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Sensitive liquid chromatography assay with ultraviolet detection for a new phosphodiesterase V inhibitor, DA-8159, in human plasma and urine

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Abstract

A sensitive high-performance liquid chromatographic (HPLC) method with ultraviolet absorption detection (292 nm) was developed and validated for the determination of the new phosphodiesterase V inhibitor, DA-8159 (DA), in human plasma and urine. A single step liquid–liquid extraction procedure using ethyl ether was performed to recover DA and the internal standard (sildenafil citrate) from 1.0 ml of biological matrices combined with 200 μl of 0.1 M sodium carbonate buffer. A Capcell Pak C_{18} UG120 column (150 mm × 4.6 mm I.D., 5 μm) was used as a stationary phase and the mobile phase consisted of 30% acetonitrile and 70% 20 mM potassium phosphate buffer (pH 4.5) at a flow rate of 1.0 ml/min. The lower limit for quantification was 5 ng/ml for plasma and 10 ng/ml for urine samples. Within- and between-run accuracy and precision were ≤15 and ≤10%, respectively, in both plasma and urine samples. The recovery of DA from human plasma and urine was greater than 70%. Separate stability studies showed that DA is stable under the conditions of analysis. This validated assay was used for the pharmacokinetic analysis of DA during a phase I, rising dose study.

Keywords: Phosphodiesterase inhibitor; DA-8159

1. Introduction

DA-8159 (5-[2-propyloxy-5-(1-methyl-2-pyrollidinylethylamidosulphonyl)phenyl]-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo(4,3-d)pyrimidin-7-one; DA; Fig. 1) is a potent and selective inhibitor of phosphodiesterase type V, which catabolizes cyclic guanosine monophosphate (cGMP) in the corpus cavernosum [1]. DA has erectogenic activity in laboratory animals, due its enhancement of nitric oxide-stimulated cGMP accumulation [2], and this activity was reported to be equal or superior to that of sildenafil (Viagra®) [1].

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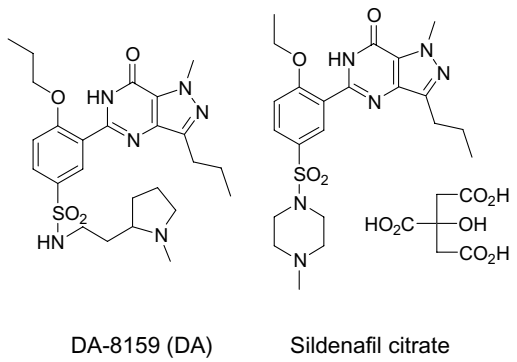

Fig. 1. Chemical structure of DA-8159 and of the internal standard, sildenafil citrate.

DA was developed by Dong-A Pharmaceutical Company Limited (Yongin, South Korea) as an oral agent to treat male erectile dysfunction, and phase I clinical trials have been completed.

Several methods for the determination of DA concentrations have been developed in pre-clinical pharmacokinetic studies in beagle dogs and rats [3,4], involving high-performance liquid chromatography (HPLC) in combination with liquid–liquid extraction. The lower limit of quantification in dog plasma was 50 ng/ml and in rat plasma 20 ng/ml [3,4]. However, these reported methods are considered to have insufficient sensitivity for the determination of DA in human biological samples. Therefore, a more sensitive assay method was needed.

We now report a reliable and sensitive assay method for the quantification of DA in human plasma and urine. The current method is based on a modification of one previously described by Shim et al. [4], using HPLC with ultraviolet (UV) detection. Assay sensitivity, as determined by the lower limit of quantification, was 5 ng/ml in human plasma and 10 ng/ml in human urine. This assay has been successfully tested in clinical phase I studies in healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

DA and sildenafil citrate (internal standard) (Fig. 1) were supplied by the Dong-A Pharmaceutical Company Limited (Yongin, South Korea). The purity of the supplied DA was confirmed to be 99.5% by HPLC analysis. Anhydrous sodium carbonate and orthophosphoric acid were purchased from Shinyo Pure Chemical (Osaka, Japan). Potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany). The acetonitrile used was of HPLC-grade, and all other reagents were of analytical quality. Milli-Q-purified water was used throughout. Drug-free human plasma from healthy donors was obtained from the Blood Bank of Seoul National University Hospital (Seoul, South Korea).

