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Radioimmunoassay for a novel lignan-related hypocholesterolemic agent, S-8921, in human plasma after high-performance liquid chromatography purification and in human urine after immunoaffinity extraction

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Abstract

The novel compound methyl-1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) has hypocholesterolemic activity in animals and is expected to exhibit a similar activity in human. Reversed-phase high-performance liquid chromatography (HPLC) separation followed by radioimmunoassay (RIA) for human plasma samples (HPLC–RIA) and immunoaffinity extraction (IAE) followed by RIA for human urine samples (IAE–RIA) were developed for investigation of S-8921 behavior in clinical studies. For the RIA, antisera from rabbit and a radioiodine-labelled S-8921 were prepared by immunizing a conjugate of S-8921 with bovine serum albumin and by the Bolton and Hunter method, respectively. HPLC–RIA using a semi-micro column was very sensitive, that is a 0.05 ng/ml limit of quantitation in human plasma, and specific for unchanged form of S-8921. IAE–RIA using a centrifugal filtration tube completely eliminated the matrix effect of human urine, and was very feasible. The limit of quantitation was 0.10 ng/ml. RIA detection following HPLC or IAE proved to be very useful for the pharmaceutical analysis of extremely low drug concentrations in body fluids. © 1997 Elsevier Science B.V.

Keywords: S-8921; Hypocholesterolemic agent

1. Introduction

A novel compound, methyl-1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6, 7, 8-trimethoxy-2-naphthoate (S-8921, Fig. 1), was found to have hypocholesterolemic activity in hamsters after oral administration [1] and is expected to exhibit a similar activity in humans. The hypocholesterolemic agent was thought to inhibit ileal Na^+/bile acid cotransporter resulting in the interruption of enterohepatic circulation of bile acids [1].

The intestinal absorption of this compound is small and, moreover, most of it is incorporated in the liver. Thus, the concentrations of the drug both in blood and urine are assumed to be very low. For investigation of the drug behavior in the human body, such as pharmacokinetic studies, it is necessary to develop a highly sensitive and specific assay. Radioimmunoassay (RIA) is suitable for its high sensitivity, but RIA may react with some cross-

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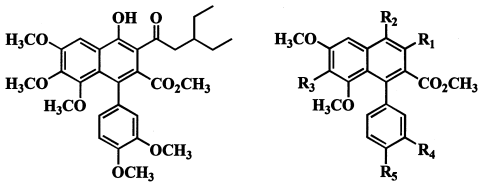

Fig. 1. Structure of S-8921 (left) and its derivatives (right). Derivatives are as follows (a position and a group substituted from S-8921, respectively); M-1 (R_1, -CO-CH_2CH(CH_2CH_3)-CH_2CH_2OH); M-3 (R_1, -CO-CH(OH)CH(CH_2CH_3)CH_2-CH_3); M-4 (R_3, -OH); M-5 (R_4, -OH); M-6 (R_5, -OH); sulfate conjugate (R_2, -O-sulfate); glucuronide conjugate (R_2, -O-glucuronide); short-chain hapten (R_3, -O-$(CH_2)_3$COOH); long-chain hapten (R_3, -O-$(CH_2)_6$COOH); and amino derivative for label (R_3, -O-$(CH_2)_3NH_2$). M-1, M-3, M-4, M-5, M-6 and the conjugates are the predicted metabolites of S-8921.

reactive metabolites as well as the unchanged drug. Furthermore, many other substances in biological fluids may interfere with the RIA, and matrix effects of urine samples, especially, are more complex between individuals than those of plasma samples.

In this study, we produced antibodies by immunizing rabbits, prepared radioiodine-labelled antigens and developed a sensitive competitive RIA. In order to eliminate the effect of cross-reactive metabolites, human plasma samples were purified with reversed-phase high-performance liquid chromatography (HPLC) before competitive RIA. The combined method of RIA after HPLC separation (HPLC–RIA) was expected to be much more specific and sensitive than conventional direct RIAs [2]. For the urine samples, immunoaffinity extraction (IAE) is thought to be most suitable for pretreatment before the RIA determination (IAE–RIA) because of its selectivity [3].

