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Determination of a novel calmodulin antagonist, 3-{2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl}-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate, DY-9760e, in human plasma using solid-phase extraction and high-performance liquid chromatography with fluorescence detection

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Abstract

A high-performance liquid chromatographic method for the determination of a novel calmodulin antagonist, 3-{2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl}-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate, DY-9760e and its major metabolite, 3-{2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl}-5,6-dimethoxyindazole, DY-9836 in human plasma has been developed. DY-9760e, DY-9836 and the internal standard (I.S.) were extracted from plasma by means of an Isolute C_{18} (EC) column. The extracts were chromatographed on a reversed-phase TSK-gel ODS-80Ts column using 0.1 *M* acetate buffer (pH 5)–CH_3CN (65:35, v/v) as the mobile phase at a flow-rate of 1.0 ml/min. Fluorescence detection at an excitation wavelength of 303 nm and an emission wavelength of 347 nm resulted in a limit of quantitation of 1.000 ng/ml for plasma. The method showed satisfactory sensitivity, precision, accuracy, recovery and selectivity. Stability studies showed that DY-9760 and DY-9836 were stable in plasma up to at least eight weeks at −80°C.

Keywords: Calmodulin antagonist; DY-9760e; DY-9836

1. Introduction

3-{2-[4-(3-Chloro-2-methylphenyl)-1-piperazinyl]ethyl}-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate, DY-9760e, is a novel calmodulin antagonist with cytoprotective effect against cell death induced by a Ca^{2+} ionophore in neuroblastoma cells [1,2]. DY-9760e possesses more potent antagonistic effect than W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, another calmodulin antagonist [3]. It is currently under development for the treatment of cerebrovascular disorders. Its major metabolite, DY-9836, *N*-dealkylated DY-9760e, has similar activity. The chemical structures of DY-9760e and DY-9836 are shown in Fig. 1.

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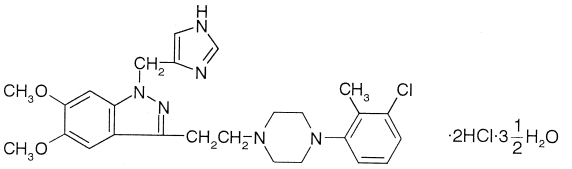
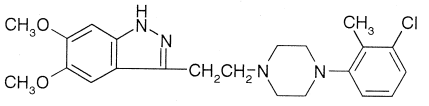
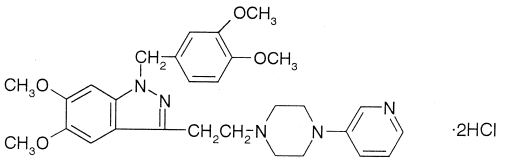


DY-9760e



DY-9836



DY-9695 (I.S.)

Fig. 1. Chemical structures of DY-9760e, DY-9836 and the internal standard (DY-9695).

On the basis of these promising pharmacological properties, DY-9760e was selected for phase I clinical evaluation. Determination of the pharmacokinetics of a drug under investigation is one of the objectives of a phase I study. A sensitive and selective method for the determination of DY-9760e is essential to support these early clinical studies.

This paper describes the method for the determination of DY-9760e and DY-9836 in human plasma using solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) with fluorescence detection.

2. Experimental

2.1. Chemicals and reagents

DY-9760e, DY-9836 were synthesized by Daiichi Pharmaceutical (Tokyo, Japan). The internal standard used for the plasma assay, 3-{2-[4-(3-pyridinyl)-1-piperazinyl]ethyl}-5, 6-dimethoxy-1-(3, 4-dimethoxybenzyl)-1*H*-indazole dihydrochloride (DY-9695) was synthesized by Sumika Chemical Analysis Service (Osaka, Japan). The chemical structures of DY-9760e, DY-9836 and DY-9695 are shown in Fig. 1. Acetonitrile (CH_3CN) and methanol (CH_3OH) were HPLC-grade solvents (Kanto Chemical, Tokyo, Japan). All other chemicals were of analytical-reagent grade and used without further purification. Purified water from a Milli-Q system (Waters, Millipore, Milford, MA, USA) was used. Human blank plasma was obtained from healthy male volunteers.

