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# Determination of a new fluoroquinolone antimicrobial agent, (*S*)-10-[(*S*)-(8-amino-6-azaspiro[3,4]octan-6-yl)]-9-fluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid hemihydrate, DV-7751a, in human serum and urine 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 new fluoroquinolone antimicrobial agent, (*S*)-10-[(*S*)-(8-amino-6-azaspiro[3,4]octan-6-yl)]-9-fluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid hemihydrate (DV-7751a, I) in human serum and urine has been developed. Compound I and the internal standard were extracted from serum and urine by means of Bond Elut C<sub>8</sub> LRC column. The extracts were chromatographed on a reversed-phase Inertsil ODS-2 column using tetrahydrofuran–50 mM KH<sub>2</sub>PO<sub>4</sub> (pH 2)–1 M ammonium acetate (19:81:1, v/v) as the mobile phase at a flow-rate of 1.0 ml/min. Fluorescence detection at an excitation wavelength of 305 nm and an emission wavelength of 530 nm resulted in a limit of quantitation of 0.0098 μg/ml for serum and 0.098 μg/ml for urine. The method showed satisfactory sensitivity, precision, accuracy, recovery and selectivity. Stability studies showed that I was stable in serum and urine for at least 1 month at –20°C and for at least 48 h at room temperature.

## 1. Introduction

(*S*)-10-[(*S*)-(8-Amino-6-azaspiro[3,4]octan-6-yl)]-9-fluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid hemihydrate (DV-7751a, I) is a new fluoro-

quinolone antimicrobial agent with high in-vitro activity against a broad spectrum of Gram-positive and Gram-negative organisms [1,2]. The chemical structure of I is shown in Fig. 1. Compound I possesses a more potent antibacterial activity against staphylococci, streptococci, and enterococci than ofloxacin and ciprofloxacin [3,4] and has an activity similar to tosufloxacin and sparfloxacin [5,6]. Based on these promising

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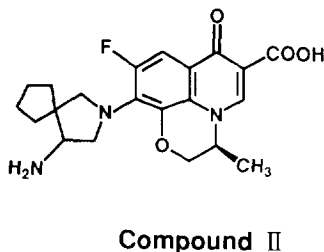
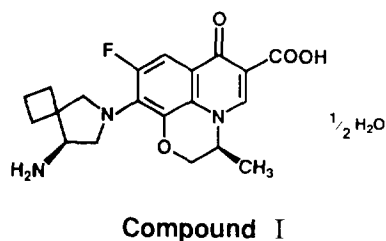


Fig. 1. Chemical structures of DV-7751a (I) and the internal standard (DY-9752, II).

pharmacological properties, I 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 I is essential to support these early clinical studies.

This paper describes a method for the determination of I in human serum and urine using solid-phase extraction and HPLC with fluorescence detection.

## 2. Experimental

### 2.1. Chemicals and reagents

Compound I and the internal standard used for the serum and urine assay, (-)-10-(4-amino-2-azaspiro[4.4]nonan-2-yl)-9-fluoro-2,3-dihydro-3-(S)-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (DY-9752, II) were synthesized by Daiichi Pharmaceutical Co. (Tokyo, Japan). The chemical structures of I and II are shown in Fig. 1. Tetrahydro-

furan (THF) was HPLC-grade from 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 Assoc., Millipore, Milford, MA, USA) was used. Consera (Nissui Seiyaku Co., Tokyo, Japan) was used as control human serum. Human blank serum and urine were obtained from healthy male volunteers.

### 2.2. Instruments and chromatographic conditions

The chromatographic system consisted of a high-performance liquid chromatograph (Model LC-10AD, Shimazu Corp., Kyoto, Japan), a degasser (ERMA CR, Saitama, Japan), a fluorescence detector (Model F1000, Hitachi, Tokyo, Japan) operated at an excitation wavelength of 305 nm and an emission wavelength of 530 nm and a reversed-phase Inertsil ODS-2 (150 × 4.6 mm I.D., 5 μm particle size) column (GL Sciences, Tokyo, Japan). A precolumn filter (Irica, Kyoto, Japan) was attached to the analytical column. The column temperature was kept at 25°C in a column oven (Model CTO-2A, Shimazu, Kyoto, Japan). Samples of 50 μl were injected automatically onto the HPLC system by an autosampler (Model AS-8000, Tosoh, Tokyo, Japan). Tetrahydrofuran–50 mM KH<sub>2</sub>PO<sub>4</sub> (adjusted to pH 2.0 with phosphoric acid)–1 M ammonium acetate (19:81:1, 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

