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Radioimmunoassay for a novel benzodiazepine inverse agonist, S-8510, in human plasma and urine

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Abstract

A radioimmunoassay (RIA) was developed for a new benzodiazepine inverse agonist, 2-(isoxazol-3-yl)-3,6,7,9-tetrahydroimidazo [4,5-d] pyrano [4,3-b] pyridine monophosphate monohydrate (S-8510), in human plasma and urine. For competitive RIA, three amino derivatives of S-8510 were labelled by the Bolton and Hunter method and rabbit antisera were prepared using three immunogens, conjugates of three carboxyl derivatives of S-8510 with bovine serum albumin. All combinations of the labelled antigens and antisera were examined and homologous combinations were selected for the competitive RIA. One of the three homologous combinations had the best selectivity after investigations of cross-reactivity using 12 related compounds and was very sensitive for S-8510. Next, a pretreatment for biological samples was developed using mixed mode solid-phase extraction (SPE) column followed by the RIA (SPE/RIA). The assay recoveries for human plasma and urine were both excellent and the limits of quantitation were extremely low, 80 and 200 pg ml^{-1}, respectively. Human plasma samples and urine samples after administration of this drug were successfully measured by the SPE/RIA. No cross-reactive metabolites were detected in any fractions after RP-HPLC separation of the plasma samples. The RIA using carefully selected antiserum and labelled antigen was highly specific for unchanged S-8510. To simplify the RIA procedure, a scintillation proximity assay (SPA) using the same labelled antigen and antiserum was developed for analyzing S-8510 in human plasma and found to be very promising.

Keywords: Benzodiazepine inverse agonist; S-8510; Competitive radioimmunoassay; Human plasma; Human urine; Solid-phase extraction

1. Introduction

A novel compound, 2-(isoxazol-3-yl)- 3,6,7,9-tetrahydroimidazo [4,5-d] pyrano [4,3-b] pyridine monophosphate monohydrate (S-8510), is characterized as a benzodiazepine partial inverse agonist with a modest GABA ratio and low efficacy. The results from many pharmacological studies suggest that S-8510 can be used as a therapeutic drug for senile dementia, including Alzheimer's disease with little risk for inducing anxiety or convulsion

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[1]. When S-8510 was given by oral administration, most of the drug was found to be extracted into the liver in preclinical studies. Plasma levels of S-8510 in clinical studies, especially in the early stage of phase I studies, were assumed to be very low, less than a few ng per ml of plasma at maximum. Our clinical studies of S-8510 were one of the first trials of this type of drug and there was little knowledge on the drug behavior in the human body. Therefore, it was necessary to develop a highly sensitive assay method for S-8510 in order to monitor drug concentrations in plasma and urine of volunteers for their safety and for the investigation of drug behavior in human body.

Radioimmunoassays (RIAs) are much more sensitive than conventional assay methods, such as high-performance liquid chromatography (HPLC), and should be very useful for measuring many samples for the pharmacokinetics of S-8510. However, an RIA sometimes responds some metabolites as well as the unchanged form of the drug. In this study, three kinds of antisera by immunizing rabbits and three different structures of radioiodine-labelled antigen were prepared in order to develop a highly selective and sensitive RIA. The best combination of the antiserum and the labelled S-8510 was selected from the results of cross-reactivity of 12 related compounds and the sensitivity of RIA standard curves.

A pretreatment procedure for biological samples was also developed using solid-phase extraction (SPE) column and then the concentrations of S-8510 in plasma and urine samples from phase I clinical studies were measured by the RIA after the SPE pretreatment (SPE/RIA).

The RIA procedure can be simplified using scintillation proximity assay (SPA) in which there is no need to separate the bound and free fractions (BF separation) of the reaction mixture. SPA has been adopted extensively in the high-throughput screening for drug discovery but only a few applications were seen when measuring drug level in biological samples [2,3]. The assay system was applied for the RIA for S-8510 and was evaluated for its feasibility.

