RADIOIMMUNOASSAY OF BENCYCLANE IN HUMAN SERUM

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A radioimmunoassay of bencyclane in human serum was developed. Male rabbits were immunized with *p*-(3-carboxy-propoxy)bencyclane-bovine serum albumin conjugate, giving antisera with high titers. ^{125}I -*p*-Hydroxybencyclane with a high specific activity was prepared as a labelled antigen by a chloramine-T method. In the radioimmunoassay procedure, a mixture of serum sample, diluted antiserum and ^{125}I -antigen solution were incubated at 4 °C for 18 h, and bound-free separation was carried out by a dextran-coated charcoal method. The detection limit of bencyclane in human serum was 1.0 ng/ml, and the cross-reactivity of the antiserum with metabolites was found to be very low. Serum samples from healthy volunteers dosed orally with bencyclane fumarate were analyzed by both of the radioimmunoassay and gas chromatography-mass spectrometric methods. An excellent correlation was observed between the values obtained by both methods.

Keywords — bencyclane; direct radioimmunoassay; *p*-(3-carboxypropoxy)bencyclane; hapten-bovine serum albumin conjugate; ^{125}I -*p*-hydroxybencyclane; specific antiserum; cross-reactivity serum level

Bencyclane fumarate, Halidor®, is clinically used as a vasodilator and antispasmodic. During clinical uses of this drug and its metabolic studies,¹⁾ it has become important to determine concentrations of the intact drug in human sera. A specific determination of this drug by gas chromatography (GC) was reported, but it was not so highly sensitive and involved tedious extraction procedure.¹⁾ A more sensitive method using gas chromatography-mass spectrometry (GC-MS) has been developed in our laboratories.¹⁾ However, this method is still time-consuming and requires extraction procedure. Consequently, more simple and sensitive method has been required.

Recently radioimmunoassay has been intensively employed to directly determine serum concentrations of various drugs. For practical use of radioimmunoassay, it is one of the most important factors to prepare an antiserum with high titer and specificity.

In this paper, we describe preparation of an antiserum which is specific to intact bencyclane (I) and development of a radioimmunoassay with high sensitivity and specificity. Evaluation of the radioimmunoassay for practical application is also reported in comparing with the specific GC-MS method.

EXPERIMENTAL

Materials — Bencyclane fumarate and 4-*cis*-hydroxybencyclane (VI) were supplied from the Pharmaceuticals Division of our company. *p*-Hydroxybencyclane (II) was prepared by the method of Ono *et al.*²⁾ 4-Oxobencyclane (VII) and monodemethylbencyclane (VIII) were prepared by the method of Kimura *et al.*¹⁾ Benzyl 4-chlorobutyrate-1- ^{14}C was prepared from potassium cyanide- ^{14}C via 4-chlorobutyronitrile-1- ^{14}C and γ -butyrolactone-1- ^{14}C by the method of Olynyk *et al.*³⁾ Carrier free Na^{125}I was purchased from Amersham International

plc (Amersham, England). Bovine serum albumin (BSA) purchased from Armour Pharmaceutical Co. (Kankakee, Ill., U.S.A.), charcoal from Merck (Darmstadt, West Germany), and Freund's complete adjuvant, dextran and papaverine hydrochloride from Nakarai Chemicals Ltd. (Kyoto, Japan). All other chemicals were of reagent grade and purchased from standard commercial sources.

Preparation of Hapten (IV) — To a solution of benzyl 4-chlorobutyrate-1- ^{14}C (170 mg, 0.8 mM, 1.68 mCi) in methyl isobutyl ketone (3 ml) were added *p*-hydroxybencyclane (II) (122 mg, 0.40 mM), potassium carbonate (132 mg, 0.96 mM) and potassium iodide (13 mg, 0.08 mM) and the mixture was refluxed for 8 h. After filtration, the mixture was extracted with chloroform. The extract was washed with 10% sodium carbonate solution and water, and dried over sodium sulfate. The extract was evaporated under reduced pressure to dryness, and the residue chromatographed on silica gel.

