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Radioimmunoassay method for DX-9065a, an anticoagulant agent. Development, evaluation and application to human plasma

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Abstract

A simple and sensitive radioimmunoassay (RIA) method was developed for determination of DX-9065a, (+)-(2*S*)-2[4-[[(3*S*)-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-[7-amidino-2-naphthyl]propanoic acid hydrochloride pentahydrate, a newly synthesized anticoagulant agent. Immunogens were prepared by condensation of a hapten with bovine serum albumin via a carboxyl group. Antisera was obtained by immunization of five rabbits with immunogen. High-titer antisera was obtained from 2 rabbits immunized with immunogen. The sensitivity of this newly developed RIA method was 100-fold greater than that of a previously used conventional HPLC method. This method was validated for determination of human plasma samples in clinical trials. The cross-reactivities of employed antisera with three steroisomers (2*R*3*R*-, 2*R*3*S*- and 2*S*3*R* forms) were 0.7, 20.2 and 43.9% respectively. The effect of cross-reactivity of postulated stereoisomers in clinical samples was evaluated by a parallelism study using human plasma samples obtained after oral administration of the drug to healthy Japanese volunteers. Results showed no effect on measured concentration. From these data, this method showed suitable accuracy and precision for the pharmacokinetic evaluation of DX-9065a in clinical study.

The method was applied to plasma samples obtained from a healthy Japanese volunteer who had orally received 12.85 mg (10 mg as DX-9065) of the drug. The maximum plasma concentration measured was 6.2 ng ml^{-1} 1 h after administration.

Keywords: Anticoagulant agent; DX-9065a; Human plasma; Radioimmunoassay; Stereoselectivity

1. Introduction

DX-9065a, (+)-(2*S*)-2-[4-[[(3*S*)-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-[7-amidino-2-naphthyl]propanoic acid hydrochloride pentahydrate, is an orally effective anticoagulant drug newly synthesized in this laboratory. It has specific inhibition activity toward factor Xa in plasma. The drug is therefore expected to be useful in the treatment of several thrombotic disorders such as

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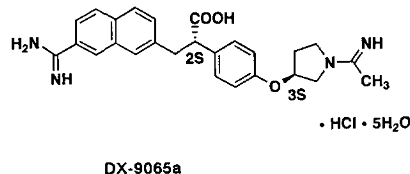
unstable angina, deep vein thrombosis and disseminated intravascular coagulation. As DX-9065a has strong activity at a low plasma concentration of 20 ng ml^{-1} [1], a sensitive method is essential for determination of the drug in tissues. However, as this drug does not have a strong fluorescent or UV absorbing group in its structure, the quantification limit obtained using a UV-detection high performance liquid chromatography (HPLC) method was only 100 ng ml^{-1} in plasma. Determination of plasma drug concentration after therapeutic dosing using conventional HPLC methods was almost impossible.

The development of a simple and sensitive method for the determination of plasma DX-9065 using radioimmunoassay (RIA) is reported in this paper. The stereospecificity of the method, a validation study and application of the method to human samples for pharmacokinetic evaluation of the drug in a clinical study are also described.

2. Experimental

2.1. Reagents

DX-9065a (Fig. 1) was synthesized in this laboratory. All concentration data in this report are expressed as DX-9065, 2*S*-2-[4-[[3*S*)-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-[7-amidino-2-naphthyl]propanoic acid. DX-9065 represents the anhydrous free base of DX-9065a. Three stereoisomers (2*R*3*R*-, 2*R*3*S*- and 2*S*3*R* forms) were synthesized in this laboratory. Bovine serum albumin (BSA) and Freund's complete adjuvant were purchased from Seikagaku Corporation (Tokyo, Japan). Dulbecco's phosphate-buffered saline (PBS) (−) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Sodium azide (NaN_3) was purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Dextran T 70 was purchased from Pharmacia Biotech (Uppsala, Sweden). Charcoal (Norit SX-II) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and solvents were commercially obtained and of reagent grade.



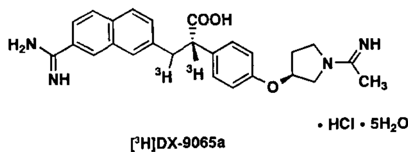


Fig. 1. Chemical structures of DX-9065a and [^3H]DX-9065a.

2.2. Animals

Japanese white male rabbits (9 weeks, $n = 5$, Oriental Co. Ltd, Tokyo, Japan) were used for immunization.

2.3. Apparatus

Melting points were measured with a Yanagimoto MP-1 melting point apparatus (Kyoto, Japan) and are uncorrected.