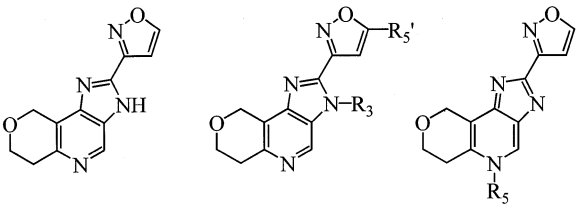

Fig. 1. Structure of S-8510 and its derivatives. S-8510 (left), S-8510-5′-butanoic acid (center; $R_{5'} = (CH_2)_3COOH$ and $R_3 =$ H), S-8510-3-butanoic acid (center; $R_{5'} =$ H and $R_3 = (CH_2)_3COOH$), S-8510-5-butanoic acid (right, $R_5 = (CH_2)_3COOH$), S-8510-5′-ethylamine (center; $R_{5'} = (CH_2)_2NH_2$ and $R_3 =$ H), S-8510-3-ethylamine (center; $R_{5'} =$ H and $R_3 = (CH_2)_2NH_2$), and S-8510-5-ethylamine (right, $R_5 = (CH_2)_2NH_2$).

2. Experimental

2.1. Reagents

S-8510, its derivatives (Figs. 1 and 3) and [^{14}C]-S-8510 were obtained from Shionogi Research Laboratories. All chemicals were of analytical grade, unless otherwise specified.

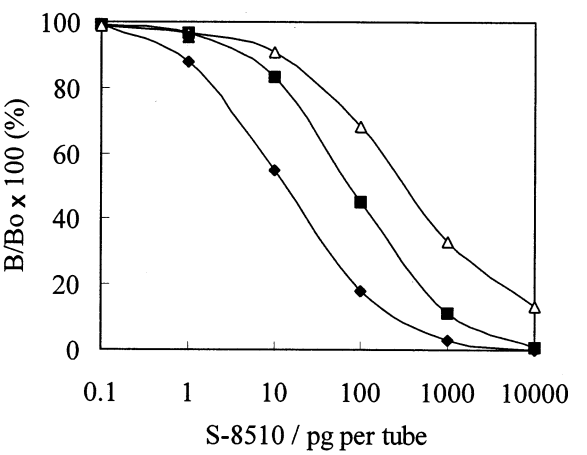

Fig. 2. RIA standard curves of three homologous systems. 5′-Homologous system (◆), 5-homologous system (■), and 3-homologous system (△) Antisera (and its 50% inhibitory concentration) used in these systems were K76 (10 pg per tube), K81 (100 pg per tube), and K79 (350 pg per tube), respectively.