Elution with chloroform-methanol (10:1 v/v) gave *p*-(3-benzyl-oxocarbonyl- ^{14}C -propoxy)bencyclane (III) (94 mg, 225 μCi); IR ν_{max} (cm^{-1} , chloroform) 1720 (CO); NMR (CDCl_3) δ : 2.25 (6H, s, N(CH_3) $_2$), 2.27 (2H, s, $-\text{CH}_2\text{-Ph-O-}$), 3.50 (2H, t, $J=4$ Hz, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{N-}$), 3.97 (2H, t, $J=4$ Hz, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO-}$), 5.12 (2H, t, $-\text{COOCH}_2\text{Ph}$), 6.70 (2H, d, $J=10$ Hz, $-\text{CH}_2\text{Ph-O-}$), 7.13 (2H, d, $J=10$ Hz, $-\text{CH}_2\text{Ph-O-}$), 7.33 (5H, s, $-\text{COOCH}_2\text{Ph}$). A mixture of *p*-(3-benzyl-oxocarbonyl- ^{14}C -propoxy)bencyclane (III) (94 mg, 0.2 mM, 225 μCi), ethanol (4 ml) and 10% Pd-C (40 mg) was stirred under hydrogen until hydrogen uptake ceased. The mixture was filtrated through celite to remove the catalyst. Evaporation of ethanol under reduced pressure afforded *p*-(3-carboxy- ^{14}C -propoxy)bencyclane (IV) (76 mg, 223 μCi) as prisms; mp 94.6–97.0 $^\circ\text{C}$; IR ν_{max} (cm^{-1} , Nujol) 1690 (CO); NMR (dimethylsulfoxide- d_6) δ : 2.25 (6H, s, $-\text{N}(\text{CH}_3)_2$), 2.67 (2H, s, $-\text{CH}_2\text{-Ph-O-}$), 3.45 (2H, t, $J=4$ Hz, $-\text{O-CH}_2\text{CH}_2\text{CH}_2\text{N-}$), 3.97 (2H, t, $J=4$ Hz,

$-\text{O-CH}_2\text{CH}_2\text{CH}_2\text{CO-}$), 6.70 (2H, d, $J=10$ Hz, $-\text{CH}_2\text{-Ph-O-}$), 7.20 (2H, d, $J=10$ Hz, $-\text{CH}_2\text{-Ph-O-}$); MS m/e : 391 (M^+), 288, 198, 102, 86, 58.

Preparation of Hapten-BSA Conjugate (V)

— To a stirred solution of *p*-(3-carboxy- ^{14}C -propoxy)bencyclane (IV) (38 mg, 96 μM , 109 μCi) in dimethylsulfoxide (2 ml) tri-*n*-butylamine (18 mg, 96 μM) and isobutyl chloroformate (13 mg, 96 μM) were added at once at 15 $^\circ\text{C}$ and the mixture was stirred at the same temperature for 20 min. The mixture was then added dropwise to a solution of BSA (70 mg) in 1 N sodium hydroxide within 20 min under ice-cooling, and the mixture stirred at 4 $^\circ\text{C}$ for 6 h. The reaction mixture was dialyzed against 0.15 M sodium chloride (2 l) at the same temperature for 48 h. The dialyzate was chromatographed on Sephadex G-50 column and eluted with distilled water. Lyophilization of the eluate gave the hapten-BSA conjugate (V) (49 mg, 20.9 μCi) as white fluffy powder. The number of haptens linked to BSA was calculated to be 25.5 from the radioactivity of carbon- ^{14}C .

Immunization — Male New Zealand white rabbits were used for immunization. The immunogen, hapten-BSA conjugate (V), was dissolved in 0.1 M phosphate buffer (pH 7.4) (0.5 ml) and emulsified with the equal volume of Freund's complete adjuvant. The emulsion was injected at multiple sites on the back of rabbits

- I: R = CH $_3$, X = H $_2$
 II: R = CH $_3$, X = OH and H
 VII: R = CH $_3$, X = O
 VIII: R = H, X = H $_2$

FIG. 1. Structures of Bencyclane, Its Metabolites and Related Compound

and boosters were given every two weeks for 15 weeks. Blood was taken a week after each booster injection for monitoring titers of antisera. Whole blood was collected after 15 weeks and allowed to clot at 25 °C for 3 h. Centrifugation of the blood at 3 000 rpm for 20 min gave the antiserum to bencyclane, which was stored at -20 °C with 0.1% sodium azide.