Stock solutions of standards were prepared by dissolving accurately weighed amounts of I (approximately 10 mg) and II (approximately 10 mg) in water (100 ml) with addition of 2 M acetic acid solution (2–3 drops) in a volumetric flask. The solutions were stored at 4°C in refrigerator for a maximum of 1 month. Serum standards were prepared at concentrations of 0.0098, 0.0489, 0.0977, 0.244, 0.489 and 0.977 μg/ml of anhydrous I by spiking the control

human serum (9.0 ml) with the standard solution (1.0 ml of 97.7  $\mu\text{g/ml}$ ) and successively diluting with control serum. Urine standards were prepared at concentrations of 0.098, 0.489, 0.977, 2.44, 4.89 and 9.77  $\mu\text{g/ml}$  of anhydrous I by spiking the blank human urine (4.5 ml) with the standard solution (0.5 ml of 97.7  $\mu\text{g/ml}$ ) and successively diluting with blank urine.

#### 2.4. Assay procedure

Bond Elut  $\text{C}_8$  LRC columns (Analytichem International, Harbor City, CA, USA) were activated prior to use by passage of methanol (4 ml), water (6 ml) and 50 mM  $\text{KH}_2\text{PO}_4$  (6 ml).

#### Serum sample preparation

A 0.2-ml aliquot of human control serum was transferred into a 13-ml disposable glass centrifuge tube and 0.4 ml of 50 mM  $\text{KH}_2\text{PO}_4$  and 0.2 ml of internal standard solution (0.1  $\mu\text{g/ml}$ ) were added. The resulting mixture was vortex-mixed and applied to a Bond Elut  $\text{C}_8$  LRC column using gentle suction. The column was washed successively with 50 mM  $\text{KH}_2\text{PO}_4$  (6 ml) and THF– $\text{H}_2\text{O}$  (20:80, v/v; 2 ml). Compound I and the internal standard were eluted from the column with THF–0.15%  $\text{H}_3\text{PO}_4$  (30:70, v/v; 3 ml). The eluate was evaporated to dryness with a centrifugal evaporator (Model CVE-200D, Tokyo Rikakikai Co., Tokyo, Japan). The residue was dissolved in THF– $\text{H}_2\text{O}$  (20:80, v/v; 0.3 ml) and a 50- $\mu\text{l}$  aliquot was injected onto the HPLC system.

#### Urine sample preparation

All assay procedures were the same as those for human serum except that a 0.2-ml aliquot of human urine and a 1.0  $\mu\text{g/ml}$  internal standard solution were used.

#### 2.5. Calibration curves

The peak area of I was divided by the peak area of the internal standard to obtain the peak-area ratio. The calibration curve for I was constructed from a least-square linear regression of the peak-area ratios of the standards versus

the drug concentrations. The drug concentrations in human serum and urine were expressed as equivalents of anhydrate of I.

#### 2.6. Recovery

The absolute recovery of I from human serum and urine was estimated by comparing the peak area obtained from injections of standards with those obtained from the injection of extracts of serum and urine samples spiked with known concentrations of I.

#### 2.7. Selectivity

Blank serum and urine 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 ( $n = 6$ ) of the serum and urine standards.

Inter-day precision and accuracy were determined by assaying the serum and urine 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 I in human serum and urine was investigated by preparing pooled serum or urine spiked with known amounts of I. The pools (10 ml) in glass tubes (13 ml) were allowed to stand at room temperature and 1.5-ml aliquots were taken at 4, 8, 24 and 48 h. Samples (2.5 ml) in capped plastic tubes (10 ml) were also stored at  $-20^\circ\text{C}$  for one month. These samples were assayed periodically by the method described above.