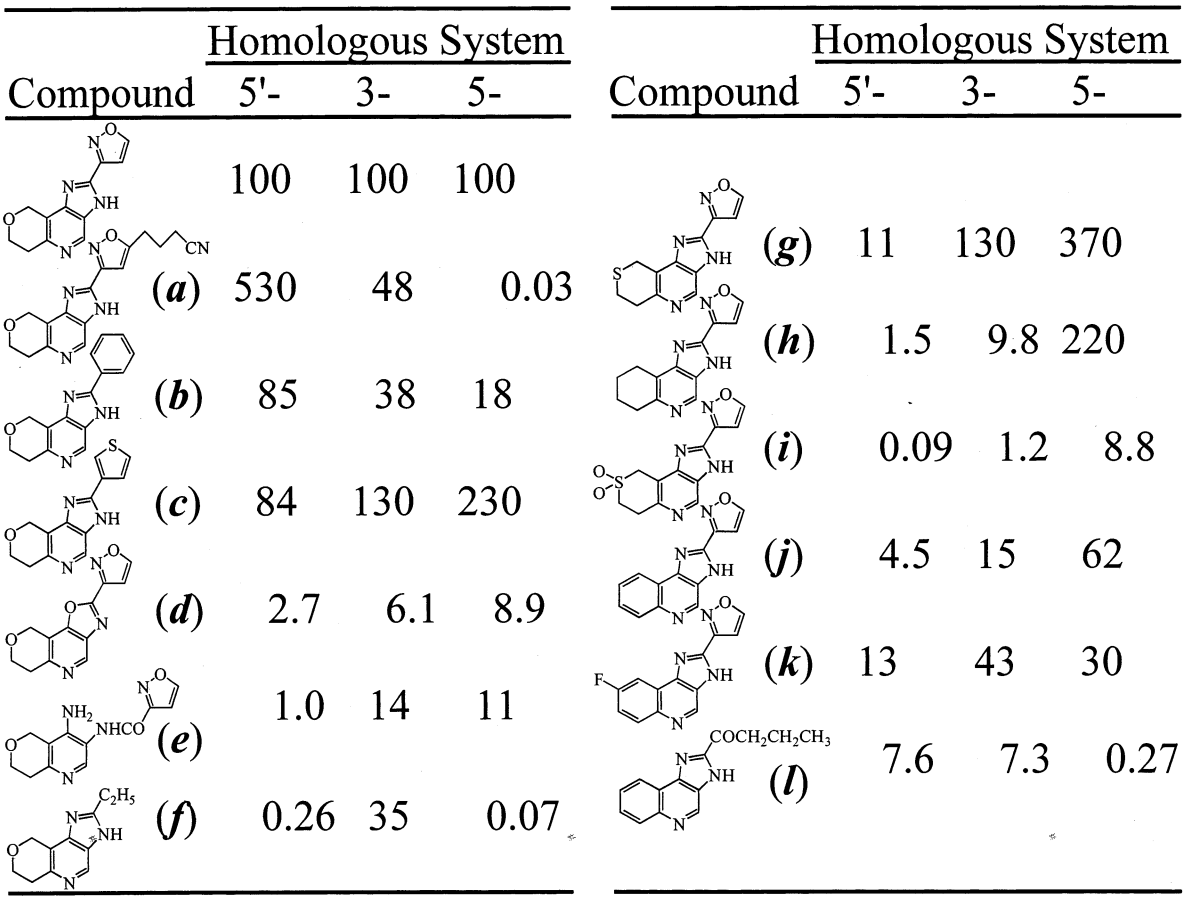

Fig. 3. Cross-reactivities of related compounds on 5′-, 3-, and 5-homologous system. Percent cross-reactivity was expressed as a ratio of the 50% inhibitory concentration of S-8510 to that of a compound in each RIA system on a molar basis.

2.2. Immunogen and antiserum

Three immunogens, conjugates of S-8510 with bovine serum albumin (BSA, lyophilized and crystallized, Sigma, St. Louis, MO, USA), were prepared by mixed anhydride method [4]. S-8510-5′-butanoic acid (Fig. 1), 33 mg (100 μmol), was dissolved in 4.5 ml of hot dry dimethylformamide (DMF). After cooling, 23.8 μl of tri-*n*-butylamine (100 μmol) and 13.2 μl of isobutyl chloroformate (100 μmol) were added to the solution with vigorous mixing at 0°C. The mixture was stirred for 35 min at 0°C to produce mixed anhydride. A BSA solution was prepared by dissolving 140 mg (2.03 μmol) in 4.0 ml of water then adding 3.0 ml of DMF, and the pH was adjusted to around 9.0 with sodium hydroxide solution (1 M). The mixed anhydride was slowly added to the BSA solution with stirring at 0°C, keeping the pH at around 8.5 by adding sodium hydroxide solution (1 M). The mixture was stirred for an additional 3 h at 4°C. The solution was dialyzed against sodium bicarbonate solution (0.1 M) for 1 day at 4°C then against distilled water several times. The conjugate solution was lyophilized to yield 133 mg and stored at 4°C.

The other two BSA conjugates of S-8510-3-butanoic acid and -5-butanoic acid (Fig. 1) were prepared in the same way and yielded 128 and 136 mg, respectively. The number of S-8510-5′-butanoic acid residues, -3-butanoic acid residues,

and -5-butanoic acid residues per BSA molecule were 47, 31, and 32, respectively, determined by the trinitrobenzenesulfonic acid method [5], which can measure the free amino groups remaining on the conjugate.