DY-9760 represents the anhydrous free base of DY-9760e and drug concentrations were expressed as the equivalents of DY-9760.

2.2. Instruments and chromatographic conditions

The chromatographic system consisted of a high-performance liquid chromatograph pump (Model L-7100, Hitachi, Tokyo, Japan), a fluorescence detector (Model L-7480, Hitachi) operated at an excitation wavelength of 303 nm and at an emission wavelength of 347 nm and a reversed-phase TSK-gel ODS-80Ts (150×4.6 mm I.D., 5 μm particle size) column (Tosoh, Tokyo, Japan). A guard column, TSK-gel ODS-80Ts (15×3.2 mm I.D.) (Tosoh) was attached ahead of the analytical column. The column temperature was kept at 40°C in a column oven (Waters temperature control module, Waters, Tokyo, Japan). Samples of 100 μl were injected automatically to the HPLC system by an autosampler (Model L-7200, Hitachi). A mixture of 0.1 *M* acetate buffer (pH 5.0)–CH_3CN (65:35, v/v) was used as the mobile phase at a flow-rate of 1.0 ml/min. The mobile phase was degassed in an ultrasonic bath before use.

2.3. Preparation of standard solutions and standard samples

Stock solutions of standards were prepared by dissolving accurately weighed DY-9760e (approximately 1 mg as DY-9760), DY-9836 (approximately 1 mg) and DY-9695(approximately 1 mg) in CH_3OH (50 ml) in a volumetric flask. The standard solution were diluted the stock solution with 50 m*M* phos-

phate buffer (pH 5). The solutions were stored at 4°C in refrigerator for a maximum of one month. Plasma standards were prepared at concentrations of 1.000, 3.000, 10.00, 30.00, 100.0 and 300.0 ng/ml of DY-9760 and DY-9836 by spiking the control human plasma (0.2 ml) with the standard solution (0.02 ml of 10.00, 30.00, 100.0, 300.0, 1000 and 3000 ng/ml).

2.4. Assay procedure

Isolute C_{18} (EC) columns (International Sorbent Technology, UK) [4–6] were activated prior to use by passage of CH_3OH (2 ml) and water (2 ml).

The procedure for plasma sample preparation was as follows: a 0.2-ml aliquot of human control plasma was transferred into a 13-ml disposable glass centrifuge tube and 2.0 ml of saline and 0.1 ml of internal standard solution (0.5 μg/ml) were added. The resulting mixture was vortex mixed and applied to a Isolute C_{18} (EC) column. The column was washed successively with water (2 ml) and CH_3OH–water (25:75, v/v; 2 ml). DY-9760, DY-9836 and I.S. were eluted from the column with trifluoroacetic acid–CH_3OH (2:98, v/v; 2 ml). The eluate was taken to dryness by centrifugal evaporator (Model EC-57C, Sakuma, Tokyo, Japan). The residue was dissolved in mobile phase and a 100-μl aliquot was injected onto the HPLC system.

2.5. Calibration curves

The peak areas of DY-9760 and DY-9836 were divided by the peak area of the internal standard to obtain the peak area ratio. The calibration curves for DY-9760 and DY-9836 were obtained by weighted [$1/conc^2$] least-squares linear regression analysis of the peak area ratios of the standards versus the drug concentrations, using the Hitachi System Manager D-7000 (Hitachi).

2.6. Recovery

The absolute recovery of DY-9760 and DY-9836 from human plasma was estimated by comparing the peak area obtained from injections of the standard solutions with those obtained from the injections of extracts of plasma samples spiked with known concentrations (3.000, 30.00, 100.0 and 300.0 ng/ml) of DY-9760 and DY-9836.

2.7. Selectivity

Blank plasma from six healthy male volunteers were assayed by the procedure described above to evaluate the selectivity of the method.

