ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 527–533

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Determination of a new phosphodiesterase V inhibitor, DA-8159, in plasma and urine by high-performance liquid chromatography

Hyun Joo Shim [a,b], Eun Joo Lee [a], Young Hee Jung [b], So Hee Kim [c], Soon Hoe Kim [b], Moohi Yoo [b], Jong Won Kwon [b], Won Bae Kim [b], Myung Gull Lee [a,*]

[a] *College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Gu, Seoul 151-742, South Korea*
[b] *Research Laboratory, Dong-A Pharmaceutical Company Limited, 47-5 Sanggal-Ri, Yongin, Kyunggi-Do 449-900, South Korea*
[c] *College of Dentistry and Research Institute of Oral Science, Kangnung National University, 123, Jibyeon-Dong, Gangnung, Gangwon-Do 210-702, South Korea*

Received 7 January 2001; received in revised form 30 April 2002; accepted 8 May 2002

Abstract

A high-performance liquid chromatographic (HPLC) method using liquid–liquid extraction for sample preparation was developed for the determination of a new phosphodiesterase V inhibitor, DA-8159, in rat plasma and urine using sildenafil citrate as an internal standard. A 100 μl aliquot of 0.1 M Na_2CO_3 (containing sildenafil citrate, 3 μg/ml as free sildenafil) and a 1 ml aliquot of ether were added to a 100 μl aliquot of biological samples (urine samples were diluted 20 times with distilled water). After vortex centrifugation at $9000 \times g$ for 3 min, the ether layer was collected and dried under nitrogen gas. The residue was reconstituted with a 150 μl aliquot of the mobile phase, centrifuged, and a 100 μl aliquot of the supernatant was injected onto a reversed-phase column. The mobile phases, 20 mM KH_2PO_4 (pH 4.7):acetonitrile (70:30, v/v for plasma and tissue samples, and 75:25, v/v for urine samples), were run at a flow rate of 1.0 ml/min. The column effluent was monitored by an ultraviolet detector set at 292 nm. The retention times for DA-8159 and the internal standard were approximately 10.7 and 9.1 min, respectively, in plasma and tissue samples and the corresponding values in urine samples were 47 and 33 min. The detection limits for DA-8159 in rat plasma and urine were 20 and 100 ng/ml, respectively. The coefficients of variation of the assay were generally low: below 10% for plasma and 9.9% for urine. No interferences from endogenous substances were found.

Keywords: DA-8159; HPLC; Pharmacokinetics

1. Introduction

Sildenafil citrate (Viagra®), a potent and selective inhibitor of cyclic guanosine monophosphate

* Corresponding author. Tel.: +82-2-880-7855/7877; fax: +82-2-889-8693
E-mail address: leemg@snu.ac.kr (M.G. Lee).

0731-7085/02/$ - see front matter
PII: S0731-7085(02)00397-7

(cGMP)-specific phosphodiesterase type V (PDE V), the predominant isozyme metabolizing cGMP in the corpus cavernosum [1], is marketed as an oral agent to treat male erectile dysfunction. A high-performance liquid chromatographic (HPLC) analysis of sildenafil and one of its metabolite in plasma was reported using automated sequential trace enrichment of dialysates (ASTED) system to prepare plasma samples [2]. Research Laboratory of Dong-A Pharmaceutical Company (Yongin, South Korea) has recently developed a new PDE V inhibitor, DA-8159, 5-[2-propyloxy-5-(1-methyl-2-pyrollidinylethylamidosulfonyl)phenyl]-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo(4,3-D)pyrimidin-7-one (Fig. 1) to treat male erectile dysfunction. The negative logarithm of acidic dissociation constants of DA-8159, pK_{a1} and pK_{a2}, were approximately 6.5 and 12.5, respectively. The log partition coefficients (octanol/buffer solutions) of DA-8159 were 0.76, 0.75, 0.81 and 1.85 for buffer pHs of 1, 3, 5 and 7, respectively. DA-8159 (m.w. of 516.6 Da and melting point of 162–164 °C) is a weak basic drug. The solubilities of DA-8159 in solutions of pHs 2, 5, 7 and 10, and in distilled water are 27.4, 12.5, 0.82, 0.033 and 0.019 mg/ml, respectively. DA-8159 is now being evaluated in phase I clinical trial to treat male erectile dysfunction.

