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Radioimmunoassay and gas chromatography/mass spectrometry for a novel antiglaucoma medication of a prostaglandin derivative, S-1033, in plasma

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Abstract

A radioimmunoassay (RIA) and a gas chromatographic/mass spectrometric (GC/MS) method for a new antiglaucoma medicament, the prostaglandin derivative sodium (5*Z*,9α,11α,13*E*)-9,11-dihydroxyprosta-5,13-dienoate (S-1033), in human and rabbit plasma were investigated. For a competitive RIA, antisera from rabbit and radioiodine-labeled S-1033 were prepared by immunizing a conjugate of S-1033 with bovine serum albumin and by the Bolton and Hunter method, respectively. Pretreatment by C_{18} solid-phase extraction (SPE) for rabbit plasma sample and further purification by high-performance liquid chromatography (HPLC) for human plasma samples followed by the RIA (SPE/RIA and HPLC/RIA, respectively) were developed. The assay recoveries of SPE/RIA and HPLC/RIA were both excellent and the limits of quantitation were 320 and 10 pg ml^{-1}, respectively. GC/MS for plasma samples after solid-phase extraction and thin-layer chromatographic purification was also developed using deuterium-labeled S-1033 as internal standard. The limit of quantitation was 100 pg ml^{-1} in human or rabbit plasma. Rabbit plasma samples after administration of this drug were measured by SPE/RIA and GC/MS and the assay results from both methods agreed well. The SPE/RIA, HPLC/RIA and GC/MS assay methods were suitable for measuring samples from preclinical studies, clincial studies and cross-validation, respectively.

Keywords: Prostaglandin derivative; Competitive radioimmunoassay; High-performance liquid chromatography; Gas chromatography/mass spectrometry; Human plasma; Rabbit plasma

1. Introduction

A new prostaglandin derivative, sodium (5*Z*,9α,11α,13*E*)-9,11-dihydroxyprosta-5,13-dienoate (S-1033) (Fig. 1), was found to lower intraocular pressure and is expected to be useful for the treatment of glaucoma [1]. When S-1033 is given by ocular administration, a small part of it is absorbed into the blood stream, and therefore its behavior in the body should be traced in preclinical and clinical studies. Its concentration was assumed to be very low, less than a few ng per ml of plasma at the maximum.

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Radioimmunoassays (RIA) 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 studying the pharmacokinetics and toxicokinetics of S-1033. However, several metabolites must be present in biological fluids and an RIA may not only react with unchanged drugs of interest but also cross-react with some metabolites. It is very difficult to estimate cross-reactivities with all the metabolites in biological samples. A combined method of HPLC separation followed by RIA should be more specific but an unchanged drug and some metabolites that have close retention times in HPLC may not be collected separately into different fractions. In this study, antibody was produced by immunizing rabbits, radioiodine-labeled antigen was prepared and a sensitive competitive RIA was developed. S-1033 in plasma samples from rabbit and human were pretreated by solid-phase extraction (SPE) and/or HPLC before RIA determination.

Gas chromatography/mass spectrometry (GC/MS) is one of the most reliable methods for both qualitative and quantitative analyses for drugs. Therefore, a GC/MS method was also developed for S-1033 in plasma and the assay results were compared for rabbit plasma by GC/MS with those obtained by RIA.

2. Experimental

2.1. Reagents

S-1033 and its derivatives (Fig. 1) were obtained from Shionogi Research Laboratories (Osaka, Japan). All chemicals were of analytical grade, unless specified otherwise.

2.2. Radioimmunoassay

2.2.1. Immunogen and antiserum

An immunogen, a conjugate of S-1033 with bovine serum albumin (BSA, lyophilized and crystallized; Sigma, St. Louis, MO) was prepared by the mixed anhydride method [2]. S-1033, 26 mg (76 μmol), was dissolved in 1 ml of dry dimethylformamide (DMF) with 20 μl of tri-*n*-butylamine (84 μmol), then 10 μl of isobutyl chloroformate (76 μmol) were added with vigorous mixing at 4°C. The mixture was stirred for 30 min at 4°C to produce active ester. A BSA solution was prepared by dissolving 50 mg (0.72 μmol) in 3.5 ml of water then adding the same volume of DMF, and the pH was adjusted to ca. 10 with 1 M sodium hydroxide. The active ester was slowly added to the BSA solution with stirring at 4°C, keeping the pH at ca. 8.5 by adding 1 M sodium hydroxide. The mixture was stirred for an additional 6 h at 4°C. The solution was dialyzed against 0.1 M sodium hydrogencarbonate for 1 day at 4°C and then against distilled water several

