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Enzyme immunoassay for 4-hydroxy-2-(4-methylphenyl)benzothiazole

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Abstract: A specific enzyme immunoassay (EIA) has been developed for 4-hydroxy-2-(4-methylphenyl)benzothiazole (KB-2714) (**1**), an active metabolite of 4-acetoxy-2-(4-methylphenyl)benzothiazole (KB-2683) (**2**) which is a promising anti-rheumatic agent. The EIA was based upon antiserum elicited against 5-(2-carboxyphenylazo)-4-hydroxy-2-(4-methylphenyl)benzothiazole (**3**)–bovine serum albumin conjugate and β-galactosidase-labelled 5-amino-4-hydroxy-2-(4-methylphenyl)benzothiazole (**5**). The sensitivity of the EIA was significantly improved by the utilization of a bridge heterologous combination system.

An appropriate dose–response curve of EIA for 4-hydroxy-2-(4-methylphenyl)benzothiazole was obtained in the range of 10 pg tube^{-1}–20 ng tube^{-1}. The specificity of EIA proved to be satisfactory in terms of cross-reactivities to 14 benzothiazole-related compounds including glucuronic acid and sulphuric acid conjugates. The proposed method was evaluated to be useful for the determination of **1** in urine and plasma with acceptable recovery and inter- and intra-assay precision.

Keywords: *4-Acetoxy-2-(4-methylphenyl)benzothiazole; 4-hydroxy-2-(4-methylphenyl)benzothiazole; anti-rheumatoid arthritis agent; enzyme immunoassay; bridge heterologous combination system.*

Introduction

Since its desirable action in adjuvant arthritis in rats was observed, 4-acetoxy-2-(4-methylphenyl)benzothiazole (KB-2683) has been expected to be effective in the treatment of rheumatoid arthritis in humans [1]. After oral administration, KB-2683 is readily hydrolysed into an active metabolite, 4-hydroxy-2-(4-methylphenyl)benzothiazole (KB-2714), during absorption from the small intestine. The metabolite undergoes further oxidative biotransformation at the *para*-methyl group and/or conjugation with glucuronic acid or sulphuric acid at the phenolic hydroxyl group as depicted in Fig. 1.

It is generally accepted that since immunoassay is favourable for the sensitive and specific determination of trace components in biological fluids with a complex matrix without tedious pretreatment, the method is useful for pharmacokinetics studies.

The present paper deals with the development of enzyme immunoassay (EIA) for 4-hydroxy-2-(4-methylphenyl)benzothiazole with a bridge heterologous combination of antiserum and enzyme-labelled antigen.

Materials and Methods

Materials

4-Hydroxy-2-(4-methylphenyl)benzothiazole (**1**), 4-acetoxy-2-(4-methylphenyl)benzothiazole (**2**) and the related compounds were prepared by New Drug Research Laboratories, Pharmaceuticals Research Center of Kanebo Ltd (Osaka, Japan) [1]. *N,O*-bis(diethylhydrogensilyl)trifluoroacetamide (DEHS-BSTFA) was prepared in these laboratories according to the method of Miyazaki *et al.* [2].

β-Galactosidase (E.C. 3.2.1.23) from *Escherichia coli* (900 units per mg protein) and bovine serum albumin (BSA) 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 IgG antiserum and normal rabbit serum were obtained from Daiichi Radioisotope Labs, Ltd (Tokyo, Japan).