2.8. Precision and accuracy

Intra-day precision and accuracy of the method were evaluated by replicate analysis ($n=5$) of the plasma calibration standards.

Inter-day precision and accuracy was determined by assaying the plasma calibration standards on three separate days. The limit of quantitation was chosen to be the concentration of the lowest calibration standard with an acceptable limit of variance.

2.9. Stability

The stability of DY-9760 and DY-9836 in human plasma was investigated by preparing plasma quality control (QC) samples of DY-9760 and DY-9836 (30.00 ng/ml).

Freeze–thaw stability was tested by assaying QC samples through one or three freeze–thaw cycles before processing.

Long-term stability was monitored by assaying QC samples that were stored at −80°C for 0 (just after preparation), two, four and eight weeks.

Stability of DY-9760 and DY-9836 in autosampler was tested by assaying QC samples stored at 4°C for 0, 24 and 48 h.

2.10. Effect of dilution

The effect of dilution was evaluated by triplicate analysis of the over-the-calibration-curve samples (1000 ng/ml) after 10-fold and 100-fold dilution with saline.

3. Results and discussion

3.1. Chromatography and selectivity

After characterization of the fluorescence spectrum of DY-9760 and DY-9836 in the mobile phase, excitation and emission wavelengths for fluorescence detection were set at 303 and 347 nm, respectively.

Well defined chromatographic peaks for DY-9760 and DY-9836 and the internal standard (I.S.) were obtained on the 5 μm TSK-gel ODS-80Ts column (150×4.6 mm I.D.), where the free silanol groups were almost completely end-capped. A representative chromatogram of extracts from control plasma is shown in Fig. 2A. This chromatogram indicated that no endogenous compounds interfered at the retention times of DY-9760, DY-9836 and I.S. A representative chromatogram of control plasma spiked with DY-9760, DY-9836 and I.S. is shown in Fig. 2B. DY-9760, DY-9836 and I.S. were well-resolved from each other, and the retention times of DY-9760, DY-9836 and I.S. were approximately 13.1, 16.4 and 6.7 min, respectively. The overall chromatographic run time was ca. 20.0 min. Plasma collected from six healthy volunteers also showed no interferences.

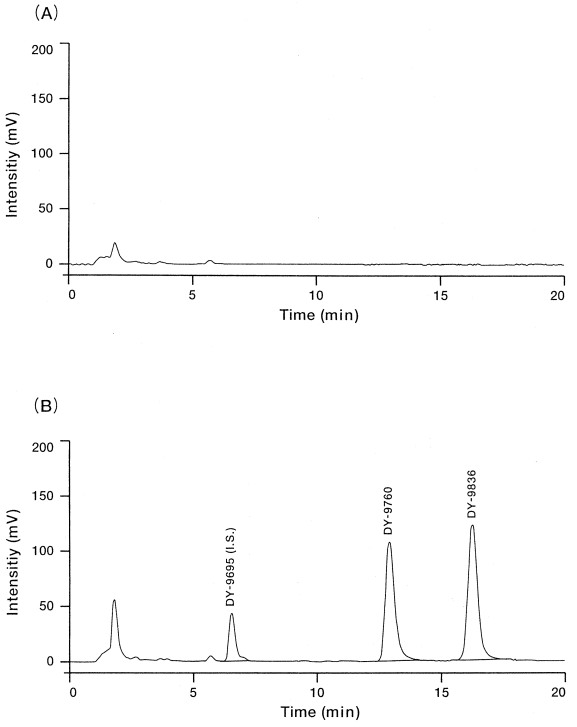


Fig. 2. Representative chromatograms of 0.2-ml plasma extracts obtained with (A) control human plasma and (B) control human plasma spiked with DY-9760, DY-9836 (100.0 ng/ml) and DY-9695 (500.0 ng/ml). The chromatograms are shown with a full scale of 200 mV.