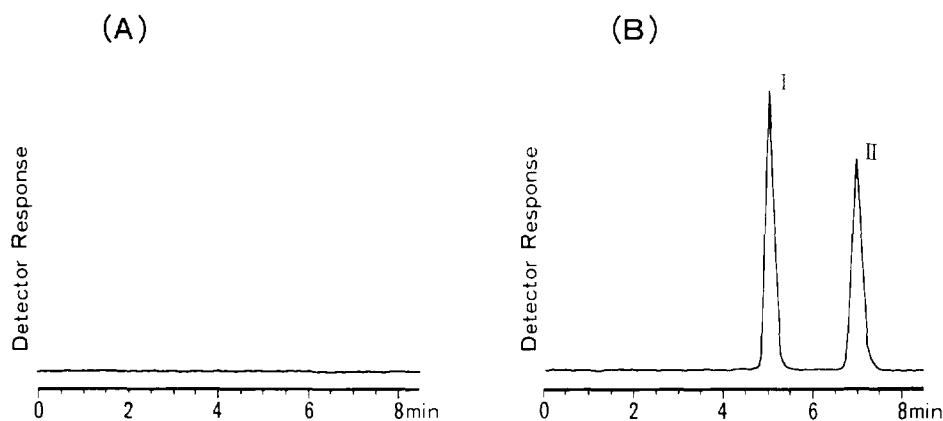


Fig. 2. Representative chromatograms of 0.2-ml serum extracts obtained from (A) control human serum and (B) control human serum spiked with I ( $0.977 \mu\text{g/ml}$ ) and II. The chromatograms are shown with a full scale of 300 mV.

### 3. Results and discussion

#### 3.1. Chromatography and selectivity

After characterization of the fluorescence spectrum of I in the mobile phase, excitation and emission wavelengths for fluorescence detection were set at 305 and 530 nm, respectively.

Well-defined chromatographic peaks for I and the internal standard were obtained on the  $5 \mu\text{m}$  Inertsil ODS-2 column ( $150 \times 4.6 \text{ mm I.D.}$ ), where the free silanol groups were almost com-

pletely end-capped. Representative chromatograms of extracts from control serum and urine are shown in Fig. 2A and 3A, respectively. These chromatograms indicated that no endogenous compounds interfered at the retention times of I and II. Representative chromatograms of control serum and urine spiked with I and II are shown in Fig. 2B and 3B, respectively. Compounds I and II were well-resolved from each other, and the retention times of I and II were approximately 5.2 and 7.2 min, respectively. The overall chromatographic run time was ca.

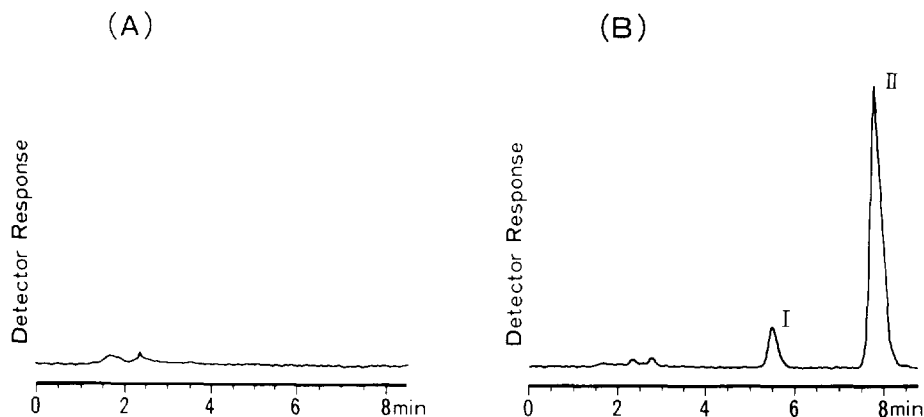


Fig. 3. Representative chromatograms of 0.2-ml urine extracts obtained with (A) blank human urine and (B) blank human urine spiked with I ( $0.098 \mu\text{g/ml}$ ) and II. The chromatograms are shown with a full scale of 300 mV.

8.5 min. Serum and urine collected from six healthy volunteers also showed no interferences.