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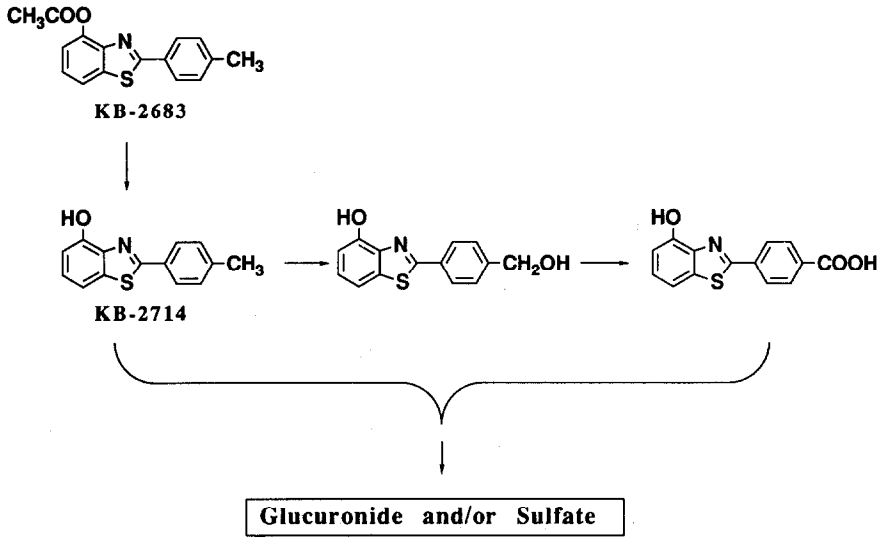


Figure 1
Presumed metabolic pathway of 4-acetoxy-2-(4-methylphenyl)benzothiazole.

Apparatus

^{1}H-Nuclear magnetic resonance (NMR) spectra were recorded on a Jeol FX-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Abbreviations used are s = singlet, d = doublet and m = multiplet. Mass spectra (MS) were obtained on a Jeol JMS-DS 303 spectrometer. Gas chromatography–mass spectrometry (GC–MS) was carried out on a MM 12030 quadrupole mass spectrometer (VG Analytical, Manchester, UK) interfaced to an HP 5790A gas chromatograph (Hewlett–Packard, Avondale, PA, USA) with a Van den Berg solventless injector. A cross-linked 5% phenylmethylsilicone fused silica capillary column (20 m × 0.3 mm i.d.) (J&W Scientific, Folsom, CA, USA) was inserted into the ion source through the direct inlet. Melting points (m.p.) were measured on an electric micro hot stage apparatus and are uncorrected. An Extrelut-1 cartridge was obtained from Merck Japan (Tokyo, Japan).

Preparation of benzothiazole-related compounds

5-(4-Carboxyphenylazo)-4-hydroxy-2-(4-methylphenyl)benzothiazole (3). To a solution of 4-aminobenzoic acid (2.0 g) in a mixture of acetic acid (20 ml), concentrated HCl (3.8 ml) and water (12.5 ml) was added aqueous $NaNO_2$ (1.1 g per 2.2 ml) dropwise at <5°C. The mixture was stirred for 3 h; to the mixture was then added **1** (3.5 g) in 40% (v/v) pyridine in dimethylformamide (72 ml). After stirring for 2.5 h, the resulting solution was poured into chilled water (1 l) and allowed to stand at 4°C overnight. The precipitate was collected by filtration, washed with water and dried. The crude product was purified by column chromatography on silica gel. Elution with chloroform–methanol (5:1, v/v) and recrystallization of the eluate from aqueous dioxane-acetic acid gave **3** (5.61 g) as orange leaflets. m.p. >300°C. Anal. Calcd for $C_{21}H_{15}N_3O_3S$: C, 64.77; H, 3.88; N, 10.79. Found: C, 64.80; H, 3.89; N, 10.70. NMR(DMSO-d_6)δ: 2.42 (3H, s, 4′-CH_3), 7.22(1H, d, J = 8.5 Hz, 6-H), 7.40 (2H, d, J = 8.5 Hz, 3′-H and 5′-H), 7.94–8.22 (7H, m, 2-H, 3-H, 5-H and 6-H of phenylazo moiety, 7-H, 2′-H and 6′-H).