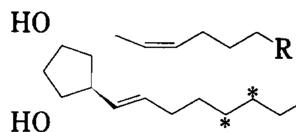

Fig. 1. Structure of S-1033 (R = COOH) and its amino derivative (R = $CONH(CH_2)_4NH_2$). Asterisks indicate positions of deuterium label ([$^2H_4$]S-1033).

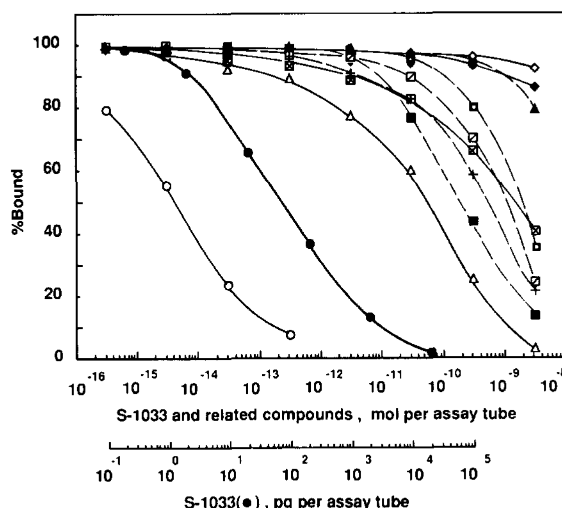

Fig. 2. RIA calibration curve for S-1033 and displacement curves for related compounds. ●, S-1033 (100); ○, amino derivative of S-1033 (8600); △, dinor form of S-1033 (0.61), ■, $PGF_{2\alpha}$ (0.14); +, 15-keto $PGF_{2\alpha}$ (0.03); ⍁, PGA_2 (0.02); ⊠, tetranor form of S-1033 (0.01); □, PGD_2 (0.01); ▲, thromboxane B_2 (< 0.01); ◆, PGB_2 (< 0.01); ◇, PGE_2 (< 0.01). Numbers in parentheses indicate cross-reactivity (%).

Table 1
Precisions and accuracies of SPE/RIA for S-1033 in rabbit plasma

S-1033 added (ng ml^{-1})	Intra-assay ($n = 4$)				Inter-assay ($n = 8$)			
	Mean (ng ml^{-1})	SD (ng ml^{-1})	RSD (%)	Bias (%)	Mean (ng ml^{-1})	SD (ng ml^{-1})	RSD (%)	Bias (%)
0.32	0.30	0.04	13	−6.3	0.30	0.04	13	−6.3
1.6	1.65	0.10	6.1	+3.1	1.63	0.11	6.7	+1.9
8.0	8.14	0.42	5.0	+1.8	7.84	0.47	6.0	−2.0
40	40.4	0.4	1.0	+1.0	39.3	2.4	6.1	−1.8

times. The conjugate was lyophilized and stored at 4°C. The number of S-1033 residues per BSA molecule was 47, determined by the trinitrobenzenesulfonic acid method [3], which can measure the free amino groups remaining on the conjugate.

The immunogen was dissolved in saline and emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, MI). A 0.5 ml volume of the emulsion containing 500 μg of immunogen was injected intradermally into 15–25 sites on the back of each of three Japanese white rabbits (Nos. F198, F199 and F200). Immunization was repeated every 3 weeks and whole blood was collected 10 days after the seventh immunization. The serum from each rabbit was stored at −20°C until use.

2.2.2. *Radioiodine-labeled antigen*

A trifluoroacetate salt of S-1033 amino derivative (Fig. 1), 4.17 μg (22 nmol), in 20 μl of DMF was incubated with 250 nmol of tri-*n*-butylamine solution for 3 h at 20–25°C. The mixture was added to a reaction tube which contained 37 MBq of ^{125}I-labeled Bolton and Hunter reagent (a benzene solution from NEN, Wilmington, DE, USA, was evaporated to dryness under a stream of dry nitrogen) [4]. The reaction mixture was stirred for 4 h at 20–25°C and then left to stand for 16 h at 4°C.