Radioiodination of *p*-Hydroxybencyclane (II)

—To a solution of Na¹²⁵I (0.44 mCi) in 0.1 N sodium hydroxide (2.0 μl) were sequentially added 0.05 M phosphate buffer (pH 7.4) (10 μl) and a solution of *p*-hydroxybencyclane (II) (5.1 μg) in methanol (10 μl), and the mixture was stirred at room temperature for 1 min. To the mixture a solution of chloramine T (60 μg) in the same buffer (30 μl) was added and stirred vigorously for 2 min. Finally, a solution of sodium metabisulfite (120 μg) in water (60 μl) was added to the mixture and mixed vigorously. The reaction mixture was then extracted with chloroform, and the extract washed with water. After dried over sodium sulfate, the extract was evaporated to dryness to give a residue. The residue was chromatographed on silica gel thin-layer plate and developed with dichloromethane-ethyl acetate-ethanol-28% ammonium hydroxide (20:10:15:1 v/v). A less polar product

with *R_f* value=0.43 (nearly identical with that of *p*-hydroxybencyclane) was scraped off from the thin-layer plate and dissolved in ethanol, giving *p*-hydroxybencyclane-¹²⁵I. The labelled ligand had a specific activity of 20.5 Ci/mM and was found to be highly immunoreactive to the bencyclane antiserum. The ethanolic solution was stored at 4 °C until use.

Radioimmunoassay Procedure — The antiserum titer was determined by adding an antiserum solution (0.2 ml) serially diluted with 0.1% gelatin buffer to a mixture of 0.1 M phosphate buffer (pH 7.4) (0.1 ml) and an ethanolic solution (0.04 ml) of *p*-hydroxybencyclane-¹²⁵I (6.3 × 10⁻³ μCi). Incubation was carried out in duplicate glass tubes (7 ml) at 4 °C for 18 h. Thereafter, bound and free *p*-hydroxybencyclane-¹²⁵I were separated by adding dextran-coated charcoal suspension (0.1 ml), containing 0.75% charcoal and 0.063% dextran in 0.1% gelatin buffer, to the incubation mixture, and the mixture was allowed to equilibrate at 4 °C for 15 min. The charcoal was removed by centrifugation at 3 000 rpm for 20 min. The supernatant was decanted into a polystyrene tube and the radioactivity was determined by Auto-gamma 240 CGD (Packard Instrument Co., Downers Grove, Ill., U.S.A.).

FIG. 2. Syntheses of the Hapten and the Hapten-BSA Conjugate Used for Immunization of Rabbits

The standard curve of bencyclane (I) in the control human serum was obtained by incubating increasing concentration of up to 1 000 ng/ml of bencyclane fumarate in the control human serum (0.1 ml), together with the same amount of *p*-hydroxybencyclane-¹²⁵I as mentioned above and 0.1 M phosphate buffer (0.3 ml), in the presence of a dilution (0.2 ml) of the respective antiserum (1:8 000) which bound nearly 50% of the radioactive tracer, as found by the titration. The incubation condition and the bound-free separation were identical with those mentioned above. For determination of bencyclane in human serum samples, the samples (0.1 ml) were used instead of the serum standard of bencyclane fumarate. Intra- and inter-assay variability of the procedure was tested by repetitive analysis of bencyclane fumarate, added to control human sera. All samples were assayed in duplicate.

The percent cross-reactions of the antiserum with bencyclane metabolites (VI, VII) and related compounds were determined by the method of Abraham.⁴⁾

Preparation of Human Serum Samples — Five healthy male volunteers were dosed orally with two 50 mg-tablets of bencyclane fumarate per man. Blood samples were collected at 0, 1, 2, 4, 6, 8, 11, and 24 h after dosing and left to stand at room temperature for 3 h. Centrifugation of the blood samples at 3 000 rpm for 10 min gave human serum samples which were stored at -20 °C until analysis.

Mass Fragmentography Using GC-MS — To a serum sample (1 ml) in a stoppered glass tubes (10 ml), 0.1 N sodium hydroxide solution (1 ml) was added and the mixture extracted with benzene (5 ml) three times. The extract was evaporated under reduced pressure to dryness. The residue was dissolved in ethyl acetate (0.2 ml), containing *n*-decane (0.5 µg/ml) as the internal standard. The ethyl acetate solution (5 µl) was assayed by GC-MS-computer system, Auto GCMS 6020-PAC 500M (Shimadzu, Kyoto, Japan). The GC column was 3% JXR-Silicone on Gaschrom Q (80–100 mesh; 2 mm × 1 m),

and the carrier gas was helium (30 ml/min). The temperature was set as follows: column 225 °C, injector 270 °C, separator 350 °C, ion source 350 °C. The energy of electron impact ionization was 20 eV. The amount of bencyclane was calculated from the relative intensities between *m/e* 102 of the base peak of bencyclane and *m/e* 99 of the internal standard.