2. Experimental

2.1. Materials

S-8921 and its derivatives (Fig. 1) were obtained from Shionogi Research Laboratories. All chemicals were of analytical grade, unless otherwise specified.

2.2. Immunogen and antiserum

An immunogen, conjugate of S-8921 with bovine serum albumin (BSA, lyophilized and crystallized, Sigma, St. Louis, MO, USA), was prepared by the mixed anhydride method [4]. S-8921 carboxyl derivative (short chain hapten, R_3; -O-$(CH_2)_3$COOH in Fig. 1), 22.7 mg (38 μmol), was dissolved in 3 ml of dry dimethylformamide (DMF) with 10 μl of tri-*n*-butylamine (42 μmol) then 10 μl of isobutyl chloroformate (76 μmol) was added with vigorous mixing at 4°C. The mixture was stirred for 35 min at 4°C to produce active ester. A BSA solution was prepared by dissolving 50 mg (0.72 μmol) in 2.5 ml of water then adding the same volume of DMF, and the pH was adjusted to around 8.5 with 1 *M* sodium hydroxide. The active ester was slowly added to the BSA solution with stirring at 4°C, keeping the pH at around 8.5 by adding 1 *M* sodium hydroxide. The mixture was stirred for an additional 3 h at 4°C. The solution was dialyzed against 0.1 *M* sodium bicarbonate for 1 day at 4°C and then against distilled water several times. The conjugate solution was lyophilized to yield 65.2 mg and stored at 4°C. The number of S-8921 residues per BSA molecule was 14.3, determined by the trinitrobenzenesulfonic acid method [5], which can measure the free amino groups remaining on the conjugate.

Another derivative, with long chain (R_3; -O-$(CH_2)_6$COOH in Fig. 1), 24.3 mg, was also conjugated to BSA in the same manner and yielded 70.4 mg, and the binding ratio per BSA molecule was 11.9.

The immunogens were dissolved in saline and emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, MI, USA). Half a milliliter of the emulsion containing 0.3 mg of immunogen was injected intradermally into 15–25 sites on the back of each of six Japanese white rabbits (nos. K101, K102, and K103 for the short-chain hapten, and K104, K105, and K106 for the long-chain hapten). Immunization was repeated every 3 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-8921 amino derivative (Fig. 1), 5 μg (8.1 nmol), in 5 μl DMF, was added to a reaction tube which contained 9.25 MBq of ^{125}I-labelled Bolton–Hunter Reagent (a benzene solution from NEN,

Wilmington, DE, USA, which 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 50 μl of acetonitrile and eluted using an ODS column (YMC-AM-302, 150×4.6 mm I.D., YMC, Tokyo, Japan) under gradient conditions with 0.1% (v/v) trifluoroacetic acid (TFA) in water (mobile phase A) and acetonitrile (HPLC grade, Merck, Darmstadt, Germany) (mobile phase B). The gradient conditions of mobile phase B were 50% (v/v) in initial and 50–100% during 0.01–40 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 around 24 min of the retention time (t_R). The radioiodine-labelled antigen of the eluate was diluted 10 times with an RIA buffer, i.e. phosphate buffer (pH 7.0; 0.1 *M*) containing 0.5% (w/v) BSA, 0.25% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS) (Dojin, Kumamoto, Japan) and 0.1% (w/v) sodium azide and stored at 4°C until use.