Each immunogen was dissolved in saline and emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, MI, USA). A half milliliter of the emulsion containing 250 μg of immunogen was injected intradermally into 15 to 25 sites each on the backs of four Japanese white rabbits (No. K73-K76, K77-K80, and K81-K84 for the BSA conjugate of S-8510-5′-butanoic acid, -3-butanoic acid, and -5-butanoic acid, respectively). Immunization was repeated every three weeks and whole blood was collected 10 days after the sixth immunization. The serum from each rabbit was stored at −20°C until use.

2.3. Radioiodine-labelled antigen

S-8510-5′-ethylamine (Fig. 1), 2.85 μg (10 nmol) in 30 μl of DMF, was added to a reaction tube which contained 18.5 MBq of [^{125}I]-Bolton-Hunter Reagent (BHR) (a benzene solution from NEN, Wilmington, DE, USA, was evaporated to dryness under a dry nitrogen stream) [6]. The reaction mixture was stirred for 3 h at 20–25°C.

The reactant was injected into an HPLC instrument (LC-6A system, Shimadzu, Kyoto, Japan) after addition of 30 μl of methanol and fractionated using an ODS column (Nucleosil 5C_{18}, 250 × 4.6 mm I.D., particle size 5 μm, Marchery-Nagel, Düren, Germany) eluted under gradient conditions with methanol (HPLC grade, Merck, Darmstadt, Germany) (mobile phase B) in water (mobile phase A). The gradient conditions of mobile phase B were 0% at the start, 0–10% during 0–5 min, 10–55% during 5–35 min, 55% during 35–45 min, 55–100% during 45–50 min, and 100% during 50–60 min. The flow rate was 1 ml min^{-1}, and the column temperature was not controlled (room temperature 20–25°C). Fractions of the eluate were collected at around 48 min of the retention time.

S-8510-3-ethylamine and -5-ethylamine (Fig. 1) were labelled with BHR and purified by HPLC in the same way. Fractions of the eluate were collected around 47 and 43 min, respectively. The radioiodine-labelled antigens of the eluate were diluted to 1 × 10^6 cpm ml^{-1} with phosphate buffer (pH 7.4, 0.1 M) containing BSA (1% (w/v)), sodium azide (0.25% (w/v)) and sodium chloride (0.75 M), lyophilized and stored at 4°C until use. The lyophilized antigen in a vial was reconsti-