Electron impact mass spectra were obtained with a JEOL JMS-HX-110 (Japan Electron Optics Laboratory Ltd., Tokyo, Japan) instrument and fast atom bombardment mass spectra were obtained with a JMS-AX505W (Japan Electron Optics Laboratory Ltd., Tokyo, Japan) instrument.

^1H-NMR spectra were obtained with a JEOL GSX-500FT-NMR spectrometer (Japan Electron Optics Laboratory Ltd., Tokyo, Japan) using Me_4Si as an internal standard.

Amino acid analysis was carried out by the *o*-phthalaldehyde method using HPLC (Shimadzu Corporation, Kyoto, Japan).

Radioactivity was measured by a liquid scintillation system LS-6000TA (Beckman, USA).

2.4. Preparation of immunogen

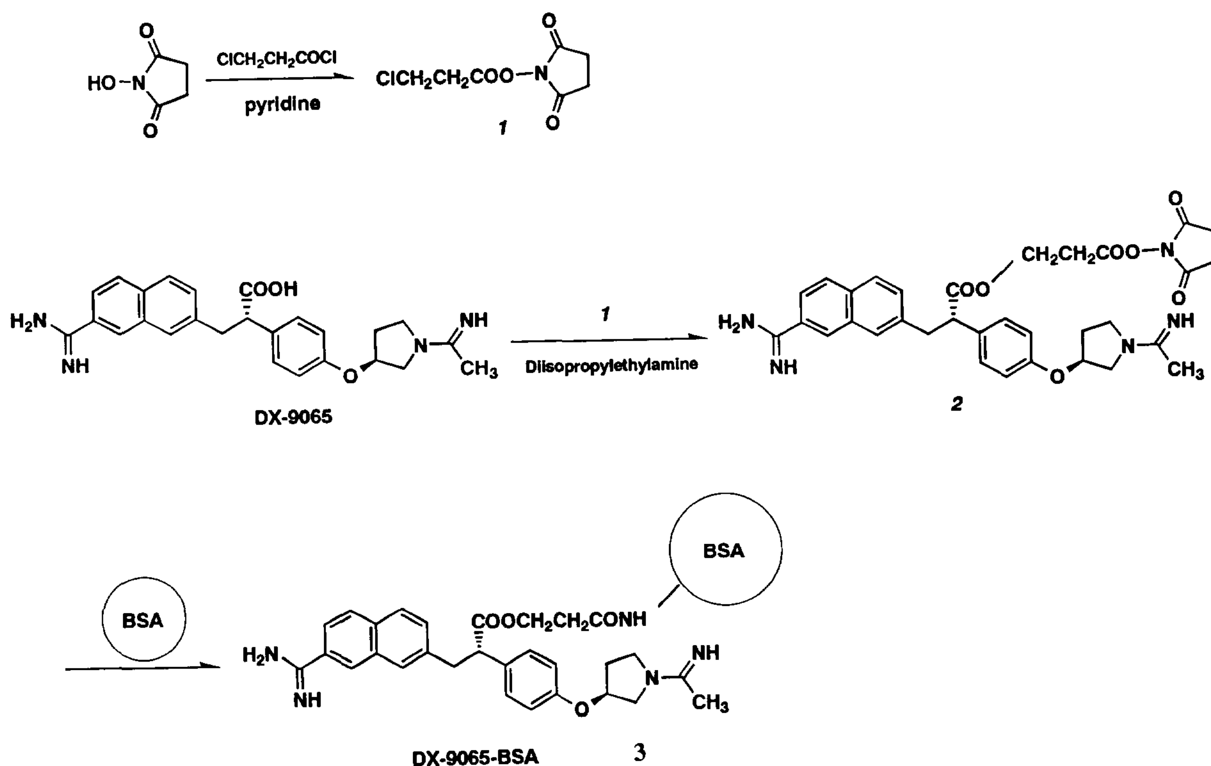
The immunogen was prepared according to the following three steps (Scheme 1).

2.4.1. Synthesis of β-chloropropionic acid succinimide ester (1)

β-Chloropropionyl chloride (2.79 ml, 22 mmol) was added to a solution of N-hydroxy succinimide (2.3 g, 20 mmol) in pyridine (1.74 ml, 22 mmol), and the solution was stirred at 4°C for 12 h. After the addition of ice-cold water (150 ml), the mixture was extracted with ethyl acetate (2 × 150 ml). The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The residue was recrystallized from acetone to give 3.25 g of β-chloropropionic acid succinimide ester (**1**), m.p. 52–54°C. ^{1}H-NMR($CDCl_3$) δ: 2.85 (4H, broad singlet, succinimidyl CH_2CH_2), 3.11(2H, t, $COCH_2$), 3.83 (2H, t, CH_2Cl). Anal. calcd for $C_7H_8NO_4Cl$: C, 40.9; H, 3.9; N, 6.1. Found: C, 40.5; H, 4.1; N, 6.1. MS m/z: 206 ($[M+H]^+$).