2.4. RIA procedure

The labelled antigen solution, 100 μl (400 000 cpm ml^{-1}, diluted with the RIA buffer), was pipetted into each RIA tube (75×12 mm I.D., glass), which contained 200 μl of the RIA buffer and 110 μl of samples from human plasma after HPLC purification or standard solutions of S-8921. The standard solutions, 2–500 pg/tube, were prepared freshly with methanol–RIA buffer (1:10) from a stock standard solution which was dissolved in methanol and stored at −20°C. Next, 100 μl of diluted antiserum (1:45 000 of K101, diluted with the RIA buffer) was pipetted to each tube, and the mixture was incubated for 16 h at 20–25°C. This was followed by adding 100 μl suspension of immobilized anti-rabbit second antibody (Amerlex-M, Amersham International, Amersham, UK) to each tube. The tubes were centrifuged for 10 min at 2000 *g* after incubation for 2 h at 20–25°C. The supernatant portions of the tubes were aspirated off and the precipitates were washed with 1 ml of the RIA buffer. Centrifugation and aspiration were repeated, and the radioactivity of the residue in each tube was measured by gamma counter (ARC-600, Aloka, Tokyo, Japan). The amount of S-8921 in each RIA tube was estimated using a simultaneously obtained RIA standard curve.

In the case of human urine, we used 100 μl of samples from human urine after IAE, or 100 μl standard solution made with the RIA buffer containing 10% (v/v) methanol instead of 110 μl of sample or standard solutions.

2.5. HPLC purification of plasma samples

Plasma, 200 μl, was mixed with 600 μl of acetonitrile and the whole supernatant, after centrifugation at 2000 *g* for 5 min, was evaporated to dryness. The residue was redissolved in 150 μl of 50% (v/v) acetonitrile containing 0.1% (v/v) TFA. The sample solution, 100 μl, was injected into an HPLC instrument (LC-10AD system, Shimadzu, Kyoto, Japan) and eluted using an ODS semi-micro column (Develosil ODS-UG-5, 250×2.0 mm I.D., Nomura Sci., Tokyo, Japan) under gradient conditions with 0.1% (v/v) TFA in water (mobile phase A) and 0.1% (v/v) TFA in acetonitrile (mobile phase B). The gradient conditions of mobile phase B were 10% in initial, 55–95% during 0.01–45 min, 95% during 45–50 min, 95–100% during 50–52 min, and 100% during 52–60 min. The flow-rate was 0.2 ml/min before 39 min, 0.2–0.1 ml/min during 39–40 min, 0.1 ml/min during 40–55 min, 0.1–0.2 ml/min during 55–56 min and 0.2 ml/min after 56 min. The column temperature was 30°C. A fraction of the eluate, 0.5 ml, was collected from 45 to 50 min, evaporated to dryness using a centrifugal vacuum concentrator (SVC 100H, Savant, Farmingdale, NY, USA) and redissolved in 30 μl of methanol with vigorous mixing for 5 min, followed by adding 300 μl of the RIA buffer. The solution, 110 μl, in double was subjected to the RIA described above.

2.6. Preparation of immunoaffinity matrix

IgG fraction of antiserum (K105), 6 mg, purified with Protein A affinity column (MAPS-II kit, Bio-Rad, Richmond, CA, USA), was incubated with 0.5 g of activated matrix (Tresyl-5PW, Toso, Tokyo, Japan) in 6 ml of phosphate buffer (pH 8.0; 0.5 *M*) for 3 h at room temperature. After centrifugation for

5 min at 2000 *g*, the supernatant was aspirated off and the solid phase was washed with 30 ml of Tris–HCl buffer (pH 8.5; 0.1 *M*) three times, with 30 ml of phosphate buffer (pH 7.0; 0.1 *M*) three times, with 30 ml of acetonitrile containing 0.1% (v/v) trifluoroacetic acid three times, and with 30 ml of 0.2% (w/v) BSA in phosphate buffer (pH 7.0; 0.1 *M*) three times. The antibody-immobilized solid phase, that is, immunoaffinity matrix, was stored in 50 ml of 0.2% (w/v) BSA in phosphate buffer (pH 7.0; 0.1 *M*) at 4°C.