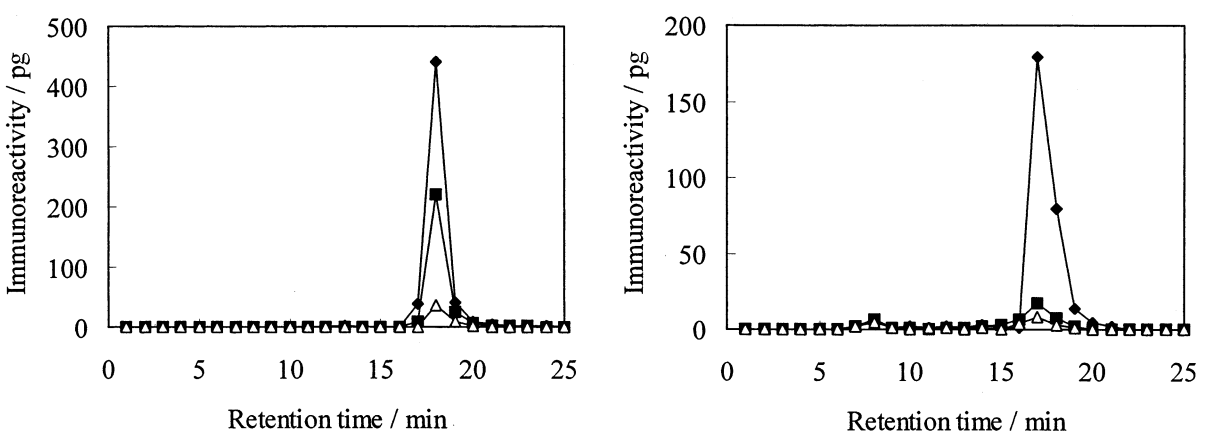

Fig. 4. Immuno-chromatogram of human plasma and urine. Plasma or urine sample was pretreated with solid-phase extraction as described in the text and 20 μl of the extract was injected to HPLC instrument (column, Nucleosil 5C_{18}, 150 × 4.6 mm I.D.; mobile phase, ammonium acetate buffer (0.3% (w/v), pH 6.0)–methanol (13:7, w/w); flow rate, 1 ml min^{-1}) after filtration. Immunoreactivity in fractions of the eluate was measured by the RIA after evaporation and dissolution in 400 μl of the assay buffer containing BSA (0.5% (w/v)). Retention time of S-8510 was 18 min. Left panel: plasma samples collected 0.5 (◆), 2 (■), and 6 h (△) after administration. Right panel: urine samples collected during 0–4 h (◆), 4–8 h (■), and 8–12 h (△) after administration.

Table 1
Assay precisions and accuracies for human plasma samples

Added (pg/ml)	Measured (Mean ± S.D.)	Precision (RSD, %)	Accuracy (bias, %)
Intra-assay (n = 5)			
80.0	88.6 ± 11.3	12.8	+10.8
320	303 ± 22	7.3	−5.3
1280	1210 ± 70	5.8	−5.5
5120	5100 ± 300	5.9	−0.4
Inter-assay (n = 5)			
80.0	82.0 ± 10.9	13.3	+2.5
320	331 ± 33	10.0	+3.4
1280	1300 ± 90	6.9	+1.6
5120	5110 ± 340	6.7	−0.2

tuted with water to make a concentration of 2×10^5 cpm ml^{-1}.

2.4. RIA procedure

All solutions in the following RIA procedure for human plasma samples were diluted with an RIA buffer, that is, phosphate buffer (pH 7.4; 0.1 M) containing sodium chloride (0.15 M), sodium azide (0.05% (w/v)), and BSA (0.5% (w/v)). Another RIA buffer which contained gelatin instead of BSA was used for human urine samples. Human plasma samples and urine samples were pre-treated by the solid-phase extraction described below. RIA standard solutions for human plasma assay contained the same amount of plasma extract from normal human plasma as from test samples. Standard solutions for human urine assay similarly contained the same amount of normal urine extract.

The labelled antigen solution, 100 μl (200 000 cpm ml^{-1} of labelled S-8510-5′-ethylamine), was pipetted into each RIA tube (75 × 12 mm I.D., polystyrene) in duplicate, which contained 100 μl of samples or standard solutions of S-8510 and 200 μl of the assay buffer. Next, 100 μl of diluted antiserum (1:300 000 of K76) was pipetted into each tube, and the mixture was incubated for 16 h at 20–25°C. This was followed by adding 100 μl of immobilized anti-rabbit second antibody (1 mg ml^{-1} suspension of Immunobead, Bio-Rad, Richmond, CA, USA) to each tube. The tubes were centrifuged for 10 min at $2000 \times g$ after incubation for 2 h at 20–25°C. The supernatant portions of the tubes were aspirated off and the radioactivity of the residue in each tube was measured by gamma counter (ARC-600, Aloka, Tokyo, Japan). The amount of S-8510 in each RIA tube was estimated using an RIA standard curve simultaneously obtained.

2.5. Scintillation proximity assay

Similar reagents to those used for the RIA for human plasma samples were used for the scintillation proximity assay except for SPA beads (SPA fluomicrospheres (yttrium-silica) linked to anti-rabbit antibody, type I, Amersham International, Buckinghamshire, England). The labelled antigen solution, 100 μl (200 000 cpm ml^{-1}), was pipetted into each tube (50 × 12 mm I.D., glass) of duplicate assays, which contained 100 μl of samples or standard solutions of S-8510 and 200 μl of the assay buffer. Next, 100 μl of diluted antiserum (1:200 000 of K76) was pipetted into each tube, and the mixture was incubated for 16 h at 20–25°C. This was followed by adding 100 μl of SPA beads (suspended SPA beads for 500 assay with 50 ml of the assay buffer) to each tube. After incubation for 30 min at 20–25°C, each tube was placed in a 20-ml vial for liquid scintillation counter (LSC) and counted by LSC (Packard LSC1900TR; window width, full).