3.2. Calibration curves

Calibration curves for plasma obtained over a three-week period ($n=6$) were linear and reproducible with mean±standard deviation values for the constants in the regression equation of $y=(24.12\pm3.51)x+(-0.42\pm0.48)$ for DY-9760, and $y=(19.92\pm2.95)x+(-0.22\pm0.44)$ for DY-9836. The weighting factor of $(1/\text{conc}^2)$ was used to improve homogeneity of variance. The correlation coefficients (r^2) were 0.9984±0.0023 and 0.9991±0.0010 for DY-9760 and DY-9836 in plasma, respectively. The inter-day coefficient of variation (C.V.) of the slope of the calibration curve was 14.6% for DY-9760 and 14.8% for DY-9836. The calibration curves for DY-9760 and DY-9836 in plasma were linear in the concentration range of 1.000 to 300.0 ng/ml.

3.3. Recovery of extraction

The absolute recoveries of DY-9760 and DY-9836 from plasma were determined by comparing the peak area of extracted standards with those of injected standards (Table 1). The recoveries of DY-9760 and DY-9836 from spiked human plasma were evaluated at the concentrations of 3.000, 30.00, 100.0 and 300.0 ng/ml in triplicates. The recoveries ranged

Table 1
Recoveries of DY-9760, DY-9836 and DY-9695 from human plasma

Concentration (ng/ml)	Recovery (%) Mean±SD	C.V.[a] (%)
DY-9760		
3.000	75.2±4.2	5.6
30.00	96.1±0.6	0.6
100.0	95.9±2.5	2.6
300.0	94.3±0.9	0.9
DY-9836		
3.000	77.4±6.4	8.3
30.00	91.6±2.7	2.9
100.0	91.9±2.4	2.6
300.0	92.1±0.9	1.0
DY-9695		
500.0	82.9±0.1	0.1

[a] C.V.=Coefficient of variation.

from 75.2 to 96.1% for DY-9760, and from 77.4 to 92.1% for DY-9836. In addition, the recovery of the internal standard from plasma was 82.9% ($n=3$).

3.4. Precision and accuracy

The intra-day precision and accuracy of the method for plasma were evaluated by analyzing human plasma spiked with DY-9760 and DY-9836 at concentrations of 1.000, 30.00, 100.0 and 300.0 ng/ml in replicates of five (Table 2). Precision was based on the calculation of the C.V. An indication of accuracy was based on the calculation of the relative error (R.E.) of the found concentration compared to theoretical. The C.V. ranged from 0.6 to 2.1% for DY-9760,and from 0.9 to 1.5% for DY-9836, and the R.E. ranged from 4.7 to 8.5% for DY-9760, from 7.5 to 10.5% for DY-9836 at concentrations above 30.00 ng/ml. The limits of quantitation (LOQs) using a 0.2-ml sample were set at the concentrations of the lowest calibration standards, or 1.000 ng/ml of plasma. At the LOQ, the C.V. and R.E. for DY-9760 were 19.9% and 5.7%, respectively, and those for DY-9836 were 14.8% and −19.0%, respectively.

Table 2
Intra-day precision and accuracy for the determination of DY-9760 and DY-9836 in human plasma

Theoretical concentration (ng/ml)	Mean found concentration (ng/ml)	C.V.[a] (%)	R.E.[b] (%)	*n*
DY-9760				
1	1.057	19.9	5.7	5
30	32.16	2.1	7.2	5
100	104.7	0.6	4.7	5
300	325.5	1.5	8.5	5
DY-9836				
1	0.810	14.8	−19.0	5
30	33.16	1.5	10.5	5
100	107.5	0.9	7.5	5
300	326.7	1.5	8.9	5

[a] C.V.=Coefficient of variation.
[b] R.E.=Relative error.

Table 3
Inter-day precision and accuracy for the determination of DY-9760 and DY-9836 in human plasma

Theoretical concentration (ng/ml)	Mean found concentration (ng/ml)	C.V.[a] (%)	R.E.[b] (%)	*n*
DY-9760				
1	1.160	17.3	16.0	3
30	32.55	3.5	8.5	3
100	107.9	6.2	7.9	3
300	330.3	3.1	10.1	3
DY-9836				
1	1.000	18.8	0.0	3
30	32.23	2.6	7.4	3
100	105.7	3.9	5.7	3
300	319.1	2.4	6.4	3

[a] C.V.=Coefficient of variation.
[b] R.E.=Relative error.