This paper describes the HPLC method with liquid–liquid extraction procedure for sample preparation of the determination of DA-8159 in rat plasma and urine. The application of the present HPLC method to the pharmacokinetics and tissue distribution of DA-8159 in rats was also reported.

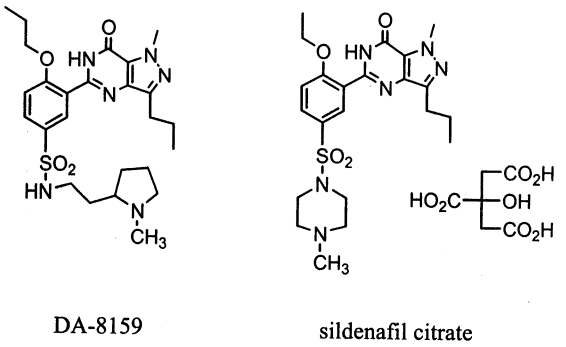

Fig. 1. Chemical structures of DA-8159 and sildenafil citrate (the HPLC internal standard).

2. Experimental

2.1. Chemicals

DA-8159 and sildenafil citrate (the internal standard of HPLC assay, Fig. 1) were donated from Research Laboratory of Dong-A Pharmaceutical Company. Various pH solutions ranging from 1 and 2 (HCl–KCl buffer), pH 3 ($KHC_8H_4O_4$–HCl buffer), pHs 4 and 5 ($KHC_8H_4O_8$–NaOH buffer), pHs 6 and 7 (KH_2PO_4–NaOH buffer), pHs 8 and 9 (H_3BO_3–KCl–NaOH buffer), pHs 10 and 11 ($NaHCO_3$–NaOH buffer), pH 12 (Na_2HPO_4–NaOH buffer), and to pH 13 (KCl–NaOH buffer) were purchased from Shinyo Pure Chemicals (Osaka, Japan). Other chemicals were of reagent grade or HPLC grade, and therefore were used without further purification.

2.2. Preparation of stock and standard solutions

A stock solution of DA-8159 was prepared in methanol (1 mg/ml). Appropriate dilutions of the stock solution were made with methanol. Standard solutions of DA-8159 in rat plasma and urine were prepared by spiking with the appropriate volume (less than 10 μl/ml of biological sample) of the diluted stock solution giving final concentrations of 0.02, 0.05, 0.1, 0.5, 1, 5, and 10 μg/ml. The internal standard solution was prepared by dissolving sildenafil citrate in 0.1 M Na_2CO_3 to give a final concentration of 3 μg/ml as free sildenafil. Only the HPLC assay results on 0.02, 0.05, and 10 μg/ml for rat plasma, and 0.1, 0.5, and 10 μg/ml for rat urine are listed in Table 1. Response factors were calculated by dividing the peak height (cm) of DA-8159 by its concentration (μg/ml)/dividing the peak height (cm) of internal standard by its concentration (μg/ml). Relative recoveries were calculated by dividing the response factor of DA-8159 in biological fluids by the response factor in distilled water. Accuracies were calculated by

Table 1
Response factors, relative recoveries, and accuracies of DA-8159 at various concentrations in rat plasma and urine using a liquid–liquid extraction procedure

Spiked concentration (μg/ml)	Response factor[a]	Relative recovery[b] (%)	Accuracy[c] (%)
Rat plasma			
10	0.532 (0.63)	115	95.9
0.05	0.576 (6.2)	110	103
0.02	0.536 (10)	114	96.6
Rat urine			
10	0.416 (7.8)	108	97.0
0.5	0.413 (6.1)	114	96.3
0.1	0.423 (8.2)	111	98.6

Values in parentheses are coefficients of variation (%); $n = 3$.
[a] [Drug peak height (cm) divided by its concentration (μg/ml)]/[internal standard peak height (cm) divided by its concentration (μg/ml)]; mean.
[b] Relative recovery compared with distilled water; mean.
[c] (Mean observed concentration/theoretical concentration) × 100; mean.

dividing mean observed concentration by theoretical concentration.