The reactant was injected into an LC-6A HPLC instrument (Shimadzu, Kyoto, Japan) after addition of 30 μl of acetonitrile and fractionated using an ODS column (Nucleosil $5C_{18}$, 150 mm × 4.6 mm i.d., particle size 5 μm; Machery–Nagel, Düren, Germany) with elution under gradient conditions with acetonitrile (HPLC grade; Merck, Darmstadt, Germany) in water, starting at 5% (v/v) for 5 min, from 5% to 55% over 30 min, at 55% for 10 min, from 55% to 100% over 5 min and at 100% for 10 min (total assay time, 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 a retention time of ca. 42 min. The radioiodine-labeled antigen of the eluate was lyophilized and stored at 4°C until use. The dried antigen in a vial was reconstituted with 10 ml of RIA buffer, i.e. phosphate buffer (pH 7.4; 0.01 M) containing sodium chloride (0.34 M), sodium azide (0.1%, w/v) and casein (0.5%, w/v), according to Hammarsten (Merck).

2.2.3. *RIA procedure*

The labeled antigen solution, 100 μl (200 000 cpm ml^{-1}, diluted with the RIA buffer), was pipetted into each RIA tube (75 mm × 12 mm i.d., made of glass), which contained 800 μl of samples from human plasma after HPLC purification or standard solutions of S-1033 (0.3–5000 pg per tube). Next, 100 μl of diluted antiserum (1:300 000 of F199-7, diluted with the RIA buffer) were pipetted to each tube and the mixture was incubated for 16 h at 20–25°C. This was followed by adding to each tube 100 μl of immobilized anti-rabbit second antibody (a 1 mg ml^{-1} suspension of Immunobeads; Bio-Rad, Richmond, CA). The tubes were centrifuged for 5 min at 2000*g*

Table 2
Precisions and accuracies of HPLC/RIA for S-1033 in human plasma

S-1033 added ($pg\ ml^{-1}$)	Intra-assay ($n = 5$)				Inter-assay ($n = 5$)			
	Mean ($pg\ ml^{-1}$)	SD ($pg\ ml^{-1}$)	RSD (%)	Bias (%)	Mean ($pg\ ml^{-1}$)	SD ($pg\ ml^{-1}$)	RSD (%)	Bias (%)
10.0	10.5	1.5	14	+5.0	10.6	2.1	20	+6.0
40.0	41.3	3.5	8.5	+3.3	40.6	5.4	13	+1.5
100	102	7	6.9	+2.0	103	10	9.7	+3.0
500	500	24	4.8	0.0	505	33	6.5	+1.0

after incubation for 2 h at 20–25°C. The supernatant portions of the tubes were aspirated off and 500 μl of the RIA buffer containing Tween 20 (0.1%, w/v) instead of casein were added to each tube. Centrifugation and aspiration were repeated, and the radioactivity of the residue in each tube was measured with a gamma counter (ARC-600; Aloka, Tokyo, Japan). The amount of S-1033 in each RIA tube was estimated using an RIA calibration curve obtained simultaneously.

In the case of rabbit plasma assay, we used 300 μl of samples from rabbit plasma after SPE or 300 μl of standard solution which contained the same amount of extract from normal rabbit plasma as those from unknown plasma sample. The other assay procedures were the same as described above.

2.2.4. Solid-phase extraction of plasma sample for RIA

A human plasma sample (1 ml), was mixed with 1 ml of citrate buffer (pH 3.0; 0.1 M) and applied to a pretreatment column for SPE (Bond Elut C_{18}, 500 mg type; Varian, Harbor City, CA) after washing with 10 ml of ethanol and 10 ml of distilled water. Next, the column was washed with 10 ml of water, 6 ml of 20% (v/v) ethanol and 10 ml of light petroleum. The retained S-1033 was eluted with 6 ml of ethyl acetate and the solvent was evaporated to dryness under a stream of nitrogen at 70°C. The residue from the human plasma sample was dissolved in 550 μl of the HPLC mobile phase for further purification as described in the next section.