2.2. Chromatographic conditions

The HPLC system consisted of a Model 307 solvent delivery system, a Model 234 autosampling device, and a Model 118 UV detector (Gilson, Villiers-le-Bel, France). The mobile phase used was 30% acetonitrile/70% 20 mM potassium phosphate buffer (pH 4.5) and this was delivered isocratically at a flow rate of 1.0 ml/min. The mobile phase was degassed under vacuum and filtered (0.45 μm) prior to use. Chromatographic separation was performed on a Capcell Pak C_{18} column UG120, 5 μm particle size, 150 mm × 4.6 mm I.D. (Shiseido, Tokyo, Japan) protected by a Brownlee guard cartridge column (15 mm × 3.2 mm) packed with reverse-phase material (C_{18}) (Perkin-Elmer, Norwalk, CT, USA). The assay was performed at ambient temperature. DA was detected using the UV detector at 292 nm. HPLC data were acquired using a computerized data analysis system (Unipoint version 1.90, Gilson).

2.3. Sample preparation

DA was extracted from biological samples by liquid–liquid extraction, as described by Shim et al. [4]. Briefly, 250 ng of internal standard (100 μl of a 2.5 μg/ml methanol solution) and 100 μl of methanol were added to a 1 ml of biological sample (urine samples were diluted 10–50 times with blank urine) in a 10 ml glass test tube. Sodium carbonate buffer (200 μl, 0.1 M) was added, the samples were mixed and 5 ml of ethyl ether was added. The mixture was vortex-mixed for 60 s and then centrifuged at 3000 × *g* for 10 min. The organic layer was then transferred into a clean tube and the solvent evaporated. The

residue obtained was reconstituted in 150 μl of mobile phase, the samples were centrifuged, and 100 μl injected onto the HPLC.

2.4. Standard curve

A stock solution (1.0 mg/ml) of DA was prepared by dissolving 10 mg of DA in 10 ml of methanol, which was then stored at −70 °C. Working standard solutions were prepared daily by serial dilution with methanol at concentrations 0.05 (included only for plasma samples), 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg/ml. A standard curve was prepared by adding 100 μl of the DA working standard solutions to 1 ml of blank plasma or urine. The eight plasma concentrations analyzed ranged from 5 to 1000 ng/ml and the seven urine concentrations ranged from 10 to 1000 ng/ml. Calibrations curves were prepared by weighted ($1/x$) least square linear regression analysis of the peak height ratio versus the nominal concentration (x). The linearity of the calibration curve was evaluated by calculating the correlation coefficient (r). The concentration of DA in the test samples was calculated using the regression parameters obtained from the standard curve.

2.5. Accuracy, precision and recovery

Five spiked plasma pools were prepared at 5, 10, 100, 800 and 4000 ng/ml and five spiked urine samples at 10, 20, 100, 800 and 4000 ng/ml were prepared as quality control standards by adding the appropriate volume (less than 1% of total volume) of the DA working standard solutions to drug-free biological fluids. Plasma or urine samples (4000 ng/ml) above the upper limit of the standard curve were analyzed after a 10-fold dilution with blank biological fluids. To determine the within-run reproducibility of the method, six replicates were run at each concentration on the same day. To confirm the between-run reproducibility, six replicates at each concentration, except for the lower limits of the quantification (LLQ) were analyzed in five separate runs. The accuracy of the assay was shown as relative error and calculated based on the difference between the mean calculated concentration and the spiked concentration. The accuracy for each concentration, excluding the LLQ, should be <15% and should be <20% at the LLQ [5]. The precision of the assay was assessed by calculating the within- and between-run relative standard deviations (R.S.D.s). The precision for each concentration, excluding the LLQ, should be <15% and should be <20% at the LLQ [5].