4-Hydroxy-2-(4-methylphenyl)-5-(4-succinimidyloxycarbonylphenylazo)benzothiazole (4). A mixture of **3** (100 mg), *N*-hydroxysuccinimide (36 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (84 mg) in dioxane (5.5 ml) was stirred at room temperature for 3.5 h. The resulting solution was diluted with ethyl acetate, washed with water, dried over anhydrous Na_2SO_4, and then evaporated. The residue was subjected to purification by column chromatography on silica gel with benzene–ethyl acetate (5:1, v/v) as an eluent. Recrystallization of the eluate from chloroform–ethyl acetate gave **4** (45 mg) as orange leaflets. m.p. 237°C (decomposition). Anal. Calcd for $C_{25}H_{18}N_4O_5S$: C,

61.72; H, 3.73; N, 11.52. Found: C, 61.45; H, 3.71; N, 11.33. NMR($CDCl_3$-CD_3OD)δ: 2.44 (3H, s, 4'-CH_3), 2.97 (4H, s, succinimidyl moiety), 7.15 (1H, d, J = 8.5 Hz, 6-H), 7.30 (2H, d, J = 8.5 Hz, 3'-H and 5'-H), 7.98 (1H, d, J = 8.0 Hz, 7-H), 8.04 (2H, d, J = 8.5 Hz, 2-H and 6-H of phenylazo moiety), 8.13 (2H, d, J = 8.5 Hz, 2'-H and 6'-H), 8.26 (2H, d, J = 8.5 Hz, 3-H and 5-H of phenylazo moiety).

5-Amino-4-hydroxy-2-(4-methylphenyl)-benzothiazole (5). To a solution of **3** (5.6 g) in a mixture of dimethylformamide (10 ml) and 5% (w/v) NaOH (500 ml) was added $Na_2S_2O_6$ (11 g). The mixture was stirred at 50°C for 2 h and then allowed to stand at 4°C overnight. The precipitate was collected by filtration and dissolved in anhydrous acetone. To the solution was added acetone saturated with dry HCl gas, and the precipitate was recrystallized from ethanol–ether to give **5**·HCl (3.4 g) as colourless needles. m.p. 185°C (decomposition). Anal. Calcd for $C_{14}H_{12}N_2OS$·HCl·1/4H_2O: C, 56.56; H, 4.58; N, 9.43. Found: C, 56.86; H, 4.46; N, 9.25. NMR(DMSO-d_6)δ: 2.41 (3H, s, 4'-CH_3), 7.00 (1H, d, J = 8.5 Hz, 6-H), 7.35 (1H, d = 8.5 Hz, 7-H), 7.39 (2H, d, J = 8.5 Hz, 3'-H and 5'-H), 7.98 (2H, d, J = 8.5 Hz, 2'-H and 6'-H).

5-(3-Carboxypropionyl)amino-4-hydroxy-2-(4-methylphenyl)benzothiazole (6). A solution of **5**·HCl (2 g) and succinic anhydride (2.05 g) in pyridine (22 ml) was stirred at room temperature for 80 min. After addition of water (3 ml), the mixture was allowed to stand for 3 h and then poured into chilled water (100 ml). The precipitate was collected by filtration and dissolved in chloroform. The soluble portion was subjected to crystallization from ethanol–hexane to give **6** (1.92 g) as colourless needles. m.p. 205°C (decomposition). Anal. Calcd for $C_{18}H_{16}N_2O_4S$·1/4H_2O: C, 59.90; H, 4.61; N, 7.76. Found: C, 59.58; H, 4.46; N, 7.65. NMR(DMSO-d_6)δ: 2.40 (3H, s, 4'-CH_3), 2.59 (4H, s, $COCH_2CH_2CO$), 6.86 (1H, d, J = 8.5 Hz, 6-H), 7.14 (1H, d, J = 8.5 Hz, 7-H), 7.36 (2H, d, J = 8.5 Hz, 3'-H and 5'-H), 7.95 (2H, d, J = 8.5 Hz, 2'-H and 6'-H), 9.96 (1H, s, 5-NHCO).