2.4.2. Synthesis of DX-9065-BSA conjugate (3)

A solution of 20% (v/v) of diisopropylethylamine in AcOEt (2.2 ml) and a solution of **1** (80 mg, 0.577 mmol) in AcOEt (1.5 ml) were added to a DX-9065 solution (200 mg, 0.525 mmol) in absolute DMSO (10 ml) and stirred at room temperature for 12 h. Compound **2**, 3-[(2,5-dioxopyrrolidinyl)oxy]-3-oxypropyl (+)-(2S)-2-[4-[[(3S)-1-acetimidoyl-3-pyrrolidinyl]oxy[phenyl]-3-[7-amidino-2-naphthyl]propionate, was obtained as a crude product and used as the starting material for the next reaction without any purification due to its instability. AcOEt was then evaporated and residual solution was added to a solution of BSA (490 mg, 7 μmol) in 50 mM phosphate buffer (pH 7.4, 10 ml) and stirred at 4°C for 2 days. The reaction solution was poured into ice-



Scheme 1. Preparation of immunogen.

cooled acetone and stirred. The resulting solution was centrifuged at 1700*g* for 20 min, and the precipitant obtained was emulsified in water and dialyzed against cold running water for 4 days using seamless cellulose tubing. The emulsion was then freeze dried to give the DX-9065–BSA conjugate (immunogen) as a fluffy powder. This conjugate was stored at 4°C and used without further purification.

2.5. Measurement of molar ratio of hapten molecule/BSA

The number of hapten molecules linked to a BSA molecule was measured by the method of Ebata et al. [2] as described below. This method includes a deamination reaction of free ε-amino groups of lysine residues by nitrite in acidic medium. 17 mg of a dried DX-9065-BSA conjugate was dissolved in 5 ml of 80% acetic acid. 1 ml of water and 1 ml of 30% $NaNO_2$ were then added to the conjugated solution at 0–4°C and the reaction mixture was stirred for 2 h at room temperature. After 2 h, a further 1 ml of 30% $NaNO_2$ was added and the mixture was stirred continuously for an additional 2 h under the same conditions. After the reaction was finished, the mixture was diluted by adding 4 ml of water, and dialyzed against water for 24 h. The deaminated and dialyzed conjugate was then lyophilized and dried in a dessicator.

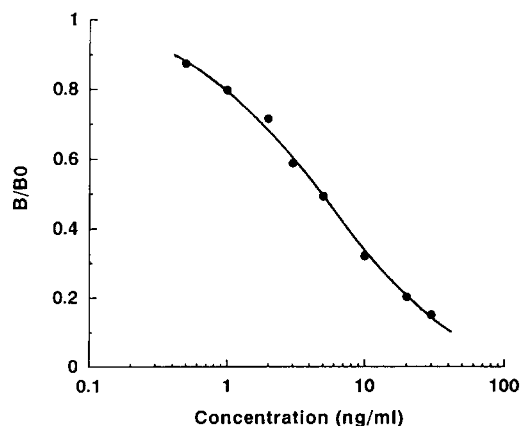


Fig. 2. Standard curve for DX-9065 in human plasma.

The deaminated conjugate was then hydrolyzed for amino acid analysis by constant boiling in hydrochloric acid at 100°C for 24 h. In the analysis, the lysine content of the conjugate could be estimated as mole residues per mole of protein based on the content of other amino acids such as aspartic acid, glycine, alanine and phenylalanine, by comparison with the amino acid content previously obtained from standard analyses of the

Table 1
Intra-assay variation of DX-9065 standard curve back-fit values

Theoretical concentration (ng ml^{-1})	Measured concentration (ng ml^{-1})							
	Assay No.					Mean ± S.D.[a]	RSD (%)	Error (%)
	1	2	3	4	5			
0.5005	0.4494	0.5478	0.5253	0.4864	0.5209	0.5022 ± 0.0434	8.6	0.3
1.001	1.095	0.9952	0.9841	1.130	1.033	1.051 ± 0.072	6.9	5.0
2.002	1.916	1.890	1.964	1.766	1.717	1.884 ± 0.084	4.5	−5.9
3.003	–[b]	3.107	3.155	3.085	3.192	3.116 ± 0.036	1.1	3.8
5.005	5.209	5.052	4.901	5.179	4.881	5.085 ± 0.140	2.8	1.6
10.01	9.696	–[b]	9.840	10.24	10.45	9.925 ± 0.282	2.8	−0.8
20.02	19.33	20.73	21.70	19.46	20.22	20.31 ± 1.12	5.5	1.4
30.03	31.36	28.95	28.27	29.96	29.29	29.64 ± 1.34	4.5	−1.3

[a]Mean of five assays.
[b]Rejected by "Immunofit".