### 3.2. Calibration curves

Calibration curves for serum obtained over a one-week period ( $n=3$ ) were linear and reproducible with mean  $\pm$  standard deviation values for the constants in the regression equation of  $y = (0.0966 \pm 0.0056)x + (0.00139 \pm 0.00178)$ . Correlation coefficients were always greater than 0.999. The inter-day coefficient of variation of the slope of the calibration curve was 5.8%. The least-square linear regression equation obtained for human urine ( $n=3$ ) over a one week period was  $y = (0.964 \pm 0.016)x - (0.0134 \pm 0.0228)$  with correlation coefficients greater than 0.999. The inter-day coefficient of variation of the slope of the calibration curve was 1.6%. The curve for serum was linear in the concentration range of 0.0098–0.977  $\mu\text{g/ml}$  and for urine 0.098–9.77  $\mu\text{g/ml}$ , respectively.

### 3.3. Recovery of extraction

The absolute recoveries of I from serum and urine were determined by comparing the peak area of extracted standards with those of injected standards (Table 1). The recoveries of spiked human serum were evaluated at the concentrations of 0.0098, 0.0977 and 0.977  $\mu\text{g/ml}$  in replicates of six. The recoveries of spiked human

urine were determined at concentrations of 0.098, 0.977 and 9.77  $\mu\text{g/ml}$  in replicates of six. The recoveries ranged from 104 to 113% in serum and from 89.9 to 97.7% in urine. In addition, the recoveries of the internal standard from serum and urine were  $94.3 \pm 3.3\%$  and  $92.8 \pm 1.2\%$  (mean  $\pm$  S.D.,  $n=6$ ), respectively.

### 3.4. Precision and accuracy

The intra-day precision and accuracy of the method for serum were evaluated by analyzing human serum spiked with I at concentrations of 0.0098, 0.0977, 0.489 and 0.977  $\mu\text{g/ml}$  in replicates of six (Table 2). Precision was based on the calculation of the coefficient of variation (C.V.). An indication of accuracy was based on the calculation of the relative error (R.E.) of the found concentration compared that of the theoretical value. The C.V. ranged from 3.5 to 6.2% and the R.E. ranged from  $-5.1$  to 1.9% of the theoretical values at concentrations above 0.0489  $\mu\text{g/ml}$ . The limit of quantitation using a 0.2-ml sample was set at the concentration of the lowest calibration standard or 0.0098  $\mu\text{g/ml}$  of serum with a C.V. of 12.1%, R.E. of  $-8.5\%$  and a signal-to-noise ratio of at least 5.

Intra-day precision and accuracy of the meth-

Table 1  
Recoveries of I from human serum and urine

Concentration ( $\mu\text{g/ml}$ )	Recovery (%)	C.V. <sup>a</sup> (%)	<i>n</i>
<i>Serum</i>			
0.0098	113	15.9	6
0.0977	107	3.4	6
0.977	104	2.6	6
<i>Urine</i>			
0.098	97.7	3.4	6
0.977	94.1	2.8	6
9.77	89.9	2.0	6

<sup>a</sup> C.V. = coefficient of variation.

Table 2  
Intra-day precision and accuracy for analysis of I in human serum and urine

Theoretical concentration ( $\mu\text{g/ml}$ )	Mean found concentration ( $\mu\text{g/ml}$ )	C.V. <sup>a</sup> (%)	R.E. <sup>b</sup> (%)	<i>n</i>
<i>Serum</i>				
0.0098	0.0089	12.1	-8.5	6
0.0977	0.0961	4.0	-1.6	6
0.489	0.492	3.5	0.5	6
0.977	0.996	6.2	1.9	6
<i>Urine</i>				
0.098	0.092	1.4	-5.7	6
0.977	0.986	1.9	0.9	6
4.89	4.89	3.9	0.0	6
9.77	9.71	4.1	-0.6	6

<sup>a</sup> C.V. = coefficient of variation.

<sup>b</sup> R.E. = relative error.