2.3. Sample preparation

A 100 μl aliquot of 0.1 M Na_2CO_3 (containing sildenafil citrate, an internal standard, 3 μg/ml as free sildenafil) and a 1 ml aliquot of ether were added to a 100 μl aliquot of biological samples (urine samples were diluted 20 times with distilled water). After vortex-mixing and centrifugation at $9000 \times g$ for 3 min, the ether layer was collected and dried under nitrogen gas. The residue was reconstituted with a 150 μl aliquot of mobile phase, centrifuged, and a 100 μl aliquot of the supernatant was injected directly onto the HPLC column.

2.4. HPLC apparatus

The HPLC system consisted of Gynkotek autosampler (Gynkotek HPLC, Munich, Germany), a model L-6000 pump (Hitachi, Tokyo, Japan), a reversed-phase (C_{18}) column [15 cm in length (25 cm in length for urine samples) × 4.6 mm, i.d.; particle size, 5 μm; Hichrom HPRPB, Berkshire, UK], a model UVIS200 UV detector (Linear, Reno, NV), and a model D-2500 integrator (Hitachi). The isocratic mobile phases, 20 mM KH_2PO_4 (pH 4.7):acetonitrile (70:30, v/v for plasma and tissue samples, and 75:25, v/v for urine samples), were run at a flow rate of 1.0 ml/min at room temperature and the column effluent was monitored by an UV detector set at 292 nm.

2.5. Pharmacokinetics after intravenous and oral administration of DA-8159

DA-8159 (the DA-8159 powder was dissolved in 0.05 M citric acid), 30 mg/kg, was infused over 1 min via the jugular vein or administered orally using feeding tubing to a male Sprague-Dawley rat. Blood (0.25 ml) was collected at 0 min (to serve as a control), 1 min (at the end of the infusion), 5, 15, 30, 60, 120, 180, 240, or 360 min after intravenous administration, and 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, or 360 min after oral administration. After centrifugation of blood samples, plasma samples were collected, and stored in the −20 °C freezer until HPLC analysis of DA-8159.

2.6. Tissue distribution after intravenous administration

The procedures are similar to those reported previously [3]. DA-8159, 30 mg/kg, was infused over 1 min via the jugular vein of a male Sprague-Dawley rat. Each rat tissue (or organ) was collected 30 min after 1 min intravenous adminis-

tration and was homogenized with 4 volume of Sørensen phosphate buffer of pH 7.4 using a tissue homogenizer (Ultra-Turrax, T25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany), and centrifuged immediately at 9000 × *g* for 10 min. After vortex-mixing centrifugation at 9000 × *g* for 10 min, two 500 μl aliquot of the supernatant were collected and stored in the −20 °C freezer until HPLC analysis of DA-8159.

2.7. Stability of DA-8159 in various pH solutions, and human and rat plasma and urine

DA-8159 stock solution in methanol was spiked (less than 10 μl/ml) in each glass test tube containing 5 ml of each pH solutions, and human and rat plasma and urine to make a DA-8159 concentration of 5 μg/ml. After vortex-mixing, each test tube was placed in a water-bath shaker kept at 37 °C and at a rate of 50 oscillations per min (opm). At 0, 1, 2, 4, 6, 8, 12, 24, and 48 h, a 50 μl aliquot was sampled from each test tube. The concentrations of DA-8159 in the above samples were analyzed as soon as the sample was collected.