Table 2
Inter-assay variation of QC parameters of calibration curve

Parameter	Calibration curve no.					Mean ± S.D.	RSD (%)
	1	2	3	4	5		
%NSB/T	3.43	3.47	3.61	3.10	3.45	3.41 ± 0.19	5.5
%B_{max}/T	91.8	90.6	90.4	92.6	91.6	91.4 ± 0.9	1.0
ED_{50} [a]	6.36	5.78	6.18	6.13	6.10	6.11 ± 0.21	3.5
M.D.D. [b]	0.236	0.151	0.137	0.148	0.148	0.164 ± 0.041	24.8
$B_{max}(A)$ [c]	3917	4090	3939	4092	4161	4040 ± 106	2.6
Slope (B) [c]	1.08	1.01	0.948	0.973	0.994	1.00 ± 0.05	5.2
Mid Pt. (C) [c]	5.71	4.55	4.97	5.09	4.79	5.02 ± 0.44	8.7
B_{min} (D)	−4.28	−5.67	−9.12	−8.27	−6.91	−6.87 ± 1.91	−27.8
Correlation	0.999	0.999	0.999	0.998	0.999	0.999 ± 0.001	0.1

[a] Dose at 50% response (ng ml^{-1}).
[b] Minimum detection dose (ng ml^{-1}).
[c] Parameters: $Y = (A - D)/(1 + (X/c)^B) + D$.

carrier protein. The remaining lysine of the conjugate thus indicated the fraction of lysine protected from deamination by conjugation with DX-9065.

2.6. Preparation of anti-DX-9065 antisera

DX-9065-BSA (immunogen) was added to saline (7 mg per 2 ml) and emulsified in Freund's complete adjuvant (5 ml). The emulsion was injected subcutaneously at multiple sites in each of five male rabbits at an amount of 1 mg (converted into protein weight) of DX-9065-BSA. Immunization was carried out eight times at 2 week intervals. 2 weeks after each immunization, 1–2 ml of blood was taken to measure to antibody titer. 2 weeks after the last booster injection, blood was collected, allowed to stand at room temperature for a few hours, and then centrifuged at 1700g for 10 min. The obtained supernatant was subdivided into amounts of 0.9 ml and lyophilized. These were stored at −80°C as anti-DX-9065 antisera until assay.

2.7. Radioligand

[^{3}H]DX-9065a (138 GBq $mmol^{-1}$) was synthesized at Daiichi Pure Chemical Co., Ltd. and used as a radioligand for assay (Fig. 1). Radiochemical purity was 99.6% and optical purity was 87.8%.

2.8. Determination of avidity constant

The avidity constant (K_a) of an anti-DX-9065 antisera was calculated by Scatchard analysis [3].

2.9. Preparation of reagent for assay

PBS was prepared by dissolving 9.6 g of Dulbecco's PBS in water (1 l) and used as a balanced salt solution for the preparation of other reagents. 0.1% BSA/PBS was prepared by dissolving 500 mg of BSA in PBS (50 ml) and used as assay buffer. Lyophilized antisera was dissolved in 0.1% BSA/PBS containing 0.05% NaN_3 (0.9 ml) and stored at 4°C until assay. The antisera solution was diluted 2000-fold with assay buffer prior to use.

[^{3}H]DX-9065a was diluted with assay buffer to a concentration of 50 000 cpm ml^{-1}, 1.66 ng ml^{-1}, before assay.

Dextran-coated charcoal was freshly prepared. Dextran T 70 (100 mg) was dissolved in PBS (100 ml), and 400 mg of charcoal was suspended in this solution.

2.10. Assay procedure

A mixture of 0.1 ml of 2000-fold diluted anti-DX-9065 antisera, 0.1 ml of drug-free plasma, 0.1 ml of standard solution of DX-9065a and 0.4 ml

Table 3
Reproducibility of QC samples

Intra-assay				Inter-assay			
Concentration of DX-9065 (ng ml^{-1})		RSD (%)	Accuracy (%)	Concentration of DX-9065 (ng ml^{-1})		RSD (%)	Accuracy (%)
Theoretical	Measured[a] mean ± SD			Theoretical	Measured[b] mean ± SD		
0.5005	0.6195 ± 0.1140	18.4	23.8	0.5005	0.4463 ± 0.1387	31.1	−10.8
1.001	1.142 ± 0.113	9.9	14.1	1.001	0.9680 ± 0.1477	1.0	−3.3
5.005	5.493 ± 0.193	3.5	9.8	5.005	5.232 ± 0.497	9.5	4.5
20.02	21.02 ± 1.05	5.0	5.0	20.02	21.19 ± 1.33	6.3	5.9
30.03	29.65 ± 1.05	3.5	−1.3	30.03	30.85 ± 2.92	9.5	2.7