2-(4-Methylphenyl)benzothiazole-4,5-quinone (7). To a solution of $NaIO_4$ (2 g) in 0.1 M HCl (100 ml) was added **5**·HCl (200 mg) in acetic acid (30 ml), and the mixture was stirred at room temperature for 1 h. The reaction mixture was extracted with chloroform (100 ml) twice and the organic layer was washed with 10% (w/v) KI and 5% (w/v) $NaHSO_3$, successively, and dried over anhydrous Na_2SO_4. The crude residue obtained was subjected to purification by column chromatography on silica gel. Elution with hexane–chloroform–ethyl acetate–acetic acid (90:30:30:1, v/v/v/v) and recrystallization of the eluate from ethyl acetate containing 0.1% (v/v) acetic acid gave **7** (15 mg) as brownish red leaflets. m.p. 205.5–206°C (decomposition). Anal. Calcd for $C_{14}H_9NO_2S$: C, 65.86; H, 3.55; N, 5.49. Found: C, 65.96; H, 3.55; N, 5.46. MS(m/z): 255 (M^+). NMR($CDCl_3$)δ: 2.43 (3H, s, 4'-CH_3), 6.88 (2H, s, 6-H and 7-H), 7.27 (2H, d, J = 8.5 Hz, 3'-H and 5'-H), 7.96 (2H, d, J = 8.5 Hz, 2'-H and 6'-H).

4,5-Diacetoxy-2-(4-methylphenyl)benzothiazole (8). The crude orthoquinone (**7**) obtained in the manner described above was subjected to reduction with $NaBH_4$ (100 mg) in methanol (10 ml). After addition of acetic acid (2 ml), the mixture was diluted with ethyl acetate, washed with water, dried over anhydrous Na_2SO_4 and then evaporated. The residue was purified by column chromatography on silica gel with chloroform–ethyl acetate–acetic acid (160:40:1, v/v/v) as the eluent, followed by acetylation with acetic anhydride in pyridine. The crude product obtained was purified by column chromatography on silica gel. Elution with hexane–chloroform–ethyl acetate (4:1:1, v/v/v) and recrystallization of the eluate from ethyl acetate–hexane gave **8** (80 mg) as light yellow needles. m.p. 138–138.5°C (decomposition). Anal. Calcd for $C_{18}H_{15}NO_4S$: C, 63.33; H, 4.43; N, 4.10. Found: C, 63.36; H, 4.45; N, 4.22. MS (m/z): 341 (M^+). NMR($CDCl_3$)δ: 2.41 (3H, s, 4'-CH_3), 2.39 and 2.48 (each 3H, s, 4-$OCOCH_3$ and 5-$OCOCH_3$), 7.18 (2H, s, 6-H and 7-H), 7.27 (2H, d, J = 8.5 Hz, 3'-H and 5'-H), 7.81 (2H, d, J = 8.5 Hz, 2'-H and 6'-H).

4,5-Dihydroxy-2-(4-methylphenyl)benzothiazole (9). A solution of **8** (30 mg) in methanol (8 ml) and 2.5 M H_2SO_4 (3 ml) was stirred at room temperature for 66 h. The reaction mixture was diluted with ethyl acetate, washed with water, 5% (w/v) $NaHCO_3$ and water,

successively, and dried over anhydrous Na_2SO_4. The residue was recrystallized from acetone–hexane to give **9** (20 mg) as light brown granules. m.p. 206–208°C (decomposition). Anal. Calcd for $C_{14}H_{11}NO_2S$: C, 65.35; H, 4.31; N, 5.44. Found: C, 65.46; H, 4.48; N, 5.44. MS(*m/z*): 257 (M^+). NMR($CDCl_3$)δ: 2.43 (3H, s, 4′-CH_3), 6.71 and 6.77 (each 1H, d, J = 8.5 Hz, 4-H and 5-H), 7.29 (2H, d, J = 8.5 Hz, 3′-H and 5′-H), 7.81 (2H, d, J = 8.5 Hz, 2′-H and 6′-H).

GC–MS analysis of **9**. A mixture of **9** and DEHS-BSTFA (50 μl) in dry pyridine (50 μl) was allowed to stand at room temperature for 1 h and then concentrated under a stream of nitrogen. The residue in hexane (100 μl) was subjected to capillary GC–MS analysis. The carrier gas was helium at a linear velocity of 70 cm s^{-1}. The injection port, column oven and ion source were kept at 220, 200 and 260°C, respectively. The ionization energy was 40 eV and the emission current was 100 μA.