2.7. Immunoaffinity extraction of urine sample

Suspension of the immobilized antibody, 2 ml, was pipetted on a centrifugal filtration unit fitted in a tube (40×10 mm I.D., maximum sample volume, 2 ml; Ultrafree CL-GV, Millipore, Bedford, MA, USA) and centrifuged for 5 min at 2000 *g*. Then, the RIA buffer, 0.9 ml, and a human urine sample, 0.1 ml, which contained 2% of Tween 20, were applied on the filter unit where the immobilized antibody was collected. The mixture was incubated for 3 h at room temperature in the filter unit and centrifuged. The immobilized antibody was washed in the same filtration unit with 2 ml of phosphate buffer (pH 7.0; 0.1 *M*) three times and with 2 ml of distilled water twice. Then, S-8921 retained on the immobilized antibody was eluted by 0.5 ml of acetonitrile containing 0.1% (v/v) TFA. After centrifugation, the eluate was evaporated to dryness under a nitrogen stream at 50°C, dissolved in 30 μl of methanol, and then 270 μl of the RIA buffer was added. The solution, 100 μl, in double was subjected to the RIA described above. The immobilized antibody was reused at least 10 times after washing with acetonitrile containing 0.1% (v/v) TFA and phosphate buffer (pH 7.0; 0.1 *M*).

3. Results

3.1. Radioimmunoassay

Radioactive iodine was successfully introduced into an amino derivative of S-8921 by the Bolton and Hunter method [6], and the labelled antigen was highly purified by reversed-phase HPLC. The labeling yield of ^{125}I was extremely high, approx. 70%. The labelled antigen was made to react with six antisera derived from two immunogens from the short-chain and long-chain haptens (Fig. 1). There was no significant difference in the properties of antisera between the two immunogens. We selected K-101 antiserum from the short-chain hapten for its high titer, 18 500 (1/v), and good affinity. Competitive binding between the labelled antigen and standard S-8921 to the antiserum was excellent, as shown in the standard curve of Fig. 2, by which S-8921 could be measured sensitively from 2 to 500 pg per assay tube.

Fig. 2 also shows the displacement curves of some related compounds and their cross-reactivities. Metabolites, M-4, M-5, and M-6, isolated from rat bile, had considerable cross-reactivities but M-3 negligible activity, which suggests that the antibody mainly binds the valeryl structure but not the ring structure in S-8921.

3.2. HPLC purification of plasma samples

We tried to purify the deproteinized plasma by reversed-phase HPLC in order to eliminate the influences from plasma components and/or metabolites. The deproteinized plasma was satisfactorily

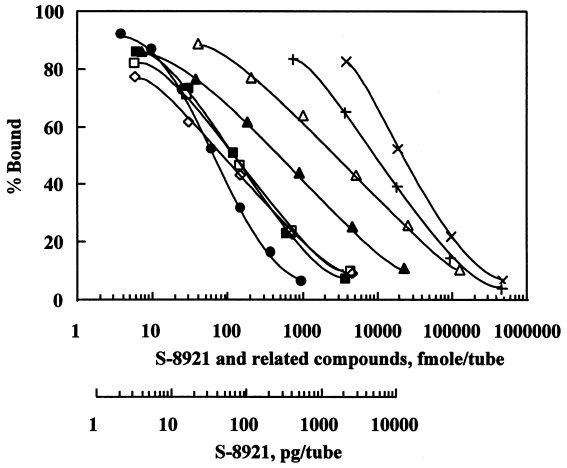

Fig. 2. RIA standard curve of S-8921 and displacement curves of related compounds. ●, S-8921 (100%); ▲, M-1 (11%); △, M-3 (2%); ■, M-4 (44%); ◇, M-5 (64%); □, M-6 (44%); +, sulfate (1%); ×, glucuronide (<1%). Percentages of cross-reactivity are in parenthesis. See structures of these compounds in Fig. 1.