A rabbit plasma sample (0.5 ml) was mixed with 0.5 ml of citrate buffer (pH 3.0; 0.1 M) and extracted as described above. The residue extracted from rabbit plasma sample was dissolved in 2 ml of the RIA buffer for the RIA.

2.2.5. HPLC purification for RIA

After the solution had been prepared by SPE from a human plasma sample as described above, 400 μl were injected into the HPLC instrument and fractionated using an ODS column (Inertsil ODS-2, 150 mm × 4.6 mm i.d., particle size 5 μm; GL Science, Tokyo, Japan), eluting under isocratic conditions with 45% (v/v) acetonitrile and 0.03% (v/v) acetic acid in water. The flow rate was 1 ml min^{-1} and the column temperature was not controlled. Fractions of the eluate were collected in the RIA tubes every 1 min for 13 to 20 min and directly evaporated to dryness under a stream of nitrogen at 70°C and the residue was dissolved in 800 μl of the RIA buffer.

2.3. Gas chromatography/mass spectrometry

2.3.1. GC/MS conditions

A MAT 44S mass spectrometer coupled to a Model 3700 gas chromatograph (Varian, Bremen, Germany) was used. The mass spectrometer was operated in the negative ion chemical ionization (NICI) mode and the ions at m/z 481 and 486 were monitored for S-1033 and the internal standard [$^{2}H_{4}$]S-1033, respectively. The gas chromatograph was equipped with a 23 m × 0.32 mm i.d. fused-silica capillary column with a 0.1 μm film thickness (MP-65-HT; Quadrex, New

Haven, CT). The injector was a moving needle injector (laboratory made). The operating temperatures were 270°C in the injector, 230°C in the column oven, 220°C in the transfer line and 160°C in the ion source. The carrier gas was helium (1.8×10^5 Pa) and the reagent gas was ammonia (45 Pa). The retention times of S-1033 (PFB-TMS) and [$^2H_4$]S-1033 (PFB-TMS) were 340 and 337 s, respectively.

2.3.2. Pretreatment of plasma samples for GC/MS

A human plasma (or rabbit plasma) sample (1 ml) was mixed with 100 μl of the internal standard solution ([$^2H_4$]S-1033, 1.00 μg ml^{-1} ethanol solution) and 1.8 ml of 10% (v/v) acetic acid. Then the solution was applied to a preconditioned SPE column (Bond Elut C_{18}, 500 mg type). After washing with 3 ml of 4% (v/v) acetic acid and then with 2.8 ml of methanol–water (1:1, v/v), the column was dried followed by washing with 2.8 ml of hexane. S-1033 was eluted with 2.8 ml of ethyl acetate. The eluate was evaporated and the residue was dissolved in 50 μl of 20% (v/v) diisopropylethylamine (DIPEA) in acetonitrile and 20 μl of pentafluorobenzyl bromide (PFBBr) were added to the solution. After shaking for 45 min at room temperature, the excess reagents were removed under a stream of nitrogen and the residue was dissolved in ethyl acetate and subjected to thin-layer chromatography (silica gel precoated plate, Art. 5715; Merck). After development with diethyl ether, S-1033 PFB ester was scraped off (R_f 0.25, ± 12 mm) and extracted with 10 ml of ethyl acetate–propan-2-ol (9:1, v/v). The extract was evaporated to dryness under vacuum and to the residue in a mini-vial 30 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were added. The solution was heated for 1 h at 80°C then subjected to GC/MS determination.

3. Results

3.1. Radioimmunoassay

Radioactive iodine was successfully introduced into an amino derivative of S-1033 by the Bolton and Hunter method and the labeled antigen was highly purified by HPLC. The labeling yield of ^{125}I was extremely high, 70–80%. The labeled antigen was made to react with antisera derived from the BSA conjugate of S-1033. F-199 antiserum after the seventh immunization (F-199-7) was selected owing to its high titer and good affinity.

Competitive binding between the labeled antigen and standard S-1033 to the antiserum was good, but the binding was strongly affected by various assay conditions, such as the carrier protein, surfactant agent and ionic strength. The composition of the RIA buffer was carefully examined and the assay conditions described under Experimental were selected. Fig. 2 shows the calibration curve from which S-1033 could be measured sensitively at levels from 3 to 5000 pg per assay tube.

