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Sensitive semi-microcolumn high-performance liquid chromatographic method for the determination of DU-6681, the active parent drug of a new oral carbapenem antibiotic, DZ-2640, in human plasma and urine using a column-switching system as sample clean-up procedure

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Abstract

DZ-2640 is a new oral carbapenem antibiotic having a dihydro-pyrroloimidazole ring as a side chain and a pivaloyloxymethyl (POM) ester prodrug of DU-6681, the active parent compound. A simple and sensitive column-switching semi-microcolumn high-performance liquid chromatographic method for the determination of DU-6681 in human plasma and urine has been developed. Human plasma was diluted with an equal volume of 1 M MOPS buffer (pH 7.0) and the mixture was filtered through an Ultrafree C3GV. The resulting filtrate was injected without further cleanup onto the HPLC system. Human urine was diluted with an equal volume of 1 *M* MOPS buffer (pH 7.0) and the mixture was directly injected onto the HPLC system. The analyte was detected by monitoring the column effluent with UV light at a wavelength of 300 nm, which resulted in the limit of quantitation of 0.008 μg/ml of plasma and 0.32 μg/ml of urine. Calibration curves were linear in the range of 0.008 to 5.85 μg/ml in plasma and 0.32 to 104.4 μg/ml in urine. The present methods showed greatly increased sensitivity for DU-6681 compared to conventional HPLC methods and also showed satisfactory recovery, selectivity, precision, and accuracy. Stability studies showed that 1 *M* MOPS buffer (pH 7.0) acted as a stabilizer. In plasma and urine diluted with equal volume of the buffer, DU-6681 showed good stability at −80°C for up to 4 weeks with no significant loss of the drug.

Keywords: Carbapenem antibiotics

1. Introduction

DZ-2640 is a new oral carbapenem antibiotic having a dihydro-pyrroloimidazole ring as a side chain and a pivaloyloxymethyl (POM) ester prodrug of (4*R*,5*S*,6*S*)-3-[[(6*S*)-6,7-dihydro-5*H*-pyrrolo[1,2-a]imidazol-6-yl]thio]-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (DU-6681, the active parent drug). The bioavailability of DU-6681 was greatly improved by esterification of the carboxy group at the C-2 posi-

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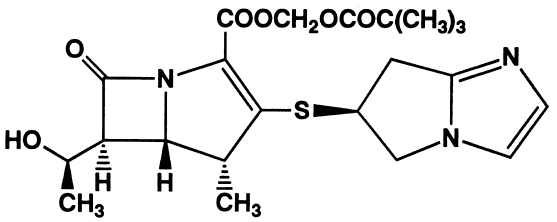
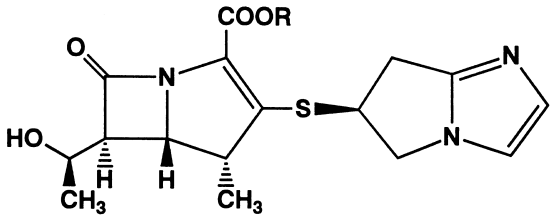
tion of the carbapenem ring with POM. DZ-2640 is believed to be hydrolyzed by nonspecific esterases in the intestinal tracts of humans to produce DU-6681, pivalic acid and formaldehyde in a manner similar to that of other prodrugs [1–4]. DU-6681 has a high in vitro activity against a broad spectrum of gram-positive and gram-negative organisms [5]. The chemical structures of DZ-2640, DU-6681a and DU-6681 are shown in Fig. 1.

On the basis of these promising pharmacological and pharmacokinetic properties, DZ-2640 was selected for phase I clinical evaluation. Determination of the pharmacokinetics of a drug under investigation is one of the main objectives of a phase I study. A sensitive and selective method for the determination of DU-6681 is essential to support these early clinical studies.