Preparation of immunogen and antisera

Preparation of immunogen. A mixture of **4** (35 mg) and BSA (100 mg) in 2.2 ml of 45% (v/v) 50 mM sodium phosphate buffer (pH 7.5) in pyridine was stirred at 4°C for 3 days. The BSA fraction was precipitated by addition of acetone followed by centrifugation at 3000 rpm for 10 min. This procedure was repeated until any unconjugated hapten was not detectable by TLC. The precipitate was redissolved in 50% (v/v) aqueous pyridine and dialysed overnight against cold running water (3 × 1 l). 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 390 nm.

Preparation of antiserum to 4-hydroxy-2-(4-methylphenyl)benzothiazole. The hapten–BSA conjugate (1 mg) 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 subcutaneously at multiple sites along back. This procedure was repeated once every fortnight. Blood withdrawn was centrifuged at 3000 rpm for 10 min. The antiserum collected was stored at 4°C in the presence of 0.1% (w/v) sodium azide.

Preparation of β-galactosidase-labelled antigen. A 10 μl volume of 0.015% (v/v) glutaraldehyde solution was added to a solution of **5**·HCl (2.2 μg) and β-galactosidase (500 μg) in 50 mM sodium phosphate buffer (pH 6.8) (0.2 ml). The mixture was vortex-mixed, then allowed to stand at 4°C with occasional shaking for 23 h. The resulting solution was dialysed against 50 mM sodium phosphate buffer (pH 7.3) (3 × 1 l) for 2 days at 4°C, diluted with the same buffer to 1 ml and, after addition of 50 mg of BSA, stored at 4°C. For immunoassay, this stock solution was diluted with buffer A, 50 mM sodium phosphate buffer (pH 7.3) containing 0.9% (w/v) NaCl and 0.1% (w/v) gelatin, in the presence of 0.5% (v/v) normal rabbit serum.

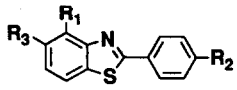
Procedure for EIA

The hapten–enzyme conjugate (0.2 μg, 0.1 ml) was mixed with **1** (0 and 10 pg–20 ng) (or related compounds for the determination of the specificity) in buffer A (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 buffer A containing 0.3% (w/v) ethylenediaminetetra-acetic acid, and allowed to stand at 4°C for 16 h. After dilution with 50 mM sodium phosphate buffer (pH 7.3) (1.5 ml) and centrifugation at 3000 rpm for 10 min, the immune precipitate was collected by aspirating off the supernatant and washed with the phosphate buffer (1.5 ml) by repeating the procedure. The precipitate was then suspended into 1 ml of the phosphate buffer (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, and then incubated at 37°C for 1 h. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml), and the absorbance was measured at 420 nm.

Determination of specificity of EIA. The specificity of the assay system was assessed by cross-reactivity to 14 benzothiazole related compounds. The cross-reactivity was expressed as the percentage of the amount of 4-hydroxy-2-(4-methylphenyl)benzothiazole (**1**) which reduced the enzyme activity in the immune precipitate by half to the amount of each the compounds listed in Table 1.

Treatment of biological specimens for EIA. The plasma or urine specimen (0.2 ml) spiked

Table 1
Cross-reactivities of the benzothiazole related compounds in EIA

Compound			% Cross-reactivity
			
R_1	R_2	R_3	
OH	CH_3	H	100
OH	CH_2OH	H	1.0
OH	CHO	H	1.6
OH	COOH	H	<0.1
O-Glucuronide	CH_3	H	0.8
O-Sulphate	CH_3	H	5.2
OCH_3	CH_3	H	6.5
OH	CH_3	OH	31
4,5-Quinone (Compound 7)			
(=O	CH_3	=O)	19
$OCOCH_3$	CH_3	$OCOCH_3$	12
$OCOCH_3$	CH_3	H	78
$OCOC_2H_5$	CH_3	H	42
H	CH_3	H	1.4
OH	H	H	1.9
$OCOCH_3$	H	H	1.8

with 4-hydroxy-2-(4-methylphenyl)benzothiazole (**1**) was diluted with 50 mM sodium phosphate buffer (pH 7.3) (0.6 ml) and then loaded on to an Extrelut-1 cartridge. The eluate with hexane (10 ml) was concentrated under a stream of nitrogen, redissolved into methanol (0.05 ml) and buffer A (0.75 ml); the mixture was subjected to EIA.