Fig. 2 also shows the displacement curves of some related compounds and their cross-reactivities. The starting material of the labeled antigen, the amino derivative of S-1033, has a very high reactivity towards the antiserum. It is thought that some antibodies against the coupling structure between a carboxyl group of the hapten and an amino group of lysine in BSA molecule were produced in the antiserum and reacted with an identical part in the ^{125}I-labeled amino derivative, which resulted in a relatively gentle slope of the calibration curve for S-1033. The cross-reactivities for some endogenous prostaglandins, especially $PFG_{2\alpha}$ which has a similar structure to S-1033, were very low and the RIA was not affected by endogenous prostaglandins in plasma.

3.2. Solid-phase extraction for RIA

S-1033 was extracted from rabbit plasma samples by the SPE method according to Powell [5] before the RIA (SPE/RIA). The extraction yield was more than 90% as estimated using [^{14}C]S-1033. Interference from rabbit plasma components with the RIA, especially inhibition by rabbit IgG to the separation procedure between antibody-bound labeled antigen and free labeled antigen (B/F separation), was negligible when using less than 25 μl of the extract. As shown in Table

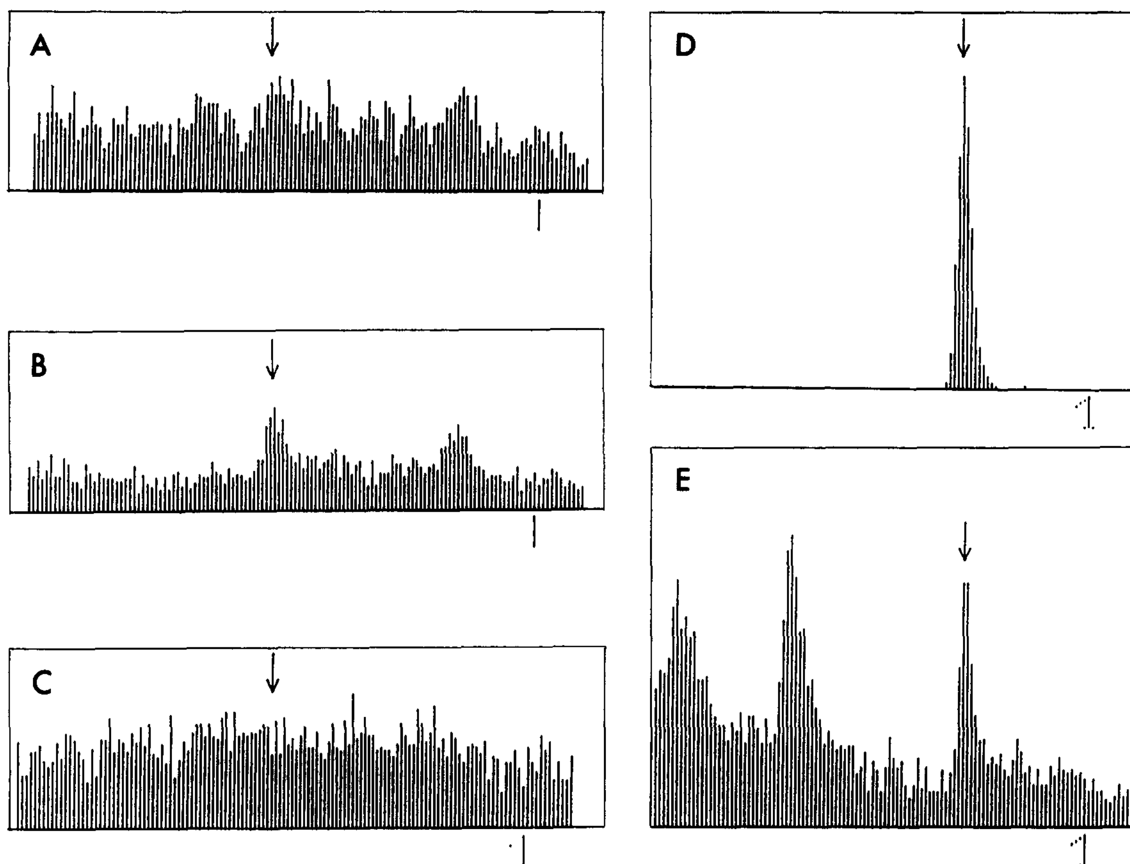

Fig. 3. Mass fragmentgram of S-1033 in plasma. Rabbit plasma blank (A, m/z 486, attenuation 2; B, m/z 485, attenuation 4; C, m/z 481, attenuation 2) and S-1033 in human plasma (D, m/z 486 of $[^2H_4]$S-1033, attenuation 1024; E, m/z 481 of S-1033, attenuation 8).