The absolute recovery of DA after extraction procedure was assessed by comparing the mean peak heights in extracted samples (at 10, 100 and 800 ng/ml in plasma and at 20, 100 and 800 ng/ml in urine) to those of reconstituted samples spiked with standard solutions, representing 100% recovery, immediately after extraction of blank matrices.

2.6. Stability

The stability of the drug during sample handling was verified by incubating six replicates at each of three concentrations (10, 100 and 800 ng/ml) at room temperature (25 °C) for 24 h and by subjecting samples to three additional freeze/thaw cycles. In addition, the stability of DA in the mobile phase while waiting for HPLC analysis was investigated by storing extracted samples on the autosampler at ambient temperature (25 °C) for 24 h. The stability of the DA was determined by comparing the concentrations of samples stored under these conditions with those of samples extracted and injected immediately.

2.7. Pharmacokinetic studies

Human plasma and urine samples generated in a phase I, single rising-dose study of DA were analyzed using the described method. DA was administered orally to six healthy volunteers at doses ranging from 25 to 300 mg. Blood samples were collected in heparinized tubes at the following times after dosing: 0 (pre-dose), 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 24 and 32 h. Blood was immediately (within 30 min) centrifuged for 10 min at 2000 × g to separate plasma. Urine was collected cumulatively for 48 h following the administration of DA. Plasma and urine samples were stored at −70 °C until thawed for analysis.

3. Results and discussion

3.1. Chromatography

Representative chromatograms of human blank and spiked samples of plasma and urine are shown in

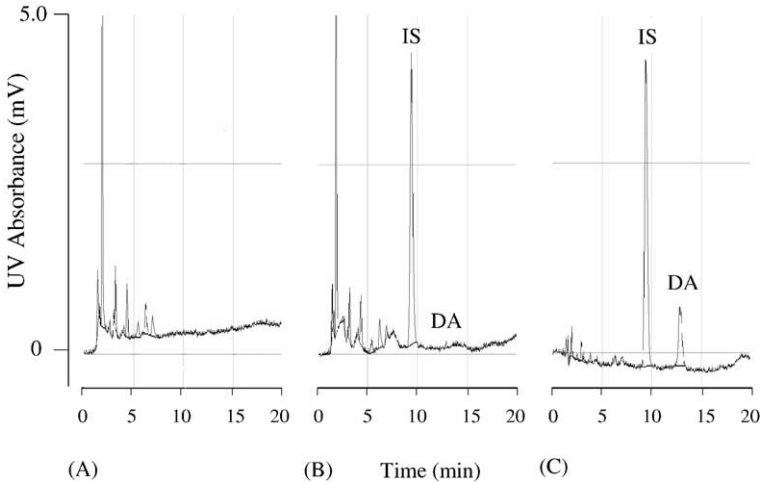

Fig. 2. HPLC chromatograms of (A) a human blank plasma sample; (B) a human plasma sample spiked with 5 ng/ml of DA and internal standard (IS); (C) an extracted plasma sample collected 24 h following the administration of a 100 mg dose of DA to a healthy volunteer, calculated to contain 47.6 ng/ml DA.

Figs. 2 and 3, respectively. No interfering peaks were observed near the retention time of DA or of the internal standard in six batches of blank human plasma or urine. The retention times for DA and internal standard were approximately 12 and 9 min, respectively. The chromatographic run time was 20 min for plasma sample analysis and 15 min for urine sample analysis.

