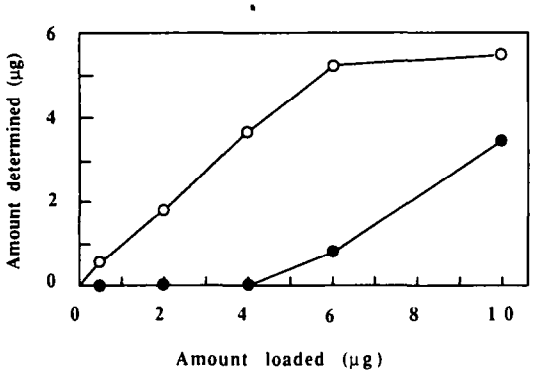


Figure 2
Amounts of 4-hydroxy-2-(4-methylphenyl)benzothiazole in the adsorbate (○) and non-adsorbate fraction (●).

tion increased with an increasing loaded amount up to 4 μg. Obviously, **1** was detected in the non-adsorbate fraction when more than 4 μg of **1** was loaded. The capacity of the adsorbent for other benzothiazole metabolites was also determined. It is evident from the data listed in Table 3 that **1** ($\mathbf{R_2}$ = $\mathbf{CH_3}$), hydroxymethyl ($\mathbf{R_2}$ = $\mathbf{CH_2OH}$), formyl ($\mathbf{R_2}$ = **CHO**), and carboxyl ($\mathbf{R_2}$ = **COOH**) derivatives were recovered at a rate of more than 87%, the loaded amount being below 4 μg. The glucuronide and sulphate were not captured on the immobilized antibody. These results indicated that 1 ml of this immobilized antibody was capable of retaining 4-hydroxy-2-(4-methylphenyl)benzothiazole and its oxygenated metabolites up to a total amount of 4 μg.

Validity of immunoaffinity extraction for group separation

The validity of immunoaffinity extraction for clean-up of these benzothiazoles in biological fluids was evaluated using human plasma spiked with **1** and its metabolites (each 50 ng) together with 4-hydroxy-2-phenylbenzothiazole (250 ng) as I.S. The adsorbate eluted from the immunoadsorption column was subjected to HPLC. The peaks corresponding to the drug and its metabolites were observed on the chromatogram without any significant interference as illustrated in Fig. 3.

The reliability of a clean-up method by immunoaffinity extraction for the drug and its metabolites was assessed. The peak-height ratio of each benzothiazole to the I.S. was plotted against the concentration of the drug; good linearity was observed in the range of 10–500 ng ml^{-1} as depicted in Fig. 4. Furthermore, as shown in Table 4, the recovery of the benzothiazole metabolites was more than 90% with an acceptable standard deviation.

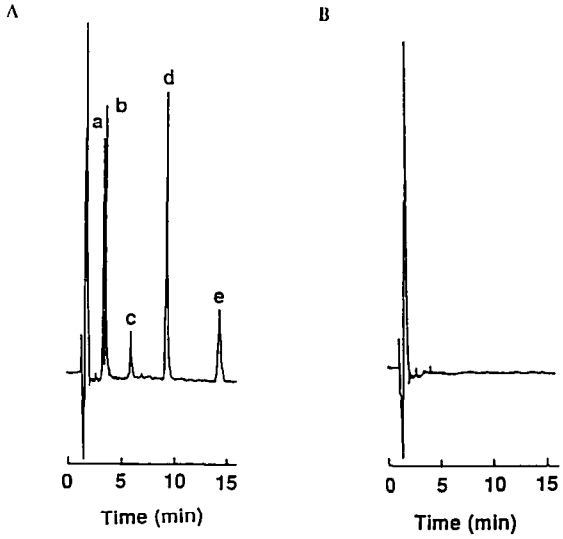
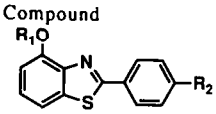


Figure 3
HPLC chromatograms of eluates from the immunoadsorption column loaded with human plasma with (A) or without (B) 4-hydroxy-2-(4-**R**-phenyl)benzothiazoles. Mobile phase: 100 mM ammonium acetate buffer (pH 3.0)–acetonitrile (1:1, v/v). Peak a: **R** = hydroxymethyl; b: **R** = carboxyl; c: **R** = formyl; d: **R** = hydrogen (I.S.); and e: **R** = methyl derivative.

Table 3
Extraction efficiency of benzothiazole-related compounds with the immunoadsorption column determined by HPLC

Compound		% Recovery Amount loaded (μg)				
		1	2	4	6	10
$\mathbf{R_1}$	$\mathbf{R_2}$					
H	$\mathbf{CH_3}$	97.6 ± 5.1*	89.5 ± 6.6	87.4 ± 8.9	85.9 ± 10.6	54.3 ± 0.9
H	$\mathbf{CH_2OH}$	95.3 ± 3.5	97.3 ± 4.9	96.0 ± 4.6	96.2 ± 4.0	65.3 ± 4.3
H	**CHO**	91.1 ± 18.0	86.5 ± 4.4	100.2 ± 4.2	88.4 ± 4.0	61.8 ± 4.6
H	**COOH**	107.8 ± 5.2	100.3 ± 3.7	89.9 ± 10.5	63.4 ± 7.5	38.0 ± 7.0
Glucuronide	$\mathbf{CH_3}$	<0.2	<0.1	—	—	<0.1
Sulphate	$\mathbf{CH_3}$	18.6 ± 4.9	7.4 ± 1.9	—	—	1.2 ± 0.6

*n = 3.