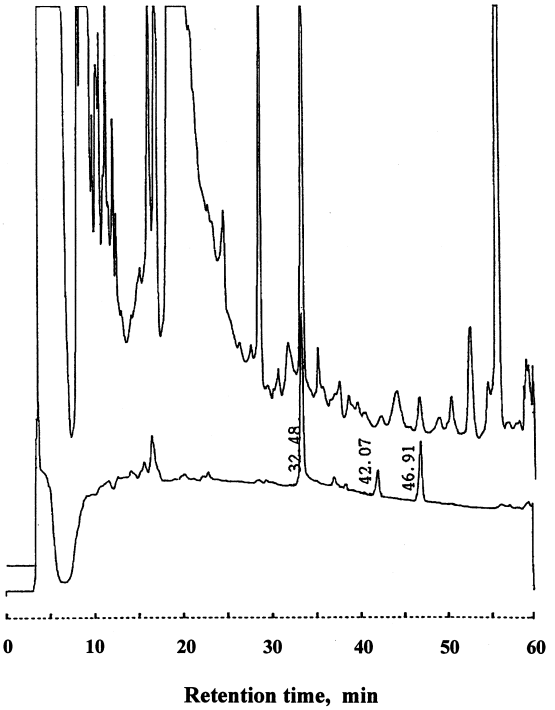

Fig. 3. Chromatogram of HPLC by UV detection at 274 nm. The lower trace was obtained from a standard mixture of S-8921 (10 ng, t_R=47 min) and M-6 (10 ng, t_R=42 min). A peak at 32.5 min was from the system. The upper trace was obtained from an extract of normal human plasma.

purified under the HPLC conditions described in Section 2, although chromatograms using ultraviolet detection (274 nm) in Fig. 3 show that some of the components in plasma were eluated around the same retention time (t_R) as S-8921 (47 min). Further, the cross-reactive metabolite, M-6, was separated from S-8921 sufficiently (t_R=42 min) and the others, M-4 and M-5, eluated before M-6. The purified fraction (t_R=45–50 min) from 500 μl of human plasma in an RIA tube could be measured by the RIA without any interference.

The relationship between the added (x ng ml^{-1}, 0.05–6 ng ml^{-1}) and the measured (y ng ml^{-1}) values of the HPLC–RIA was linear, $y=0.941x+0.27$ ($r=0.999$, $n=42$), and the recovery, 94.1%, was used to correct assay values. As shown in Table 1, the precisions and the accuracies were very good and the LOQ was 0.05 ng ml^{-1}.

In addition, S-8921 in human plasma was stable for 15 weeks at −40°C, and also stable during five freeze–thaw cycles (data not shown). Moreover, we did not find any differences in performances of the semi-micro column among three lots.

3.3. Immunoaffinity extraction of urine samples

We tried to extract S-8921 in urine by immunoaffinity centrifugal filtration in order to eliminate

Table 1
Precision and accuracy of HPLC–RIA for S-8921 in human plasma

	S-8921 added (ng ml^{-1})	S-8921 measured, mean±S.D. (ng ml^{-1})	Precision R.S.D. (%)	Accuracy bias (%)
Intra-assay (n=6)	0.05	0.053±0.005	9.4	+6.0
	0.1	0.105±0.009	8.6	+5.3
	0.3	0.277±0.011	4.0	−7.7
	1	0.890±0.057	6.4	−11.0
	3	2.75±0.11	4.0	−8.3
	6	5.97±0.37	6.3	−0.4
	10	10.52±0.60	5.7	+5.0
Inter-assay (n=6)	0.05	0.054±0.007	13	+8.0
	0.1	0.106±0.013	12	+6.0
	0.3	0.306±0.016	5.2	+2.0
	1	1.02±0.08	7.8	+2.0
	3	2.92±0.23	7.9	−2.7
	6	5.81±0.40	6.9	−3.2
	10	10.2±0.6	5.9	+2.0

Table 2
Precision and accuracy of IAE–RIA for S-8921 in human urine

	S-8921 added (ng ml^{-1})	S-8921 measured, mean±S.D. (ng ml^{-1})	Precision, R.S.D. (%)	Accuracy bias (%)
Intra-assay (n=6)	0.1	0.098±0.012	12	−2.0
	0.2	0.220±0.024	11	+10
	1.0	0.990±0.042	4.2	−1.0
	5.0	5.43±0.26	4.8	+8.6
	10	9.70±0.44	4.5	−3.0
Inter-assay (n=4)	0.25	0.251±0.034	14	+0.40
	1.0	1.03±0.17	17	+3.0
	5.0	5.54±0.26	4.7	+11

influences from urine matrix. S-8921 was satisfactorily purified under the conditions described in Section 2. The purified extract from 100 μl of human urine in a RIA tube could be measured without any interference.