Results and Discussion

Preparation of the hapten (Fig. 2)

4-Acetoxy-2-(4-methylphenyl)benzothiazole (**2**) is known to be hydrolysed completely in plasma as well as in the small intestine, yielding 4-hydroxy-2-(4-methylphenyl)benzothiazole (**1**) as an active metabolite. It is, therefore, important to develop a reliable method for the determination of the deacetylated metabolite (**1**) rather than the parent drug (**2**) itself to study the pharmacokinetics. EIA has been recognized as a sensitive and specific method that is widely used for the trace analysis of drugs in biological fluids. In the immunoassay the antibody should discriminate between the drug and the metabolites to establish a specific method.

It is sufficiently substantiated that the specificity of the antibody raised against a small molecule is remarkably influenced by the position on the hapten molecule used for conjugation to the carrier protein [3]. On the other hand, oxidative biotransformation of the *para*-methyl group on the benzene moiety of **1** by the hepatic cytochrome P-450 would be predominant according to general xenobiotic metabolism. Therefore, a compound having a 4-carboxyphenylazo group at the 5-position on the benzothiazole ring was designed as a pertinent hapten. After subjection to the diazo coupling reaction with 4-carboxyphenyldiazonium halide obtained from 4-aminobenzoic

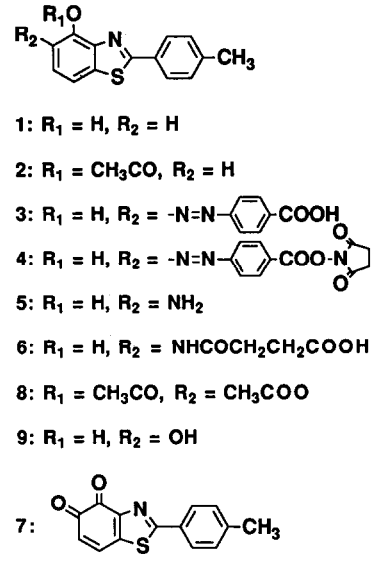


Figure 2
Structures of 4-hydroxy-2-(4-methylphenyl)benzothiazole and related compounds.

acid, 4-hydroxybenzothiazole (**1**) was transformed solely into the 5-(4-carboxyphenyl)azo derivative (**3**) in spite of the availability of two sites (5- and 7-positions).

Characterization of the hapten (3)

To determine the position of the azo group introduced on the benzothiazole ring, the hapten (**3**) was converted into the hydroxylated compound (**9**) through the amino derivative (**5**). The azo group was reduced by hydrogenation with sodium dithionate [4] to **5**, which in turn was subjected to sodium metaperiodate oxidation followed by sodium borohydride reduction to yield **9**.

It has been well established that on treatment with a novel silylating agent, *N,O*-bis(diethylhydrogensilyl)trifluoroacetamide (DEHS-BSTFA), an isolated hydroxyl group is readily led to the diethylhydrogensilyl (DEHS) ether, whereas a vicinal glycol is transformed into the cyclic diethylsilylene (DES) derivative, providing the characteristic ions on MS analysis [2, 5]. Accordingly, **9** was subjected to derivatization with DEHS-BSTFA followed by GC–MS analysis. The formation of a fragment ion at *m/z* 340, one mass unit smaller than the unique diethyl cyclic siliconide depicted in Fig. 3, indicated the presence of a vicinal glycol moiety in **9**. This result is compatible with the mass spectrum of toluene which gives a base peak at *m/z* 91 corresponding to $[M—H]^+$ [6]. The structure of the hapten (**3**) was thus unambiguously characterized.