3.2. Validation

The standard curves of DA in human plasma and urine were linear over the ranges 5–1000 ng/ml and 10–1000 ng/ml, respectively, and the regression correlation coefficients (r) were over 0.999 from each standard curve on six separate runs. In order to prevent

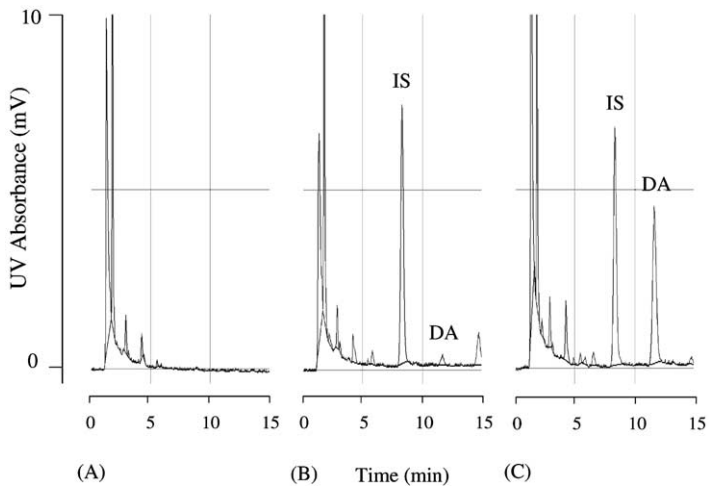

Fig. 3. HPLC chromatograms of (A) a human blank urine sample; (B) a human urine sample spiked with 10 ng/ml of DA and internal standard (IS); (C) an extracted urine sample collected from 24 to 32 h following the administration of a 100 mg dose of DA to a healthy volunteer, calculated to contain 192.3 ng/ml DA.

Table 1
Reproducibility of the standard curve ($n = 6$)

Spiked concentration (ng/ml)	Plasma		Urine	
	Calculated concentration (mean ± S.D.) (ng/ml)	R.S.D.[a] (%)	Calculated concentration (mean ± S.D.) (ng/ml)	R.S.D.[a] (%)
5	5.05 ± 0.534	10.6		
10	10.0 ± 0.521	5.2	10.1 ± 0.81	8.0
20	20.1 ± 0.366	1.8	20.3 ± 1.21	6.0
50	50.3 ± 1.270	2.5	49.1 ± 1.53	3.1
100	99.9 ± 2.390	2.4	100.9 ± 1.83	1.8
200	199.3 ± 2.831	1.4	199.8 ± 2.82	1.4
500	501.6 ± 4.123	0.8	503.1 ± 4.38	0.9
1000	998.8 ± 7.784	0.8	996.4 ± 2.84	0.3
Slope	4.804 ± 0.113	2.3	4.564 ± 0.298	6.5
y intercept	0.00147 ± 0.00438		0.0072 ± 0.00930	
Correlation coefficient	0.99998 ± 0.00001		0.99997 ± 0.00003	

[a] R.S.D. = 100% × (S.D./mean).

bias caused by the higher concentrations, it was necessary to use 1/concentration weighted linear regression analysis. As Table 1 shows, the slopes of standard curves (mean ± S.D.) were 4.804 ± 0.113 and 4.564 ± 0.298 in plasma and urine, respectively. R.S.D.s of the slopes for six different standard curves were 2.3% in plasma and 6.5% in urine samples. The between-run precision of the standard curve was evaluated using each standard curve concentration point after back-calculation. R.S.D.s were less than 10.6% and less than 8.0% in plasma and urine, respectively (Table 1). The LLQ was defined as the lowest concentration at which the percent deviation from the spiked concentration and the relative standard deviation were both less than 20% in the within-run validation. The LLQ was 5 and 10 ng/ml in plasma and urine, respectively. Within- and between-run accuracy and precision were examined by performing replicate analyses of quality control samples ($n = 6$) at five different known concentrations of DA. As Table 2 shows, the accuracy in human plasma for within- and between-run was from −12.9 to 2.2% and from −10.0 to −1.6%, respectively. The within- and between-run precision in plasma ranged from 0.8 to 5.1% and from 1.4 to 7.9%, respectively. As Table 3 shows, the accuracy in human urine for within- and between-run was from −9.1 to 1.4% and from −3.5 to 4.1%, respectively. The within- and between-run precision in urine ranged from 1.3 to 3.3% and from 2.4 to 3.5%, respectively. To obtain good extraction efficiency, the absolute recovery for DA was determined using six replicates at three different concentrations in both plasma and urine after the extraction procedure. The recoveries (mean±S.D.) of DA in plasma were 73.8±7.9% at