Table 2
Assay precisions and accuracies for human urine samples

Added (pg/ml)	Measured (Mean ± S.D.)	Precision (RSD, %)	Accuracy (bias, %)
Intra-assay (n = 5)			
200	215 ± 32	14.9	+7.5
800	852 ± 53	6.2	+6.5
3200	3120 ± 90	2.9	−2.5
12 800	11 700 ± 400	3.4	−8.6
Inter-assay (n = 5)			
200	217 ± 35	16.1	+8.5
800	810 ± 38	4.7	+1.3
3200	3260 ± 120	3.7	+1.9
12 800	12 300 ± 600	4.9	−3.9

2.6. Solid-phase extraction of plasma sample and urine sample for RIA

A human plasma or urine sample, 250 μl, was mixed with 250 μl of citrate buffer (pH 3.0; 0.1 M) and 400 μl of the mixture was applied to a pretreatment column for solid-phase extraction (Bond Elut Certify, 300 mg type, Varian, Harbor City, CA, USA, pre-washed with 6 ml of methanol, 6 ml of distilled water, and 1 ml of the citrate buffer). Next, the column was washed with 10 ml of water, 10 ml of methanol, and 2 ml of ethyl acetate containing diluted ammonia water (2.5% (v/v)). The retained S-8510 was eluted by 6 ml of ethyl acetate containing the diluted ammonia water and the solvent was evaporated to dryness under a nitrogen stream at 40°C. The residue from human plasma sample or urine sample was dissolved in 1.6 or 4 ml of the respective assay buffer.

3. Results and discussion

3.1. Radioimmunoassay

Radioactive iodine was successfully introduced into each of three amino derivatives of S-8510 (Fig. 1) by the Bolton and Hunter method and the labelled antigens were highly purified by HPLC. Each labelled antigen was eluted as a main radioactive peak separated from the respective amino derivative and a hydrolyzed Bolton and Hunter reagent. The labeling yield of ^{125}I was high, 50–70%.

Three kinds of rabbit antiserum were derived from three BSA conjugates with carboxyl derivatives, S-8510-5′-butanoic acid, -3-butanoic acid, and -5-butanoic acid (Fig. 1). Antibodies in these antisera should bind to different sites on S-8510 and have different specificities [7,8]. It was also expected that a site-heterologous combination of the labelled antigens and the antisera would be

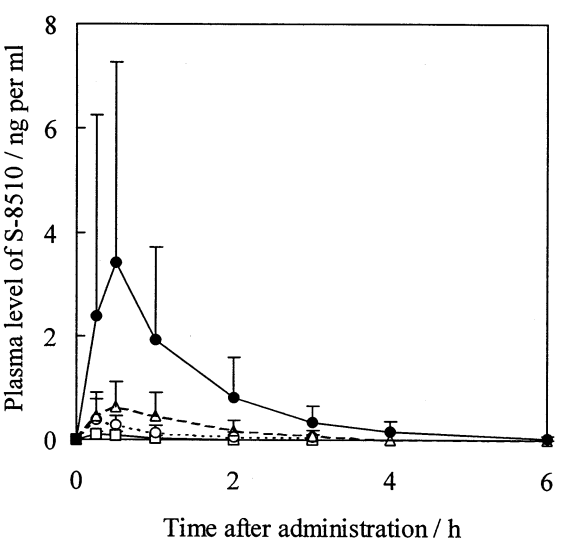

Fig. 5. Plasma levels of S-8510 after single oral administration to healthy volunteers. Dose: 1 mg per person (□) ($n = 4$), 2.5 mg per person (○) ($n = 6$), 5 mg per person (△) ($n = 6$), and 10 mg per person (●) ($n = 6$). Each point represents the mean ± S.D.