Table 4
Freeze–thaw stability of DY-9760 and DY-9836 in human plasma

Initial concentration	Percent difference from the initial concentration at the indicated cycle[a]		
(ng/ml)	0-cycle	1-cycle	3-cycle
DY-9760			
30.00	0.0	−5.1	−1.6
DY-9836			
30.00	0.0	−4.1	−2.3

[a] Results are reported as the mean percent difference of three determinations.

The inter-day precision and accuracy of the method for plasma were assessed by the analysis of calibration standard samples at concentrations of 1.000, 30.00, 100.0 and 300.0 ng/ml of plasma in triplicates on three separate days (Table 3). The C.V. ranged from 3.1 to 3.5% for DY-9760, and from 2.4 to 3.9% for DY-9836, and the R.E. ranged from 7.9 to 10.1% for DY-9760, and from 5.7 to 7.4% for DY-9836 at concentrations above 30.00 ng/ml. At the quantitation limits of 1.000 ng/ml, the method showed acceptable precision and accuracy with C.V. of 17.3 and 18.8%, and R.E. of 16.0 and 0.0% for DY-9760 and DY-9836, respectively.

3.5. Stability

DY-9760 and DY-9836 exhibit acceptable stability in human plasma when exposed up to three freeze–thaw cycles with the compounds loss of ≤5.1% (Table 4).

The long-term stability of DY-9760 and DY-9836 in plasma was assessed at −80°C (Table 5). They were found to be stable for up to at least eight weeks at −80°C.

Stability of DY-9760 and DY-9836 in human plasma extracts was tested at 4°C in the autosampler for 0, 24 and 48 h (Table 6). DY-9760 and DY-9836

Table 5
Stability of DY-9760 and DY-9836 in human plasma at −80°C

Initial concentration	Percent difference from the initial concentration at the indicated time[a]			
(ng/ml)	0 week	2 week	4 week	8 week
DY-9760				
30.00	0.0	2.5	2.3	7.8
DY-9836				
30.00	0.0	−2.5	−0.3	5.2

[a] Results are reported as the mean percent difference of three determinations.

Table 6
Process stability of DY-9760 and DY-9836 in human plasma extract stored at 4°C in autosampler

Initial concentration	Percent difference from the initial concentration at the indicated time[a]		
(ng/ml)	0 h	24 h	48 h
DY-9760			
30.00	0.0	2.4	−0.5
DY-9836			
30.00	0.0	2.9	−0.5

[a] Results are reported as the mean percent difference of three determinations.

Table 7
Dilution of human plasma spiked with DY-9760 and DY-9836 into the calibration range

Theoretical concentration (ng/ml)	Percent difference from the theoretical concentration			
	100-fold		10-fold	
	C.V.	R.E.	C.V.	R.E.
DY-9760				
1000	1.3	−3.8	1.9	11.8
DY-9836				
1000	2.8	−6.9	1.7	3.4

showed acceptable stability for up to at least 48 h with loss of the compounds of ≤0.5%.

These studies indicated that the samples containing DY-9760 and DY-9836 could be handled under normal laboratory conditions without significant loss of the compounds.

3.6. Sample Dilution

The intra-batch C.V. and R.E. in the QC plasma samples at concentration of 1000 ng/ml were≤2.8% and ≤11.8% after 10-fold and 100-fold dilution, respectively (Table 7). There was no effect of sample dilution on the concentrations of DY-9760 and DY-9836 in human plasma.

4. Conclusions

SPE has been successfully applied to the extraction of a novel calmodulin antagonist, DY-9760e, from human plasma. The method is satisfactory with respective to sensitivity, precision, accuracy, recovery and selectivity, and will be useful for human pharmacokinetic studies. The results of these studies will be reported elsewhere.

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