RESULTS

Synthesis of Hapten-BSA Conjugate (V) as Immunogen

Hapten-BSA conjugate (V) was synthesized from *p*-hydroxybencyclane (II) by the procedure illustrated in Fig. 2. Condensation of *p*-hydroxybencyclane (II) with benzyl 4-chlorobutyrate-1-¹⁴C gave *p*-(3-benzyloxy-carbonyl-¹⁴C-propoxy) bencyclane (III). Catalytic hydrogenolysis of III with hydrogen using Pd-C yielded the desired hapten, *p*-(3-carboxy-¹⁴C-propoxy)bencyclane (IV), whose chemical structure was confirmed by spectral analysis. The radioactive hapten (IV) was coupled with BSA by mixed anhydride method, giving the required hapten-BSA conjugate (V). In this conjugate, the number of haptens linked to BSA was found to be 25.5 by radiochemical analysis.

*Preparation of *p*-Hydroxybencyclane-¹²⁵I as Radioactive Tracer*

p-Hydroxybencyclane (II) was labelled with Na¹²⁵I by Greenwood's method⁵⁾ using chloramine T. Purification of the reaction mixture by preparative thin-layer chromatography on silica gel plates afforded *p*-hydroxybencyclane-¹²⁵I, which showed high immunoreactivity to the antiserum.

Production of Antiserum

Production of a specific antiserum to bencyclane was achieved by immunization of hapten-BSA conjugate (V) to male New Zealand white rabbits. A typical antiserum dilution curve is shown in Fig. 3 and the dilution of the antiserum which exhibited 50% binding to *p*-hydroxybencyclane-¹²⁵I was found to be 1:8 000.

Sensitivity and Specificity of the Radioimmunoassay

A typical standard curve of bencyclane in the control human serum is shown in Fig. 4. Intra- and inter-assay coefficients of variation ($n=6$) were less than 10% in a range of 1.0 to 200 ng/ml. The detection limit of the radioimmunoassay for bencyclane in human sera was found to be 1.0 ng/ml when defined as the concentration of unlabelled bencyclane producing 20% inhibition of maximal *p*-hydroxybencyclane- ^{125}I binding.

The cross-reactivity of the antiserum to metabolites (VI, VII) of bencyclane, related compounds (II, VIII) and papaverine was studied

by the method of Abraham;⁴⁾ and the results are shown in Table I. 4-Hydroxybencyclane (VI), 4-oxobencyclane (VII) and monodemethylbencyclane (VIII) showed insignificant cross-reactions with 0.1, 1.4 and 5.1%, respectively, whereas, as anticipated, the antiserum did not discriminate essentially *p*-hydroxybencyclane (II) from bencyclane. Papaverine did not bind to any mea-

FIG. 3. Dilution Curve for the Specific Antiserum to Bencyclane

FIG. 4. Standard Curve of Bencyclane in the Control Human Serum

The relative percentage of *p*-hydroxybencyclane- ^{125}I bound to the antiserum in the presence of various amounts of unlabelled bencyclane is plotted on a semilogarithmic scale.

TABLE I. Percent Cross-Reaction of Anti-bencyclane Antiserum with Metabolites of Bencyclane, Related Compounds and Papaverine

Compound	% cross-reaction (50%) ^{a)}
Bencyclane (I)	100
<i>p</i> -Hydroxybencyclane (II)	90.4
4- <i>cis</i> -Hydroxybencyclane (VI)	0.1
4-Oxobencyclane (VII)	1.4
Monodemethylbencyclane (VIII)	5.1
Papaverine	0.01

a) Percent cross-reaction was determined by the method of Abraham.⁴⁾

surable degree to the antiserum. Concentrations were determined by the Radioimmunoassay applied directly to serum samples.

Comparison of the Radioimmunoassay with the Mass Fragmentgraphic Assay

The validity of the radioimmunoassay for bencyclane in human serum samples was demonstrated by comparison with mass fragmentgraphic assay. Twenty-four human serum samples were tested by both methods. As shown in Fig. 5, the regression line calculated for the serum concentration of bencyclane determined by radioimmunoassay versus that measured by mass fragmentgraphic assay, showed a slope of 1.07 and an intercept of nearly -3.0 . The correlation coefficient was 0.97 over a concentration range of 5 to 56 ng/ml.