The relationship between the added (x ng ml^{-1}, 0.1–10 ng ml^{-1}) and the measured (y ng ml^{-1}) values of the IAE-RIA was linear, $y=0.722x+0.14$ ($r=0.995$, $n=24$), and the recovery, 72.2%, was used to correct assay values. As shown in Table 2, the precisions and the accuracies were very good. The LOQ was estimated at 0.10 ng ml^{-1} although inter-assay validation data were not fully obtained around the LOQ.

3.4. Determination of S-8921 in human plasma and urine

S-8921 was administered orally to volunteers who gave us written consent and their plasma and urine were collected with some intervals. The concentrations in the plasma and urine were measured by the HPLC–RIA and IAE–RIA, respectively. The results are shown in Figs. 4 and 5.

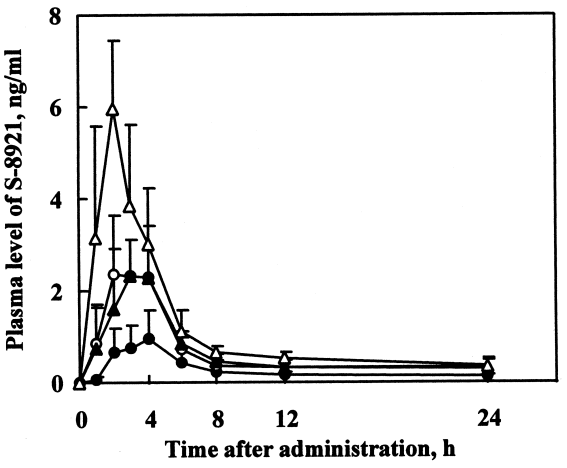

Fig. 4. Plasma levels of unchanged S-8921 after single oral administration to healthy volunteers. Each point represents the mean±S.D. ●, 2.5 mg per person (n=6); ○, 5 mg per person (n=6); ▲, 7.5 mg per person (n=5); △, 10 mg per person (n=5).

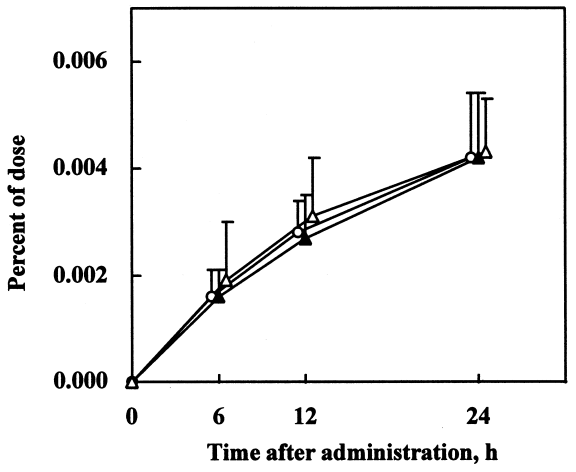

Fig. 5. Cumulative excretion of S-8921 immunoreactivity in urine after single oral administration to healthy volunteers. Each point represents the mean±S.D. ○, 5 mg per person (n=6); ▲, 7.5 mg per person (n=5); △, 10 mg per person (n=5).

4. Discussion

We have developed a competitive RIA for S-8921 using antiserum from rabbit and radioiodine-labelled S-8921. In order to measure the unchanged form of S-8921 in human plasma, we developed a specific HPLC–RIA which measured the HPLC fraction of the parent drug. The recovery, precision, and accuracy of the HPLC–RIA were excellent. Especially, HPLC–RIA was not only specific but also very sensitive, 0.05 ng ml^{-1} of LOQ [2]. A highly sensitive method was desired for clinical studies because doses per body weight of S-8921 in clinical studies were much lower than those in preclinical studies, especially at the first stage of phase 1 studies. The phase 1 study was started with the lowest dose, 2.5 mg of S-8921 per person, and the plasma levels were very low, below 1 ng ml^{-1}, and were successfully measured by HPLC–RIA as shown in Fig. 4.