[a] Measured mean of six triplicate assays.
[b] Measured mean of four triplicate assays.

of assay buffer was incubated with 0.1 ml of diluted [^{3}H]DX-9065a in a plastic RIA tube (Eiken Kizai Company Ltd., Tokyo, Japan) at 4°C for 16 h. Drug-free plasma was diluted before assay with assay buffer to the same dilution as the samples to be analyzed. All samples were assayed in triplicate. Dextran-coated charcoal suspension (0.5 ml) was added to each tube and vortexed. After further incubation at 4°C for 1 h, the mixture was centrifuged at 1700*g* for 15 min. All supernatant was carefully withdrawn into counting vials. After the addition of 10 ml of Aquasol-2 (NEN, Boston, MA), the tritium radioactivity bound to the antibody was calculated by liquid scintillation counting.

2.11. Calibration and calculation

All data were captured on a Beckman data capture system using the software DATA CAPTURE™ version 1.0 (Beckman, USA), and reduced using the data analysis software, IMMUNOFIT™ EIA/RIA version 3.0 (Beckman, USA). The standard curve was fitted from the bound labeled drug vs log dose by four-parameter logistic curve fit regression.

2.12. Assay validation

The lower limit of quantitation should show both a relative standard deviation (measure of precision) and percent deviation from the nominal concentration (measure of accuracy) of less than 20%.

Accuracy and intra-assay precision were calculated from a single run of six replicates of quality control (QC) samples with concentrations of 0.5, 1, 5, 20 and 30 ng ml^{-1} of DX-9065 in plasma (QC samples). Inter-assay precision was calculated from four separate runs of six replicates with concentration of 0.5, 1, 5, 20 and 30 ng ml^{-1} of DX-9065 in plasma.

Reproducibility of the standard curve for DX-9065 was evaluated from the deviation of parameters of standard curves of five separate runs.

To study the effect of dilution on the calibration range, plasma samples with high concentration were prepared and diluted (5-, 20- and 100-fold) with assay buffer before assay. Accuracy was determined by comparing obtained with theoretical values.

To evaluate the specificity of the assay for human plasma samples, drug-free plasma samples from six individuals were spiked with DX-9065 at a concentration of 1 ng ml^{-1}. Accuracy and precision were calculated from a single run of six replicates.

Plasma samples at concentrations of 1, 5 and 20 ng ml^{-1} were investigated for stability under three sets of conditions relevant to clinical phar-

Table 4
Accuracy of RIA for DX-9065 by dilution test in drug-free human plasma spiked with DX-9065

Theoretical conc. of Dx-9065 ($ng\ ml^{-1}$)	Dilution (fold)	Theoretical conc. of DX-9065 after dilution ($ng\ ml^{-1}$)	Measured conc. of DX-9065[a] ($ng\ ml^{-1}$)	Measured conc. of DX-9065 × dilution ($ng\ ml^{-1}$)	Accuracy (%)
20.02	1	20.02	20.87	20.87	4.2
20.02	5	4.004	4.283	21.42	7.0
20.02	20	1.001	1.113	22.26	11.2
200.2	20	10.01	11.11	222.2	11.0
200.2	100	2.002	1.966	196.6	−1.8

[a] Measured mean of six triplicate assays.

macokinetic research, namely storage periods prior to analysis of 1 day at room temperature, 1 week at 4°C and 1 month at −20°C.

In cases where the extent of standard deviation or accuracy for another validation test is similar to that of the intra-assay validation test, the resulting variance estimated is defined as negative.

2.13. *Cross-reactivity of antisera with stereoisomers*

Cross-reactivity of anti-DX-9065 antisera with its stereoisomers was measured. Cross-reactivity was defined as the ratio of concentration of DX-9065 to concentration of stereoisomers at the 50% intercept of the respective dose response curve, multiplied by 100.

2.14. *Parallelism study*

To study the effect of cross-reactivity with stereoisomers in clinical samples, plasma samples were serially diluted (two-, four- and eight-fold) in drug-free human plasma and assayed for the determination of parallelism. In addition, to elucidate whether substances in patients' plasma (endogeneous or metabolites) interfere with determination of the drug, recovery of the assay was checked by determination of samples before and after the addition of 3.003 $ng\ ml^{-1}$ of DX-9065 solution. For this study, plasma samples were obtained from healthy Japanese volunteers who had been orally administered 12.85 mg of the drug (10 mg as DX-9065).