3. Results and discussion

There was a strong UV absorption at 216 and 292 nm for DA-8159 using spectrophotometer. Due to higher background at 216 nm, 292 nm was chosen in the present study. The deproteinization procedure (by adding 2 volume of acetonitrile) for sample preparation caused poor detection limit (50 ng/ml, our unpublished data), therefore a liquid–liquid extraction procedure was employed. Fig. 2 shows typical chromatograms of drug-free rat plasma, drug standards in rat plasma, and plasma collected at 2 h after intravenous administration of 30 mg/kg of DA-8159 to a rat using a liquid–liquid extraction procedure; the corresponding chromatograms for rat urine and rat testis homogenates are shown in Figs. 3 and 4, respectively. No interferences from endogenous substances were observed in any of the biological samples (Figs. 2–4). The retention times for DA-8159 and the internal standard were approximately 10.7 and 9.1 min, respectively, in rat plasma (Fig. 2) and rat

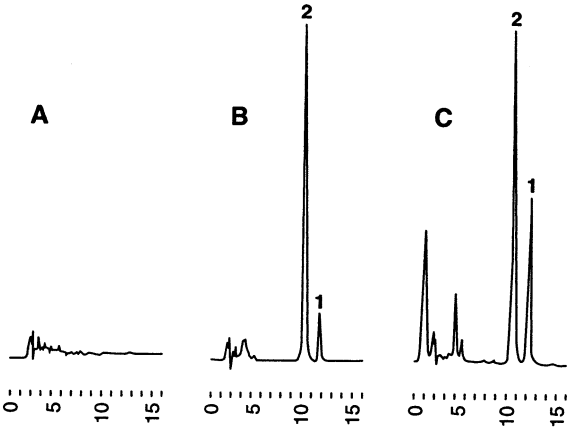

Fig. 2. Chromatograms after extraction of drug-free rat plasma (A), rat plasma spiked with 0.2 μg/ml of DA-8159 and 3 μg/ml of internal standard (B), and plasma collected 2 h (0.650 μg/ml) after 1 min intravenous administration of 30 mg/kg of DA-8159 to a male Sprague-Dawley rat (C). Peaks: 1—DA-8159 (10.7 min); 2—internal standard (9.1 min). The "0" marks the point of injection. The detector sensitivity was set at 0.02 AUFS and the recorder sensitivity was set at 16 mV.

tissue samples (Fig. 4). Although the extraction procedure was employed, the interference from urine sample could not be excluded, therefore, the mobile phase was changed to decrease acetonitrile content. Hence the retention times of DA-8159 and the internal standard in urine samples were much longer than those in plasma and tissues samples. The retention times for DA-8159 and the internal standard in rat urine were approximately 47 and 33 min, respectively.

The detection limits for DA-8159 in rat plasma and urine were 20 and 100 ng/ml, respectively (Table 1), based on a signal-to-noise ratio of 3.0. The detection limits were enough to study pharmacokinetics of DA-8159 in rats as will be discussed in pharmacokinetics in rats. Reproducibility of the present method was fairly good. The ranges of coefficients of variation (CVs) of DA-8159 in rat plasma and urine were 0.48–10 and 6.1–9.9%, respectively (Table 1). The accuracies ((mean observed concentration/theoretical concentration) × 100) of DA-8159 were 95.9–104 and 96.3–102% for rat plasma and urine, respectively (Table 1). The relative recoveries of DA-8159 were 110–117 and 106–114% for rat plasma