Experiment in Humans

The radioimmunoassay was applied to determine concentrations of bencyclane in human serum samples collected from male healthy volunteers, who received single oral dose of 100 mg of bencyclane fumarate. Mean serum levels of bencyclane in five subjects are illustrated in

FIG. 5. *Comparison of Radioimmunoassay with Mass Fragmentgraphic Assay to Determine Concentrations of Bencyclane in Human Serum*
 $Y = 1.07X - 2.95$
 Correlation coefficient $r = 0.97$

Fig. 6. The serum levels were maximal at 6 h after dosing and the mean peak concentration was 41.0 ng/ml. The levels decreased gradually from the peak to about 8 ng/ml at 24 h after dosing.

DISCUSSION

The purpose of present studies has been to develop and evaluate a radioimmunoassay for determination of bencyclane (I) in human serum, and to prove the applicability in clinical and pharmacokinetic studies.

Kimura *et al.*¹⁾ reported that the major identified metabolic pathway of bencyclane in human was the oxidation of its cycloheptane ring at C-4 position to yield 4-*cis*-hydroxy-bencyclane (VI) and 4-oxobencyclane (VII). The metabolites, VI and VII, were excreted in human urine to the extent of 23.5 and 3.4% dose in the first 24 h, respectively, when 100 mg of bencyclane fumarate was dosed orally. They also suggested that there was found monomethylation of VII as a minor metabolic pathway, though the amount of the corresponding metabolite was found much less than 1% dose.

Therefore, in order to produce a specific anti-

FIG. 6. *Mean Serum Concentration in Male Healthy Humans (n=5) after Single Oral Dosing of 100 mg of Bencyclane Fumarate*

serum which discriminates intact bencyclane from its metabolites, the hapten derivative for BSA conjugate was designed with modification of *p*-position of the phenyl ring, because the position was far in the distance from the metabolic sites of both the cycloheptane and the dimethylamino group. Thus, *p*-hydroxybencyclane (II) was selected as the starting material and converted to *p*-(3-carboxy-¹⁴C-propoxy)bencyclane (IV). Conjugation of the hapten (IV) with BSA by mixed anhydride method provided the desired hapten-BSA conjugate (V). The analysis of the radioactivity of V revealed that satisfactory numbers of the hapten molecules (25.5) were covalently bound to one molecule of BSA.

Immunization of rabbits with the hapten-BSA conjugate (V) in the usual manner afforded antisera exhibiting remarkable binding activity to bencyclane. The dilution curve of the antiserum possessing the highest titer is shown in Fig. 3. The 1:8 000 dilution of the antiserum which was able to bind 50% of *p*-hydroxybencyclane-¹²⁵I was employed for the radioimmunoassay.

The specificity of the antiserum towards closely related compounds including the metabolites, and papaverine which may be co-mediated in the therapeutic treatment was examined. As shown in Table I, the metabolites (VI, VII) and papaverine did not appreciably cross-reacted with the antiserum. On the other hand, the antiserum slightly cross-reacted with monodemethylbencyclane (VIII), and did not differentiate *p*-hydroxybencyclane (II) from bencyclane. However, the monodemethyl analogue of VIII was found to be only a minor metabolite as mentioned above, and *p*-hydroxybencyclane has not been detected as a metabolite in human.¹⁾ These facts, therefore, indicate that the antiserum is, as expected, sufficiently specific to bencyclane and suitable for the practical use of the radioimmunoassay.

The standard curve of bencyclane in the control serum (Fig. 4), with low coefficients of variation (below 10%) in the intra- and inter-assay, suggests that the measurable range of the radi-

oimmunoassay is 1 to 200 ng/ml for bencyclane, which makes the assay satisfactory in terms of sensitivity and precision for measurement of serum concentrations.

The excellent correlation, between the serum concentrations measured directly by the radioimmunoassay and those measured after extraction by the mass fragmentographic assay (Fig. 5), demonstrates that the radioimmunoassay gives a quantitative measure of bencyclane without any extraction procedure. As an example, the radioimmunoassay was successfully applied to the determination of serum levels of bencyclane in clinical samples (Fig. 6).

In conclusion, the radioimmunoassay developed here offers distinct advantages such as the precise, accurate and sensitive determination with a small volume (0.1 ml) of serum samples, and the simple procedure without extraction enabling to assay a large number of samples at once. Therefore, this method is expected to facilitate the monitoring of serum levels of bencyclane in clinical subjects, possibly offering more detailed information on the relationship between the serum levels and clinical effects or side effects of this drug.

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