Table 5
Effect of drug-free plasma on precision of RIA

Drug-free plasma (Initial subject)	Theoretical conc. ($ng\ ml^{-1}$)	Measured conc.[a] ($ng\ ml^{-1}$)	Accuracy (%)	RSD (%)
S.S.	1.001	1.080	7.9	
K.S.	1.001	1.173	17.2	
M.T.	1.001	1.078	7.7	
N.M.	1.001	1.123	12.2	4.1
T.A.	1.001	1.046	4.5	
T.K.	1.001	1.080	7.9	

[a] Measured mean of six triplicate assays.

Table 6
Stability of DX-9065a in human control plasma

Theoretical conc. ($ng\ ml^{-1}$)	Storage	Measured conc.[a] ($ng\ ml^{-1}$)	Difference from theoretical conc. (%)
1.001	Initial	1.190	18.9
	1 day (r.t.[b])	0.9777	−2.3
	1 week (4°C)	0.8702	−13.1
	1 month (−20°C)	0.9775	−2.4
5.005	Initial	5.001	−0.1
	1 day (r.t.[b])	4.896	−2.2
	1 week (4°C)	5.020	0.3
	1 month (−20°C)	4.887	−2.4
20.02	Initial	23.33	16.5
	1 day (r.t.[b])	21.18	5.8
	1 week (4°C)	19.11	−4.6
	1 month (−20°C)	19.92	−0.5

[a] Mean of six triplicate assays.
[b] Room temperature.

Table 7
Freeze–thaw stabillity of DX-9065a in human plasma

QC level (ng ml^{-1})	Freeze cycle	Measured conc.[a] (ng ml^{-1})	Difference from pre-cycle (%)
1.001	Pre-cycle	1.081	–
	1	1.145	5.9
	3	1.138	5.3
4.004	Pre-cycle	3.900	–
	1	3.673	−5.8
	3	4.073	4.4
30.03	Pre-cycle	30.39	–
	1	27.68	−8.9
	3	26.54	−12.7

[a] Measured mean of six triplicate assays.

2.15. Application to clinical samples

The pharmacokinetics of DX-9065a after a single oral administration were investigated in a healthy Japanese male volunteer. The volunteer received 12.85 mg (10 mg as DX-9065) of DX-9065a with 150 ml of water after overnight fasting. Blood samples were collected in heparinized syringes before drug administration and 0.25–48 h after administration. After centrifugation, plasma was separated and frozen at −20°C until analysis. The concentration of unchanged DX-9065 in plasma was determined by the RIA method described, and the pharmacokinetic parameters C_{max}, T_{max}, AUC, MRT and $T_{1/2}$ were calculated. T_{max} and C_{max} values were obtained from the raw data. AUC and MRT values were calculated by non-compartment analysis. $T_{1/2}$ values were obtained by regression analysis of the linear portion of the transformed mean plasma concentration into the logarithmic time curve. AUC, MRT and $T_{1/2}$ values were obtained using the PC software TOP-FIT™ version 2.0 (Gustav Fischer Verlag, Stuttgart, Germany).

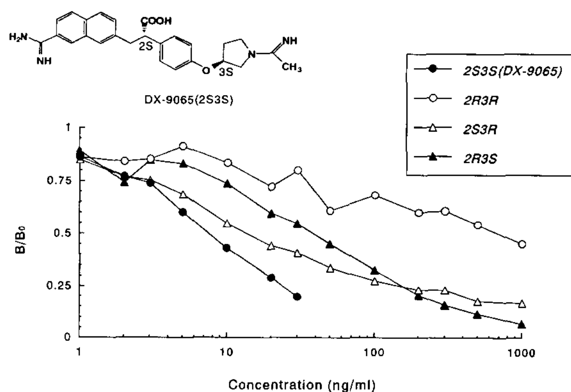


Fig. 3. Cross-reactivity of anti-DX-9065 antisera with stereoisomers.

3. Results and discussion

3.1. Preparation of antisera

In order to derivtize DX-9065 to an active ester, a conventional N-succinimide method was tried but produced no conjugates. Therefore, alkylation with β-chloropropionic acid succinimide ester, an active ester, was designed for the preparation of the hapten. The molar ratio of hapten molecule/BSA was 8.9. The avidity constant measured by Scatchard plot analysis was 4.45×10^9 M^{-1}.