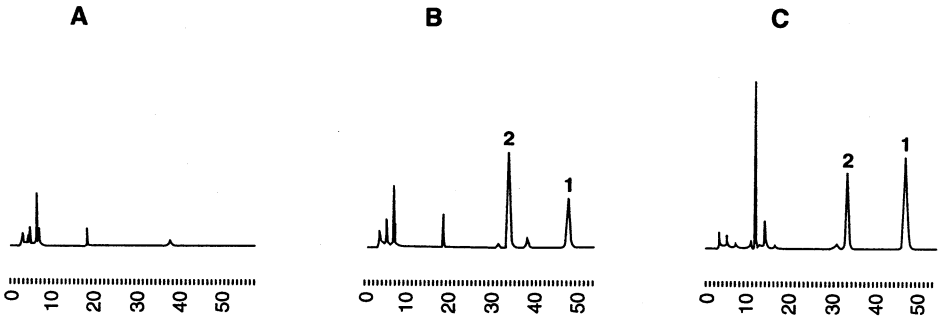

Fig. 3. Chromatograms after extraction of drug-free rat urine (A), rat urine spiked with 1.0 μg/ml of DA-8159 and 3 μg/ml of internal standard (B), and urine collected between 0 and 24 h (2.63 μg/ml) after 1 min intravenous administration of 30 mg/kg of DA-8159 to a male Sprague-Dawley rat (C). Peaks: 1—DA-8159 (47 min); 2—internal standard (33 min). The "0" marks the point of injection. The detector sensitivity was set at 0.02 AUFS and the recorder sensitivity was set at 16 mV.

and urine, respectively (Table 1). The exact reason why the accuracies for rat plasma and urine are greater than 100% is not clear. This could be due to pH differences in human plasma and urine, and distilled water. The log partition coefficients of DA-8159 were also dependent on buffer pHs as mentioned earlier.

The present HPLC method was successful for the pharmacokinetic studies after intravenous or oral administration of DA-8159 to a rat (Fig. 5) and determination of DA-8159 in rat tissues (or organs). The plasma concentrations of DA-8159 declined in a polyexponential fashion with a terminal half-life of 115 min after intravenous

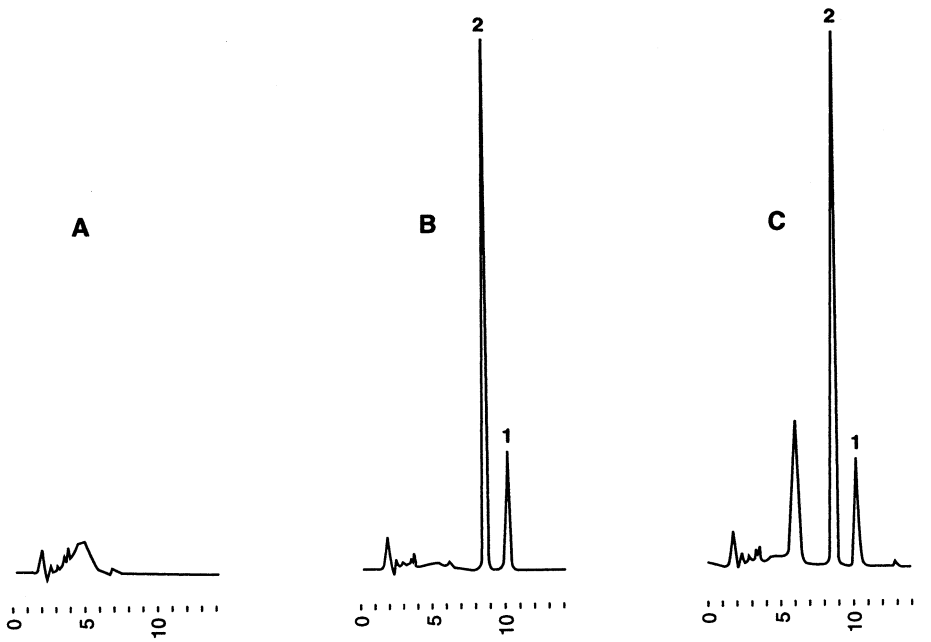

Fig. 4. Chromatograms after extraction of drug-free rat testis homogenates (A), rat testis homogenates spiked with 1 μg/ml of DA-8159 and 3 μg/ml of internal standard (B), and testis homogenate collected 30 min (0.962 μg/ml) after oral administration of 30 mg/kg of DA-8159 to a male Sprague-Dawley rat (C). Peaks: 1—DA-8159 (10.7 min); 2—internal standard (9.1 min). The "0" marks the point of injection. The detector sensitivity was set at 0.02 AUFS and the recorder sensitivity was set at 16 mV.