Table 3  
Inter-day precision and accuracy for analysis of I in human serum and urine

Theoretical concentration ( $\mu\text{g/ml}$ )	Mean found concentration ( $\mu\text{g/ml}$ )	C.V. <sup>a</sup> (%)	R.E. <sup>b</sup> (%)	<i>n</i>
<i>Serum</i>				
0.0098	0.0113	18.5	16.1	3
0.0977	0.0911	4.9	1.4	3
0.489	0.490	4.9	0.2	3
0.977	0.981	4.1	0.4	3
<i>Urine</i>				
0.098	0.084	27.7	-13.9	3
0.977	0.978	0.8	0.06	3
4.89	4.85	0.9	-0.7	3
9.77	9.79	2.4	0.2	3

<sup>a</sup> C.V. = coefficient of variation.

<sup>b</sup> R.E. = relative error.

od for urine were evaluated by analyzing human urine spiked with I at concentrations of 0.098, 0.977, 4.89 and 9.77  $\mu\text{g/ml}$  in replicates of six (Table 2). C.V.s were  $\leq 4.1\%$  and the R.E. ranged from -1.4 to 1.2 of the theoretical values at concentrations above 0.489  $\mu\text{g/ml}$ . The limit of quantitation using a 0.2-ml sample was set at the concentration of the lowest calibration standard, or 0.098  $\mu\text{g/ml}$  of urine with C.V. of 1.4%,

R.E. of -5.7% and a signal-to-noise ratio of at least 30.

The inter-day precision and accuracy for serum were assessed by the analysis of six samples at concentrations of 0.0098 to 0.977  $\mu\text{g/ml}$  of serum on three separate days (Table 3). The C.V. was  $\leq 7.9\%$  and the R.E. ranged from -0.8 to 1.4% at concentrations above 0.0489  $\mu\text{g/ml}$ . At the quantitation limit of 0.0098  $\mu\text{g/ml}$ , the method showed acceptable precision and accuracy with C.V. of 18.5% and R.E. of 16.1%. The inter-day precision and accuracy for urine was assessed by the analysis of six samples at concentrations of 0.098-9.77  $\mu\text{g/ml}$  of urine on three separate days (Table 3). The C.V. was  $\leq 4.9\%$  and the R.E. ranged from -2.4 to 0.2%. At the quantitation limit of 0.098  $\mu\text{g/ml}$ , the precision and accuracy would be less satisfactory with C.V. of 27.7% and R.E. of -13.9%. Accuracy and precision could possibly be improved by using a larger volume ( $> 0.2$  ml) of urine sample.

### 3.5. Stability

The stability of I in serum and urine was assessed at room temperature and  $-20^\circ\text{C}$  (Tables 4 and 5). Compound I was found to be stable for at least 48 h at room temperature and

Table 4  
Stability of I in human serum and urine at room temperature

Initial concentration ( $\mu\text{g/ml}$ )	Percent difference from the initial concentration at the indicated time <sup>a</sup>				
	0 h	4 h	8 h	24 h	48 h
<i>Serum</i>					
0.017	0.0	5.8	4.6	15.2	23.3
0.101	0.0	-1.9	-3.5	-3.1	-4.4
1.02	0.0	-0.6	0.0	-1.4	-0.2
<i>Urine</i>					
0.166	0.0	-2.1	-3.5	-3.2	-0.2
1.17	0.0	-2.9	-5.2	-4.4	-2.6
10.1	0.0	-2.0	-2.1	-2.2	-0.3

<sup>a</sup> Results are reported as the mean percent difference of six determinations.

Table 5  
Stability of I in human serum and urine at  $-20^{\circ}\text{C}$

Initial concentration ( $\mu\text{g/ml}$ )	Percent difference from the initial concentration at the indicated time <sup>a</sup>	
	0 h	1 month
<i>Serum</i>		
0.094	0.0	0.5
1.01	0.0	-5.5
<i>Urine</i>		
0.058	0.0	-12.1
0.970	0.0	-2.4
10.1	0.0	4.9

<sup>a</sup> Results are reported as the mean percent difference of six determinations.

for at least one month at  $-20^{\circ}\text{C}$ . These studies indicated that the samples containing I could be handled under normal laboratory conditions without significant loss of the compound.

#### 4. Conclusions

Solid-phase extraction has been successfully applied to the extraction of a new antimicrobial

agent, DV-7751a, from human serum and urine. The method is satisfactory with respect 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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