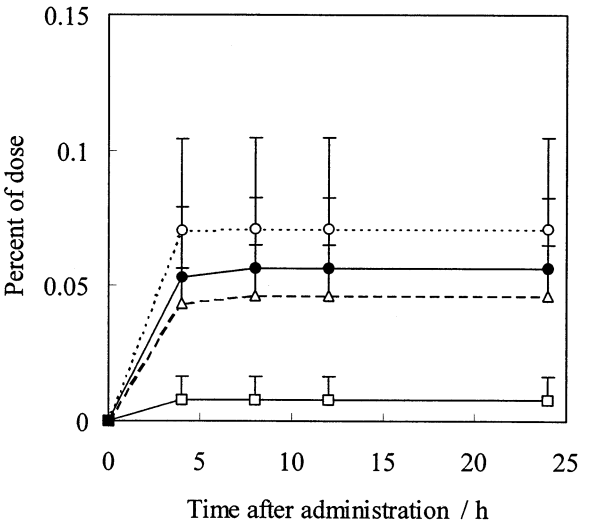

Fig. 6. Cumulative excretion of S-8510 in urine after oral administration to healthy volunteers. Dose: 1 mg per person (□) ($n=4$), 2.5 mg per person (○) ($n=6$), 5 mg per person (△) ($n=6$), and 10 mg per person (●) ($n=6$). Each point represents the mean ± S.D.

more sensitive than a homologous combination [9].

Each labelled antigen was made to react with every antisera and bindings between them were observed only in the three homologous combinations. The bindings in all site-heterologous combinations were none or very small at some practical dilutions (more than several thousands fold dilution) of antisera. It was thought that the distance between the iodine-labelled position and and the antibody binding region on S-8510 was not enough far compared with the distance between the iodine-labelled position and the protein coupled position, and therefore, the binding of labelled antigen to the antibody did not occur effectively in these site-heterologous combinations. In order to develop a sensitive site-heterologous RIA for a relatively small molecule such as S-8510, distances among the three positions should be carefully considered.

Homologous RIAs of the 5′-position homologous system, 3-position homologous system, and 5-position homologous system were, thus, selected for further investigation, in which the most sensitive antiserum in each system was used (K76, K79, and K81, respectively). As shown in Fig. 2, the RIA standard curve of the 5′-position homologous system was the most sensitive among the three systems.

3.2. Cross-reactivities at three homologous RIA systems

Cross-reactivities of 12 related compounds were estimated for the three systems and the results are shown in Fig. 3. In the 5′-position homologous system, the binding with labelled antigen to antibody was competed for derivatives of isoxazol ring (***a***, ***b***, and ***c***). But cross-reactivities to the dihydropyran (pyran) ring derivatives (***g***, ***h***, ***i***, ***j***, and ***k***) and imidazole ring derivatives (***d***, ***e***, and ***f***) were rather low and the antibody could bind to the structure from the pyran ring to the imidazole ring. The cross-reactivity values of the 3′-position homologous system did not decrease much with some changes of the structure (except ***i***) and the binding region seems to be broad. The binding region of the 5′-position homologous system is also relatively broad but the inhibition did not occur in some 5-position derivatives (***a***, ***f***, and ***l***) which have alkyl chains at the 5′-position or 2-position of imidazole ring. Interestingly, replacement of the oxygen with sulfur (***c*** and ***g***) showed high cross reactivity in the three homologous systems. These results agreed with previous investigations about immunoassay specificity which was determined largely by the chemical groups of the hapten far from the point of attachment of the original compound to the carrier protein [7].

S-8510 was suggested to be metabolized mainly around the isoxazol ring in animal (data not shown) and it was thought that the 5′-position homologous RIA system was the most suitable for clinical studies. The 3-position homologous system has a relatively wide spectrum for S-8510 derivatives and is useful for group-specific analysis, such as a detection system of the combined HPLC/RIA method and an immunoaffinity extraction with immobilized antibody column.