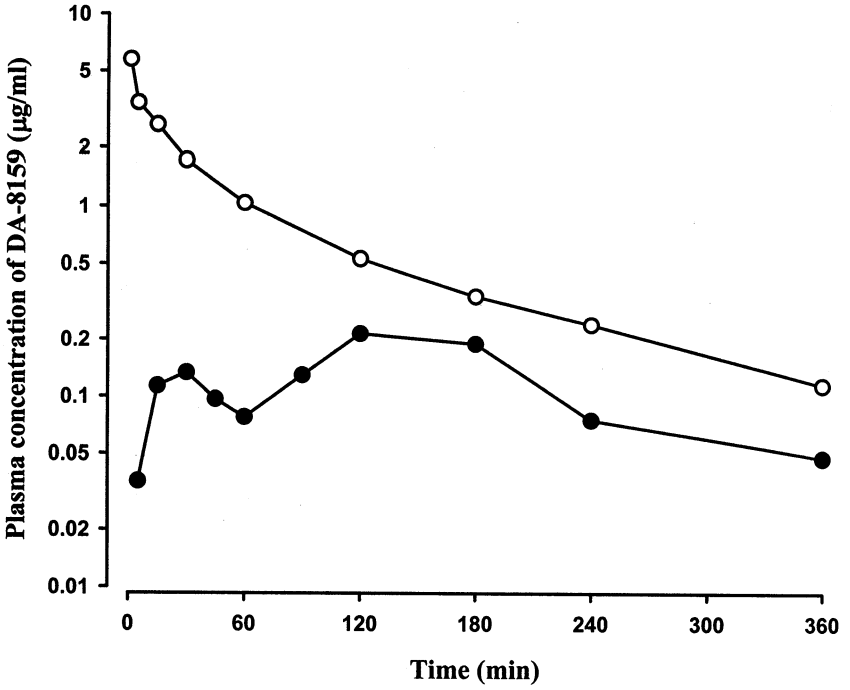

Fig. 5. Plasma concentration–time profiles of DA-8159 after intravenous (○) or oral (●) administration of the drug, 30 mg/kg, to a rat.

administration to a rat (Fig. 5). Absorption of DA-8159 from rat gastrointestinal tract was fast; plasma concentration of DA-8159 was detected from the first blood sampling time (5 min) and similar plasma concentrations were maintained from 30 to 360 min (Fig. 5), and this could be due to continuous absorption of the drug from rat gastrointestinal tract. The detection limit for DA-8159 was approximately 100 ng/ml for rat tissues (or organs) studied. The amounts of DA-8159 recovered from each gram of tissue (or organ) were 0.085 μg/ml for plasma, and 0.332, 0.758, 2.35, 0.477, 0.293, 16.5, 8.06, 9.93, 198, 141, and 2.72 μg/g for brain, eye, heart, testis, penis, liver, lung, kidney, stomach, small intestine, and large intestine, respectively.

DA-8159 was relatively stable in each pH solutions, and human and rat plasma and urine at DA-8159 concentration of 5 μg/ml for up to 48 h incubation; more than 90% of the spiked amount of DA-8159 was recovered after 48 h incubation.

4. Conclusion

The present HPLC method was enough to study pharmacokinetics of DA-8159 by measuring the concentrations (or amounts) of the drug in rat plasma, urine, and tissues. The present HPLC method employed a liquid–liquid extraction procedure for sample preparation, not ASTED system, and required less than 100 μl of plasma sample, which is proper volume for pharmacokinetics studies on small animals since it is not easy to collect much blood in small animals.

Acknowledgements

This work was supported in part by the Ministry of Health and Welfare (HMP-99-D-07-0003) 1999–2000.

References

[1] M.M. Goldenberg, Clin. Ther. 20 (1998) 1033–1048.
[2] J.D. Cooper, D.C. Muirhead, J.E. Tayler, P.R. Baker, J. Chromatogr. B 701 (1997) 87–95.
[3] S.H. Kim, W.B. Kim, M.G. Lee, Drug Metab. Dispos. 27 (1999) 710–716.