HPLC–RIA is laborious and, thus, some improvement of the method has been expected. In this study, we introduced a semi-micro column which uses much less mobile phase than a conventional column, and the volume of the fraction that we collected was very small. Consequently, the evaporation time of the fraction was dramatically decreased. We may perform an HPLC–RIA without evaporation of the fraction, in cases of some improvement in HPLC conditions (much smaller flow-rate) and/or development of other RIAs that are hardly interfered with the mobile phase. In this study, the sample solutions, 110 μl, for RIA contained 10 μl of methanol with which S-8921 in the residue, after evaporation, was dissolved. The organic solvent lowered the percentage of antigen–antibody binding, but we cancelled the effect by adding the same amount of methanol to the standard solutions.

LOQ directly depends on the injection volume to the semi-micro column, which should preferably be more than 100 μl. We selected the gradient conditions described in Section 2, which concentrated S-8921 on the front of the column and separated the parent drug from the cross-reactive metabolite, M-6. We had to collect a wide fraction to avoid losing the analyte by adventitious variation of retention time. It was necessary for M-6 to be eluted at least 5 min earlier than the parent drug. Therefore, the total assay time became rather long and only 50 samples per week were processed. However, much of the time for the manual operation of the assay procedure was saved by using an autosampler and an autosample collector, i.e. a kind of column switching for the detector. Briggio et al. developed semi-automated HPLC–RIA which is a very sophisticated method using a two-parallel-column system and scintillation proximity RIA [7]. We did not investigate further improvement of our HPLC–RIA system as it is simple and labor-saving enough for our small scale assays.

Although an on-line immunoassay detection system with a fast and simple operation has been developed recently [8,9], measuring each fraction by RIA is much more sensitive and has wider applicability at this stage. Liquid chromatography–tandem mass-spectrometry (LC–MS–MS) is as sensitive and specific as HPLC–RIA and will be used more extensively in the field of pharmaceutical assay. However, it needs very expensive machines and extremely careful maintenance. We think RIA following HPLC fractionation is still valuable for drug analysis due to its high sensitivity, specificity and simplicity. Especially, if we can obtain an antibody which is specific for a certain group or residue in a drug structure, we will easily be able to develop a group-specific HPLC-RIA, having an identical assay procedure to the HPLC–RIA but without using any expensive machines.

The composition of urine largely depends on food and drink and varies in a more complex way than serum, in which major components are kept in homeostasis. The pretreatment of urine samples is very important for sensitive assay. We developed IAE–RIA for S-8921 in human urine samples. An IAE matrix was successfully prepared by the method described in Section 2; we had to wash the immobilized antibody entirely in order not to leak any antibody from the matrix, which seriously inhibits the immunoreaction of the next step. This method was very effective for reducing interferences, not only from urine components but also from Tween-20 that had been added to prevent adsorption of S-8921 to the walls of the sampling vessel. Immunoaffinity columns are usually packed with a large excess of immunoaffinity matrix, about 0.5 g per column, for rapid and quantitative extraction. Our procedure with

a centrifugal filtration tube used only 20 mg of immunoaffinity matrix per tube and more than 20 samples can be treated at once. This IAE pretreatment proved to be very useful for biological samples.

Although the measured values from IAE-RIA may include some cross-reactive metabolites, we did not separate unchanged S-8921. Because the urinary excretion had been expected to be so small, less than 0.01% of dose, that it was not very important to measure the unchanged form separately. As shown in Fig. 5, the cumulative excretion for 24 h in volunteer urine after oral administration was about 0.004% of dose and the drug was considered to be hardly excreted in the urine.

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