Table 2
The accuracy and precision of the determination of DA in human plasma

Spiked plasma concentration (ng/ml)	Accuracy (relative error[a], %)		Precision (R.S.D.[b], %)	
	Within-run ($n = 6$)	Between-run ($n = 5$)	Within-run ($n = 6$)	Between-run ($n = 5$)
5	−12.9		4.6	
10	−4.3	−10.0	5.1	7.9
100	−2.9	−3.5	1.2	3.1
800	2.2	−1.6	0.9	1.4
4000[c]	0.7	−1.9	0.8	1.9

[a] Relative error = 100% × (spiked concentration − mean calculated concentration)/spiked concentration.
[b] R.S.D. = 100% × (S.D./mean).
[c] Analyzed after 10-fold dilution with control plasma.

Table 3
The accuracy and precision of the determination of DA in human urine

Spiked plasma concentration (ng/ml)	Accuracy (relative error[a], %)		Precision (R.S.D.[b], %)	
	Within-run ($n = 6$)	Between-run ($n = 5$)	Within-run ($n = 6$)	Between-run ($n = 5$)
10	−9.1		1.3	
20	−4.2	1.9	2.9	3.1
100	1.4	4.1	3.3	3.5
800	−3.6	2.2	2.5	2.4
4000[c]	−4.8	−3.5	2.7	3.5

[a] Relative error = 100% × (spiked concentration − mean calculated concentration)/spiked concentration.
[b] R.S.D. = 100% × (S.D./mean).
[c] Analyzed after 10-fold dilution with control urine.

10 ng/ml, 73.1±3.1% at 100 ng/ml and 70.4±3.0% at 800 ng/ml. The recoveries of DA in urine were 69.1± 2.9% at 20 ng/ml, 81.2±5.5% at 100 ng/ml and 75.7± 2.7% at 800 ng/ml. The overall recoveries from plasma and urine found to be 72.4 and 75.4%, respectively.

3.3. Stability

The stability of DA under various conditions is shown in Table 4. DA was stable in both human plasma and urine after 24 h incubation at room temperature (25 °C). No significant decrease of DA in plasma or urine was detected after exposing samples to three freeze/thaw cycles. Extracted samples in mobile phase were also found to be stable at ambient temperature (25 °C) upon standing on an autosampler tray for at least 24 h. Since internal standard, sildenafil, in plasma sample was reported to be stable within 95% for as long as 6 h at room temperature [6], the entire sample treatment was performed within 2 h for every analysis.

3.4. Application of the assay in pharmacokinetic studies

The developed assay was applied to a phase I clinical study in which DA was orally administered to five dosage groups (25–300 mg) comprised of six healthy volunteers in each group. The mean plasma concentration–time profile of DA is shown in Fig. 4. The assay method was able to detect the relatively low (less than 20 ng/ml) plasma concentrations of DA

Table 4
Stability of DA ($n = 6$)

Storage conditions	Plasma			Urine		
	Spiked concentration (ng/ml)	Recovery (%)	R.S.D.[a] (%)	Spiked concentration (ng/ml)	Recovery (%)	R.S.D.[a] (%)
24 h at 25 °C in plasma or urine	10	109.0	5.1	20	100.4	4.7
	100	100.5	1.4	100	104.7	2.2
	800	99.1	1.5	800	100.0	3.9
Three freeze/thaw cycles in human plasma or urine	10	106.1	6.1	20	104.2	2.6
	100	102.1	1.8	100	99.1	2.7
	800	101.6	2.0	800	100.0	2.5
24 h at 25 °C in mobile phase after extraction	10	104.7	3.6	20	104.1	5.3
	100	97.7	7.4	100	105.9	5.7
	800	99.5	5.1	800	98.5	2.4

[a] R.S.D. = 100% × (S.D./mean).