1, the precisions and accuracies were good enough to validate this SPE/RIA. The limit of quantitation (LOQ) in plasma was 0.32 ng ml^{-1}.

3.3. HPLC purification for RIA

Attempts were made to purify the plasma extract by reversed-phase HPLC in order to eliminate the influence from the plasma components or metabolites and also to load more plasma extract with the RIA (HPLC/RIA). The plasma extract was satisfactorily purified under the HPLC conditions described under Experimental. The chromatogram of the immunoreactivity showed one peak which identified S-1033 by its retention time, 16.5 min. The purified extract from 727 μl of human plasma in an RIA tube could be measured without the interference.

The relationship between the added (x pg ml^{-1}, 10–1000 pg ml^{-1}) and the measured (y pg ml^{-1}) values in HPLC/RIA was linear, $y = 0.99x + 0.78$ ($r = 0.999$, $n = 32$), and the difference between them was not significant according to regression analysis ($p < 0.01$). The recovery for this assay was considered to be 100%. As shown in Table 2, the precisions and accuracies were very good and the LOQ was 10 pg ml^{-1}.

When the sampling volume of plasma or the HPLC injection volume of the plasma extract was decreased, the measured values of this assay decreased proportionally. The suitable volume for each step must be selected for high-level samples. Moreover, S-1033 in human plasma was found to be stable for over 12 weeks when stored at −40°C.

Table 3
Inter-assay precisions and accuracies of GC/MS for S-1033 in rabbit and human plasma

S-1033 added (ng ml^{-1})	Rabbit plasma ($n = 5$)				Human plasma ($n = 5$)			
	Mean (ng ml^{-1})	SD (ng ml^{-1})	RSD (%)	Bias (%)	Mean (ng ml^{-1})	SD (ng ml^{-1})	RSD (%)	Bias (%)
0.10	0.092	0.011	12	−8.0	0.083	0.010	12	−17
0.20	0.206	0.015	7.2	+3.0	0.19	0	0	−5.0
0.30	0.308	0.016	5.2	+2.7	0.288	0.010	3.5	−4.0
0.50	0.538	0.031	5.8	+7.6	0.538	0.019	3.5	+7.6
1.00	1.05	0.06	5.7	+5.0	1.04	0.04	3.8	+4.0
5.00	5.20	0.17	3.3	+4.0	5.18	0.04	0.8	+3.6
10.0	10.2	0.2	2.0	+2.0	10.3	0.1	1.0	+3.0
50.0	49.8	0.7	1.4	−0.4	50.4	1.5	3.0	+0.8

3.4. Selected-ion monitoring

In each mass spectrum of S-1033 (PFB-TMS) and [$^2H_4$]S-1033 (PFB-TMS) obtained in the GC/NICI mode, the fragment $[M - PFB]^-$ was observed as the base peak and the fragment $[PFB]^-$ of m/z 181 as a very weak peak. The background of the mass fragmentogram of normal rabbit plasma sample and a typical mass fragmentogram for the determination of S-1033 in human plasma sample are shown in Fig. 3. The chromatogram of the ion at m/z 485 ([$^2H_4$]S-1033 (PFB-TMS)-PFB) showed existence of an interference peak from rabbit plasma. We chose m/z 486 $[M - PFB^{+1}]^-$, a weaker peak, as the monitoring mass for the internal standard.

The calibration curves for rabbit plasma samples were found to be linear over the range 0.1–50 ng ml^{-1} ($y = 0.0372x - 0.00006$, $r = 0.9991$, $n = 12$, where x is the concentration of S-1033 (ng ml^{-1}) and y is the internal standard ratio). The inter-assay precisions and accuracies of GC/MS were very good, as shown in Table 3, and the LOQ was 100 pg ml^{-1}. The sensitivity would become better if a larger amount of sample were loaded into the MS system. However, we used the assay conditions described under Experimental to avoid heavy contamination of the instrument.