3.2. Calibration curve

A typical calibration curve is shown in Fig. 2. The detection limit was estimated to be 1 or 0.5

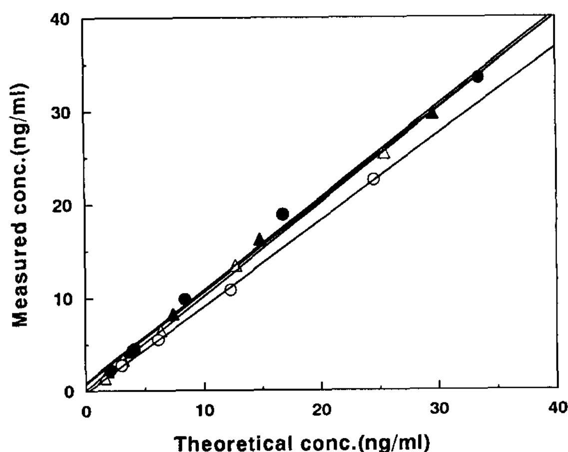


Fig. 4. Parallelism studies with various concentrations of DX-9065 in human plasma. ○ A, $y = 0.923x - 0.224$, $r = 1.000$; ● B, $y = 0.995x + 0.886$, $r = 0.997$; △ C, $y = 1.005x + 0.118$, $r = 0.999$; ▲ D, $y = 0.990x + 0.719$, $r = 0.999$.

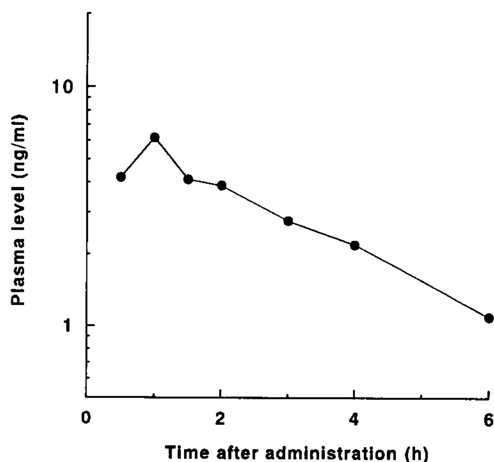


Fig. 5. Concentration of DX-9065 in human plasma.

ng ml^{-1}, which was almost 100-fold more sensitive than a previous HPLC method.

3.3. Accuracy and precision of the calibration curve

The coefficient value of a back-calculated amount of DX-9065 in plasma calibration standards varied from 8.6% at a nominal concentration of 0.50 ng ml^{-1} to 1.1% at a nominal concentration of 3.0 ng ml^{-1} (Table 1). The absolute values of the percent error value from theoretical concentration were less than 5.9. Reproducibility of quality control parameters of the calibration curve was good (Table 2).

3.4. Intra- and inter-assay validation (Table 3)

3.4.1. Intra-assay validation

The coefficient values of QC samples at concentrations of 1, 5, 20 and 30 ng ml^{-1} were between 3.5 and 9.9. The accuracy of QC samples at concentrations of 1, 5, 20 and 30 ng ml^{-1} were between −1.3 and 14.1%. However, the RSD values and accuracy for a 0.5 ng ml^{-1} QC sample were 18.4 and 23.8% respectively.

3.4.2. Inter-assay validation

The coefficient values of QC samples at concentrations of 1, 5, 20 and 30 ng ml^{-1} were less than 15.3%. However, the RSD value of a 0.5 ng ml^{-1} QC sample was 31.1%.

The limit of quantification was therefore set at 1 ng ml^{-1} of plasma and the calibration range at 1–30 ng ml^{-1}.

3.5. Effect of dilution on calibration range

Results obtained from plasma samples diluted 5-, 20- and 100-fold prior to analysis are shown in Table 4. Accuracy was from 11.2 to −1.8%. These results show the validity of determination of DX-9065a in over-range samples.

3.6. Specificity of the assay to drug-free plasma

Specificity of the assay to drug-free plasma is shown in Table 5. Accuracy was 4.5–17.2%. In comparison with the values for intra-assay validation, these results are acceptable for determination of the drug in human plasma.

3.7. Stability in drug-free human plasma

The stability of DX-9065a in plasma is shown in Table 6. The difference from the theoretical value was −13.1% to 5.8%.

The stability of DX-9065a to a freeze–thaw cycle is shown in Table 7. The difference from the theoretical value was −12.7% to 5.9%.

The percentage difference from theoretical concentration and the difference from initial concentrations were approximately equal to those of the inter-assay test. In comparison with the values for intra-assay validation, these results are acceptable for stability of the drug in human plasma under these conditions.