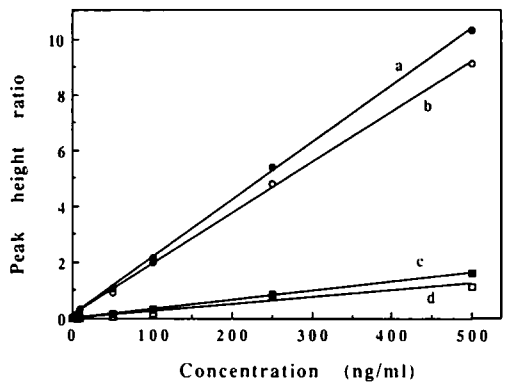


Figure 4
Relationship between the loaded and recovered amounts of 4-hydroxy-2-(4-**R**-phenyl)benzothiazoles for the immunoadsorption column. Mobile phase: 100 mM ammonium acetate buffer (pH 3.0)–acetonitrile (1:1, v/v). Line a: **R** = carboxyl; b: **R** = hydroxymethyl; c: **R** = methyl; d: **R** = formyl derivative.

Table 4
Recovery* of the benzothiazole metabolites added to human plasma from the immunoadsorption column

Compound	Added (ng ml^{-1})	Found (mean ± SD) (ng ml^{-1})
R = CH_3	10	10.7 ± 3.0
	100	101 ± 3.6
	500	499 ± 7.1
R = CH_2OH	10	10.3 ± 2.5
	100	102 ± 2.1
	500	498 ± 20
C = **COOH**	10	10.9 ± 1.9
	100	103 ± 1.3
	500	498 ± 24

*n = 4.

It is to be noted that the immunoadsorption column prepared from antiserum raised against 4-hydroxy-2-(4-formylphenyl)benzothiazole O-carboxymethyloxime (**5**)–BSA conjugate will serve for efficient clean-up of the drug and its metabolites in biological fluids. In addition, the immunoaffinity extraction method, combined with HPLC, will be useful for the pharmacokinetic and pharmacodynamic study of 4-acetoxy-2-(4-methylphenyl)benzothiazole.

Conclusions

A group extraction method through the use of an immunoaffinity adsorbent has been developed for the determination of 4-hydroxy-2-(4-methylphenyl)benzothiazole and its major metabolites in plasma by HPLC. The antibody elicited from an immunogen, 4-hydroxy-2-(4-formylphenyl)benzothiazole O-carboxymethyloxime–BSA conjugate, exhibited a specificity for metabolites oxidized at the 4-methyl group of the benzene moiety as well as the parent compound.

The use of an immunoadsorption column prepared from an antibody having a broad affinity spectrum for both a drug and its metabolites would be favourable for their purification and enrichment in pharmacokinetic and pharmacodynamic studies. Furthermore, it is possible that the binding property of an unknown metabolite to the immunoaffinity adsorbent gives a clue to its structure.

Acknowledgements — The authors are indebted to the staff of the central analytical laboratory of the Pharmaceutical Institute of Tohoku University for the elemental analyses and spectral measurements. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

[1] R.G. Glencross, S.A. Abeywardene, S.J. Corney and H.S. Morris, *J. Chromatogr.* **223**, 193–197 (1981).
[2] S.J. Gaskell and B.G. Brownsey, *Clin. Chem.* **29**, 677–680 (1983).
[3] Y. Hayashi, F. Shono, S. Yamamoto, W. Takasaki, A. Nakagawa, K. Watanabe, K.Yamashita and H. Miyazaki, *Anal. Biochem.* **187**, 151–159 (1990).
[4] H.H.D. Meyer, H. Sauerwein and B.M. Mutayoba, *J. Steroid Biochem.* **35**, 263–269 (1990).
[5] G. Mackert, M. Reinke, H. Schweer and H.W. Seyberth, *J. Chromatogr.* **494**, 13–22 (1989).
[6] C. Chiabrando, V. Pinciroli, A. Campoleoni, A. Benigni, A. Piccinelli and R. Fanelli, *J. Chromatogr.* **495**, 1–11 (1989).
[7] N. Hori, G. Tsukamoto, A. Imamura, M. Ohashi, T. Saito and K. Yoshino, *Chem. Pharm. Bull.* **40**, 2387–2390 (1992).
[8] S. Miyairi, H. Shimada, H. Morita, N. Awata, J. Goto and T. Nambara, *J. Pharm. Biomed. Anal.* **11**, 469–476 (1993).
[9] M. Bradford, *Anal. Biochem.* **72**, 248–254 (1976).
[10] A.M.G. Bosch, F.C. den Hollander and G.F. Woods, *Steroids* **23**, 699–711 (1974).
[11] D.H.R. Barton, P.D. Magnus and T. Hase, *J. Chem. Soc.* **C**, 2215–2225 (1971).
[12] J. Horejsi and R. Smetana, *Acta Medica Scand.* **155**, 65–70 (1956).

[Received for review 14 June 1993; revised manuscript received 20 July 1993]