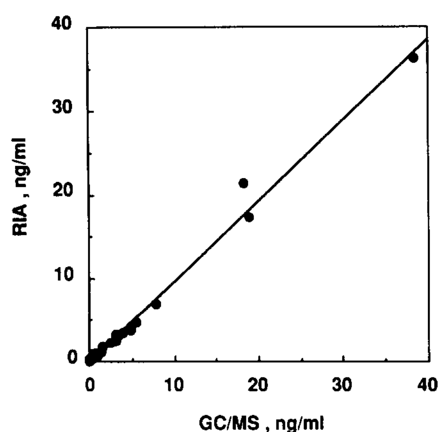

Fig. 4. Correlation between GC/MS and SPE/RIA for S-1033 in rabbit plasma samples. $y = 0.967x - 0.0644$, $r = 0.9996$, $n = 29$, where x (ng ml^{-1}) = value obtained by GC/MS and y (ng ml^{-1}) = value obtained by SPE/RIA.

3.5. Comparison of RIA with GC/MS

S-1033 in amounts of 50–100 μg was administered ocularly to 12 female rabbits and blood was collected at several points in time. Each sample of the rabbit plasma was measured by SPE/RIA and GC/MS. Fig. 4 shows a good correlation between the results from both methods, with no significant difference according to regression analysis ($p < 0.01$).

4. Discussion

A competitive RIA was developed for the unchanged form of S-1033 using antiserum from rabbit and radioiodine-labeled S-1033. In order to

measure S-1033 in plasma, it was necessary to extract S-1033 from a plasma sample using a reversed-phase SPE column (SPE/RIA). We also developed a specific HPLC/RIA which measures only the HPLC fraction of the parent drug. The recoveries, precisions and accuracies of both SPE/RIA and HPLC/RIA were found to be excellent. Surprisingly, HPLC/RIA was much more sensitive, with an LOQ of 10 pg ml^{-1}, than SPE/RIA, 320 pg ml^{-1}. HPLC could eliminate some components that remained in plasma extract which disturb immunoreaction and improve the sensitivity with an increase in the amount of sample loaded into the RIA tube.

A highly sensitive method was desired for clinical studies because the doses per unit body weight of S-1033 in clinical studies were much lower than those in preclinical studies, especially in the first state of phase 1 studies. Plasma levels of S-1033 in phase 1 studies were successfully measured by HPLC/RIA and the drug was found to adsorb rapidly in the bloodstream. The details will be presented elsewhere. However, HPLC/RIA is laborious and one operator can treat only 30 plasma samples in 3 working days. As SPE/RIA is more efficient, handling 50 samples in 2 working days, it was used for mass routine assays in preclinical studies.

Both quantitative and qualitative analysis by GC/MS is the most reliable approach in many sensitive analytical methods for drugs because it can discriminate just one chemical species. A GC/MS method was successfully developed here for determining plasma levels of unchanged drug. The limit of quantitation was 100 pg ml^{-1} in plasma, which was more sensitive than SPE/RIA but less sensitive than HPLC/RIA. GC/MS could not used for routine assays because of the complicated pretreatment and troublesome maintenance of the instrument. Quantitative GC/MS is useful for important and limited samples in preclinical studies and, especially, for validation of RIA [6].

Rabbit plasma samples after administration of S-1033 were measured by both SPE/RIA and GC/MS and the two methods showed no significant difference. The concentrations in most plasma samples from early phase 1 clinical studies were too low to measure by GC/MS. Although the assay values for human plasma samples obtained by HPLC/RIA, which has some possibility of measuring unseparated cross-reactive substances, could not be compared with those obtained by GC/MS, HPLC/RIA is much more specific than SPE/RIA and hence fewer problems would be found in HPLC/RIA than SPE/RIA.

In conclusion, three sensitive assays were developed, SPE/RIA for preclinical studies, HPLC/RIA for clinical studies and GC/MS for cross-validation, and could be used effectively for the development of the new drug S-1033.

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