3.8. Cross-reactivity and parallelism study

In the development of RIA methods for drugs, the most important determinant of the specificity of the assay procedure is cross-reactivity of the used antisera with the metabolites of the drug. Designing immunogens for the preparation of specific antisera requires prior knowledge of the metabolism of the drug. However, any metabolites were identified in experimental animal plasma

Table 8
Precision of RIA for DX-9065 by recovery test with plasma sample spiked with 3.003 ng ml^{-1} of DX-9065[a]

Sample name	Concentration (ng ml^{-1})				Difference	
	Before addition	Added DX-9065	After addition		%	Mean ± SD
			Theoretical	Measured		
2–0.25 h	0.5380	3.003	3.541	3.820	7.9	
2–0.5 h	4.195	3.003	7.198	7.698	6.9	
2–1 h	6.168	3.003	9.171	9.340	1.8	
2–1.5 h	4.122	3.003	7.125	7.400	3.9	
2–2 h	3.881	3.003	6.884	7.330	6.5	2.5 ± 6.4
2–3 h	2.755	3.003	5.758	6.306	9.5	
2–4 h	2.192	3.003	5.195	4.577	−11.9	
2–6 h	1.092	3.003	4.095	4.072	−0.6	
2–8 h	0.8160	3.003	3.819	3.695	−3.2	
2–12 h	0.6680	3.003	3.671	3.826	4.2	

[a] Plasma samples were obtained from healthy male Japanese volunteers after oral administration of DX-9065a at a dose of 12.85 mg (10 mg as DX-9065).

or urine samples (unpublished data). Because DX-9065a has two chiral centers in its molecule, four possible stereoisomers can exist in a biological matrix. Many stereoselective methods, e.g. HPLC, gas chromatography and immunoassay [4], have been used for stereoselective determination of chiral drugs and their metabolites, and the implications of stereoselectivity in the pharmacokinetics of these drugs should be generally understood. In the present study, cross-reactivities of employed antisera with enantiomer (2*R*3*R* form) and two epimers (2*R*3*S*- and 2*S*3*R* forms) were 0.7, 20.2 and 43.9% respectively (Fig. 3).

As employed antisera did not have sufficient stereospecificity, stereoisomers which might have been generated under physiological conditions would have affected concentration measurement by the proposed RIA method. As no data were available concerning chiral inversion of DX-9065a under physiological conditions, additional validation tests were carried out.

First, the results of parallelism studies were plotted as theoretical concentrations vs. measured concentrations (Fig. 4). All stereoisomers showed nonparallel displacement curves which could have caused significant overestimation of the drug at lower plasma concentrations if the isomers were present in appreciable amounts (Fig. 3). However, analysis by least-squares regression revealed high correlations ($r = 0.997–1.000$) for the diluted plasma samples.

Second, to elucidate whether substances in the patient's plasma (endogenous or metabolites) interfered with determination of the drug, recovery of the assay was checked by determination of the apparent endogenous concentration in plasma samples before and after the addition of 3.003 ng ml^{-1} of DX-9065 to plasma samples from healthy Japanese volunteers after oral administration of the drug. The differences in measured concentrations before and after the addition of DX-9065 solution were nearly equal to theoretical values (Table 8).

These results suggest that the generation of stereoisomers in human plasma was negligible. Further, no significant effect resulting from postulated metabolites or endogenous substances on values was observed.

The stereoselectivity of antibody for the C2 position of the carboxyl moiety is higher than that for the C3 position of the pyrrolidine ring. In general, specificity of an antibody is significantly

influenced by position on the molecule involved in conjugation to the carrier protein. It is well known that an antibody has specific recognition toward the position distant from the introduced carrier protein of the molecule [5]. In contrast, the antibody employed in this study showed higher stereoselectivity for the chiral carbon closer to the position of the carrier protein. The reason for this was considered to be that (1) the distance between the pyrrolidine ring and the carrier protein was less than expected due to interaction generated by the electrosteric force of hetero atoms of the acetimidoyl moiety or pyrrolidine ring of DX-9065 with BSA, and (2) the bridge of the introduced immunogen had a suitable carbon length for stereospecificity at the C2 position.

3.9. Application to clinical study

The plasma concentration-time profile of unchanged substance for a volunteer who had received a single oral 12.85 mg (10 mg as DX-9065) dose of DX-9065a is shown in Fig. 5. Plasma concentration reached a C_{max} value of 6.2 ng ml^{-1} at 1 h and decreased with a half-life of 2.3 h. AUC and MRT values were 20.9 ng h ml^{-1} and 3.58 h respectively. These data suggest that DX-9065a was rapidly absorbed from the gastrointestinal tract after oral administration and was cleared immediately from the plasma.

4. Conclusion

A sensitive and simple RIA method was developed. The sensitivity, precision and accuracy of the method were sufficient for determination of DX-9065a in human plasma samples. Using this method, the pharmacokinetic property of DX-9065a in humans was clarified for the first time.

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