Preparation of the antiserum

The activated ester (**4**) prepared from **3** by condensation with *N*-hydroxysuccinimide in the presence of water-soluble carbodiimide was subjected to conjugation with BSA [7]. The immunogen thus obtained was administered to rabbits with complete Freund's adjuvant subcutaneously. The appropriate antiserum was obtained from rabbits at 6 months after the initial administration.

Preparation of the enzyme-labelled antigen

It is well known that azo compounds bound to protein exhibit strong antigenicity [8]. However, too strong antigenicity usually reduces the sensitivity in hapten immunoassays owing to the disadvantageous affinity of the antibody to the bridge portion. In fact, the sensitive EIA system was unsuccessful when the same azo hapten (**3**) was used for preparing both the labelled antigen and the immunogen, that is a homologous combination system.

On the other hand, the utility of a bridge heterologous combination of the enzyme-labelled antigen and the antibody has been demonstrated for the development of sensitive EIA for steroids [9] and carcinogenic γ-carboline [10]. Therefore, a bridge heterologous assay system, using haptenic derivatives with different bridge structures on the benzothiazole ring for preparing both the enzyme-labelled antigen and the immunogen, was chosen to develop a sensitive EIA.

An initial attempt was made with 5-(3-caroboxypropionyl)amino-4-hydroxy-2-(4-methylphenyl)benzothiazole (**6**). The amino hapten (**5**) described above was treated with succinic anhydride to form **6**, which in turn was subjected to reactions with *N*-hydroxysuccinimide in the presence of water-soluble carbodiimide or with *N,N'*-disuccinimidyl carbonate [11]. However, in both cases **6** gave solely the unexpected product, which was identified as a compound cyclized intramolecularly with the hydroxyl group at the 4-position on the basis of MS and ^{1}H-NMR data. In the MS analysis, an intensive base peak at *m/z* 340 was assignable to the molecular ion of the cyclized compound. On the NMR spectrum, the proton signal of the 6-position was shifted from 6.86 to 7.02 ppm (doublet, J = 8.5 Hz) by cyclization while the proton at 7-position showed almost the same value as that of **6**. The signal of the succinamide moiety in the product was shifted to the lower field by 0.39 ppm (at 2.98 ppm, singlet) by the ring current effect. These

Figure 3
Derivatization of 4,5-dihydroxy-2-(4-methylphenyl)benzothiazole with DEHS-BSTFA.

findings also lent support in assigning the structure of the azo hapten (**3**).

Accordingly, an enzyme-labelled antigen was prepared by coupling of the amino derivative (**5**) with β-galactosidase using the glutaraldehyde method [12]. Compound **5**, β-galactosidase and glutaraldehyde were used in a molar ratio of 2:1:4 for the preparation of the hapten–enzyme conjugate. Sufficient enzyme activity was obtained in the immune precipitate formed by 1:2,500 (v/v) diluted anti-serum.

EIA for 4-hydroxy-2-(4-methylphenyl)benzothiazole

Separation of the bound and unbound antigens in EIA was performed by the second antibody method using goat anti-rabbit IgG antiserum. This EIA system was evaluated to be sufficiently sensitive, according to the dose–response curve for 4-hydroxy-2-(4-methylphenyl)benzothiazole (**1**) in the range of 10 pg–20 ng per tube (Fig. 4). The dynamic range of this EIA system was judged to be useful for the pharmacokinetic study. The amount of **1** to reduce the enzyme activity in the immune precipitate by half was determined to be 500 pg per tube.

Specificity of the EIA system

The specificity of the EIA system was assessed by ascertaining the ability of various related compounds to compete with the enzyme-labelled antigen for binding to the antibody, and was expressed as per cent cross-reactivity in Table 1. This antibody was characteristic of discriminating the metabolites oxygenated at the *para*-methyl group (R_2 = CH_2OH, CHO and COOH) from the intact compound (**1**) as well as the demethylated compound (R_2 = H) with their cross-reactivities of a few per cent or less. As for the conjugates at 4-position, the cross-reactivity of glucuronide was only 0.8% while those of the sulphate and methyl ether were significant, 5.2 and 6.5%, respectively. 4-Acetoxy-2-(4-methylphenyl)benzothiazole (R_1 = $OCOCH_3$) (KB-2683) and the homologous propionate (R_1 = $OCOC_2H_5$) showed cross-reactivities of 78 and 42%, respectively.