3.3. Pretreatment of biological samples for RIA

S-8510 was extracted from human plasma and urine samples by SPE method before the RIA (SPE/RIA). Bond Elut Certify was chosen rather than the usual C18 SPE column. Bond Elut Certify has an ion exchange separation mode as well as a C18 separation mode. The extraction yield was more than 95% as estimated by [^{14}C]-S-8510 and the interferences with the RIA from plasma or urine components were markedly eliminated by the conditions described in the experimental section. However, RIA binding percentages with plasma or urine extract were a little lower than those with the assay buffer. In addition, no differences in the RIA binding percentage were observed among individual plasma and urine samples. Thus, amounts of normal plasma or urine extract equal to those of unknown samples were added to the respective RIA standard solutions.

3.4. Specificity, precision and accuracy of the SPE/RIA

In order to search for cross-reactive metabolites in human plasma and urine, immunoreactivities were measured by the RIA for all the fractions after RP-HPLC separation of some human plasma and urine samples from the phase I clinical study described below. As shown in Fig. 4, no cross-reactive metabolites were found in the plasma and some in the urine, although there may possibly be another cross-reactive metabolite which has a similar retention time to S-8510. These results suggest that the SPE/RIA is specific enough to measure unchanged S-8510 in human plasma samples, as expected from the cross reactivities. On the other hand, SPE/RIA for urine may measure some metabolites, especially after 4 h from the administration, which was estimated as 30–40% of the total immunoreactivity in Fig. 4. However, it was not thought to be very important to measure unchanged drug concentration in urine separately because the excretion of S-8510 was assumed to be very low, less than 0.1% of the dose, from preclinical studies (data not shown) and the concentration after 4 h was so low that the cumulative excretion value was hardly affected by cross-reactive metabolites in urine. It should be no matter with using the SPE/RIA for urine samples as well as plasma samples.

As shown in Tables 1 and 2, the precision and accuracy values were good enough to validate this SPE/RIA. The limit of quantitation (LOQ) was 0.08 ng ml^{-1} in human plasma and 0.2 ng ml^{-1} in human urine.

3.5. Scintillation proximity assay

SPA is a homogeneous RIA without BF separation and is expected to reduce assay steps and time. The standard curve of the SPA was identical with those of the conventional RIA described above. Human plasma samples were measured by both the RIA and the SPA after SPE pre-treatment. The correlation of assay results between the RIA (x ng ml^{-1}) and the SPA (y ng ml^{-1}) was excellent, $y = 1.004 \times -0.019$, $r = 0.997$, $n = 63$. The assay performances of SPA for S-8510 were the same as those of the RIA, showing that it could be used in place of the RIA. However, from the practical aspect, there was only a conventional LSC in our laboratory and few advantages were shown in the overall SPA procedures because it is necessary to separately discarded radioactive liquid waste and the SPA tubes in the LSC vials after completion of the experiment. Thus, in the present study, the conventional RIA was used for measurement of clinical samples. SPA should be very useful if special equipment is available.

3.6. Determination of S-8510 in human plasma and urine

S-8510 was administered orally to healthy volunteers who gave us written consent in phase I studies and their plasma and urine were measured by the SPE/RIA. Plasma and urine levels of S-8510 in phase I studies were successfully measured by the SPE/RIA and some results are shown in Figs. 5 and 6. Considerable differences in time course curves among individuals were observed, while the assay reproducibility were confirmed with some samples.

This sensitive RIA enabled pharmacokinetic analyses of S-8510 in the human body even at the lowest dose and precise assessment of the β-phase elimination rate of lower concentrations, although conventional high-performance liquid chromatography could not determine less than 5–10 ng of S-8510 per ml of plasma sample. The results of the pharmacokinetic analyses will be reported elsewhere.

Highly sensitive analytical methods to measure drug concentrations in body fluids are indispensable for estimating pharmacokinetics in the human body and immunoassays are playing increasingly important roles in developing new drugs used at low dosages.

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