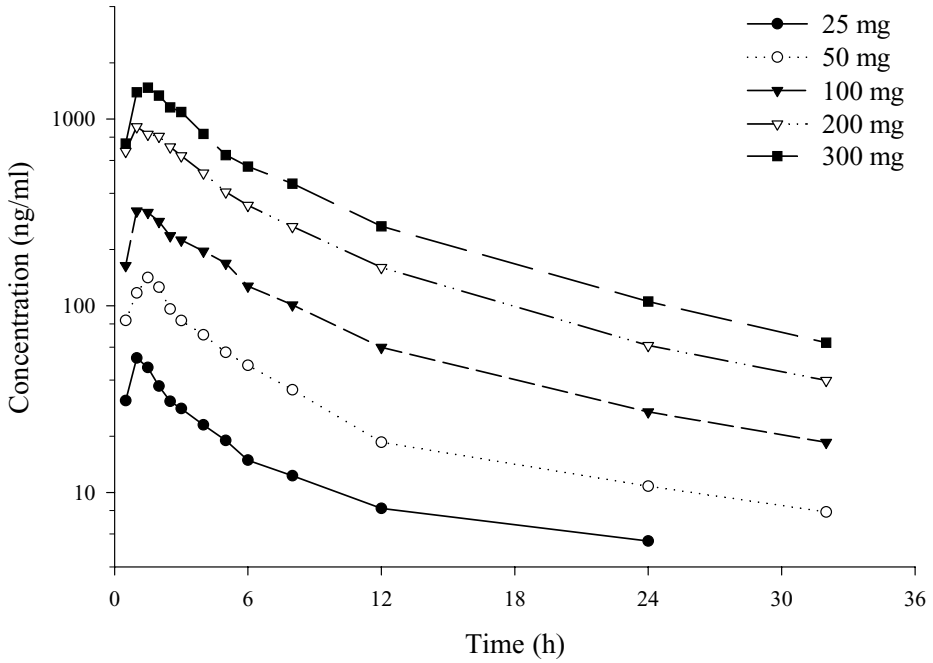

Fig. 4. Mean plasma concentration–time profile of DA after oral administration of DA to six healthy volunteers at doses ranging from 25 to 300 mg.

for at least 32 h in all dose groups, except in the lowest dose group (25 mg), which was nevertheless followed for at least 24 h. In addition, AUC%Extrap was calculated to evaluate the importance of measuring the last concentration using WinNonlin Professional version 3.1 (Pharsight Co., Mountain View, CA, USA) and the following equation: AUC%Extrap $= (AUC_{0-\infty} - AUC_{0-t})/AUC_{0-\infty} \times 100$, t means time of last measurable concentration. The AUC%Extrap values in the 25, 50, 100, 200 and 300 mg dosage groups were 23.8 ± 4.7, 12.1 ± 1.7, 11.3 ± 2.5, 7.7 ± 3.3 and 7.4 ± 1.8%, respectively (mean $\pm$ S.D., $n = 6$). These results indicated that the described method with an LLQ of 5 ng/ml in human plasma is suitable for deriving pharmacokinetic parameters with an acceptable accuracy.

4. Conclusion

We describe a sensitive and validated HPLC method for the quantification of a new phosphodiesterase V inhibitor, DA, in human plasma and urine. The assay covers the concentration range of interest and has a satisfactory accuracy, precision and lower quantification limit. Practical testing of the developed assay on samples from subjects orally administered 25–300 mg of DA also showed adequate sensitivity for clinical studies.

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