On the other hand, the cross-reactivities of catechol (R_3 = OH) and orthoquinone derivatives, possible metabolites, were also significant (31 and 19%). Based upon the result of diazo coupling reaction, the electron density of 4-hydroxy-2-(4-methylphenyl)benzothiazole (**1**) would be higher at the 5-position than at the 7-position. The 4,5-catechol derivative (**9**) seems to be a possible metabolite formed from the phenol by the hepatic cytochrome P-450. However, the formation of the catechol was not detected by high-performance liquid chromatography, when **2** was incubated with rat liver microsomes. It has been confirmed that the newly developed EIA for 4-hydroxy-2-(4-methylphenyl)benzothiazole (KB-2714) is fairly specific and applicable to the metabolic study.

Validity of the clean-up method

For obtaining reliable results, it is important to establish the appropriate method for purification and enrichment of an analyte in biological specimens. The procedure for clean-up of 4-hydroxy-2-(4-methylphenyl)benzothiazole (**1**) in human plasma or urine using an Extrelut-1 cartridge was evaluated with respect to the recovery rate and reproducibility. The acceptable recovery rates (95.5–114 and 84.7–116%) of **1** added to plasma and urine at four

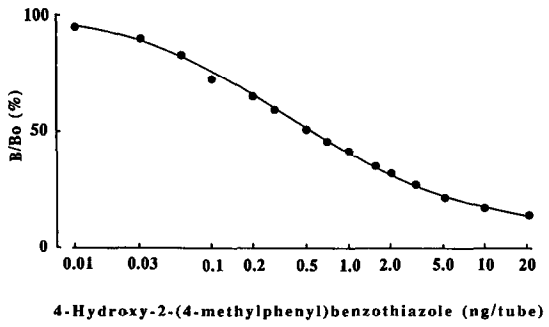


Figure 4
Dose–response curve of EIA for 4-hydroxy-2-(4-methylphenyl)benzothiazole.

Table 2
Recovery* of 4-hydroxy-2-(4-methylphenyl)benzothiazole added to human plasma and urine

	Plasma		Urine	
Added (ng ml^{-1})	Found (ng ml^{-1})	Recovery (%)	Found (ng ml^{-1})	Recovery (%)
0.97	1.11	114	1.13	116
3.89	4.09	105	4.25	109
31.1	29.7	95.5	31.3	101
497	493	99.2	421	84.7

*n = 5.

Table 3
Relative standard deviation for the assay of 4-hydroxy-2-(4-methylphenyl)benzothiazole in human plasma and urine

	Plasma		Urine	
Added (ng ml^{-1})	Intra-assay* (%)	Inter-assay† (%)	Intra-assay* (%)	Inter-assay† (%)
3.89	27.9	12.7	15.3	12.6
31.1	13.8	7.0	21.0	4.8
497	18.7	6.5	8.8	17.6

*, $n = 5$; †, $n = 3$.

levels justified the reliability of the proposed EIA method (Table 2). The reproducibility (intra- and inter-assay) was examined using biological specimens spiked with various amounts of **1**. It is evident from the RSD values listed in Table 3 that the precision of the present method is satisfactory in the range of 3.89–497 ng ml^{-1} of plasma and urine.

It should be emphasized that the antiserum elicited against the immunogen, in which the hapten is linked to the carrier protein through the 5-position of the benzothiazole moiety remote from *para*-position of the benzene ring, is capable of discriminating 4-hydroxy-2-(4-methylphenyl)benzothiazole from its major metabolites. The newly developed EIA method will be useful for the study of the pharmacokinetics of this benzothiazole drug.

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