High-performance liquid chromatography (HPLC) plays a very important role in the trace analysis of drugs and their metabolites. Several HPLC methods have been reported for the quantitation of cabarpenems including imipenem and panipenem in human plasma and urine [6–10]. Carbapenems exhibit limited stability in biological fluids and special precautions are necessary to minimize artificial degradation during analytical procedures [6–10]. The column switching technique allows direct injection of biological fluids in to a HPLC system, which avoids labor intensive sample preparation and block or slow the degradation of analytes with finite stability.



DZ-2640



R=Na: DU-6681a
R=H : DU-6681

Fig. 1. Chemical structures of DZ-2640, DU-6681a and DU-6681.

All the methods previously reported employed conventional HPLC system and UV detection, which resulted in quantitation limit of 0.3–0.05 μg/ml of plasma [6–10]. Higher sensitivity of the method for the determination of DU-6681 specially in plasma was required because of its higher antimicrobial activity and expected lower starting dose in the phase I study.

Microcolumn LC was introduced in the pioneering studies by Scott and Kucera [11], Tsuda and Novotny [12] and Ishii et al. [13]. Considerable improvements in column technology and instrumentation have been made with this technique [14]. Semi-microcolumns with a diameter of 1.0 to 2.0 mm are being accepted as a compromise between conventional and microcolumn HPLC. Microcolumn and semi-microcolumn HPLC have many advantages over conventional-size HPLC such as higher sensitivity and reduced mobile phase consumption. However, low sample loadability (in volume) has limited their applicability. Recently, it has been reported that the column switching technique which uses a low dead volume valve unit could solve this problem [15–17].

This paper describes a sensitive and selective column-switching semi-microcolumn HPLC methods for the determination of DU-6681 in human plasma and urine, which are simple, reliable, and applicable for the analysis of plasma and urine samples in the phase I study of DZ-2640.

2. Experimental

2.1. Chemicals and reagents

The sodium salt of (4*R*,5*S*,6*S*)-3-[[(6*S*)-6,7-dihydro-5*H*-pyrrolo[1,2-a]imidazol-6-yl]thio]-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabi-

cyclo[3.2.0]-hept-2-ene-2-carboxylic acid (DU-6681a) was synthesized by Daiichi Pharmaceutical Co. (Tokyo, Japan). Acetonitrile and methanol were an HPLC-grade solvent (Kanto Chemical, Tokyo, Japan). 3-(N-Morpholino)propanesulfonate (MOPS) was purchased from Sigma (St. Louis, MO, USA). 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.

2.2. Instruments and chromatographic conditions for plasma assay

Schematic diagram of the column-switching semi-microcolumn HPLC system is shown in Fig. 2. The HPLC system consisted of the components of the nanospace SI-1 series (Shiseido, Tokyo, Japan), which were two 2001 pumps, 2009 degasser, a 2004 column oven, and a 2002 variable-wavelength UV detector. The UV detector was operated at 300 nm. An 2003 autosampler (Shiseido) and a 2011 high-pressure two position six-port switching valve (Shiseido) were used to provide automated on-line extraction and analysis. The Bioptic AV-2 (33×4.6 mm I.D., 5 μm particle size) (GL Sciences, Tokyo, Japan) was used as an extraction column. One hundred m*M* phosphate buffer (pH 6.5) was used as an eluent for the extraction column. A CAPCELL Pak C18 UG120 (35×1.5 mm I.D., 5 μm particle size) (Shiseido) was used as an intermediate column in the three-column system and attached ahead of the analytical column, which was Inertsil ODS-2 (150×1.5 mm I.D., 5 μm particle size) column (GL Sciences). A short intermediate column was placed between pre and analytical columns to minimize loss in separation efficiency. A mixture of 100 m*M* phosphate buffer (pH 6.5)/acetonitrile (95:5, v/v) was used as the analytical mobile phase at a flow rate of 0.1 ml/min (pump B). The eluents were degassed in an ultrasonic bath before use. The column temperature was kept at 40°C in a column oven.

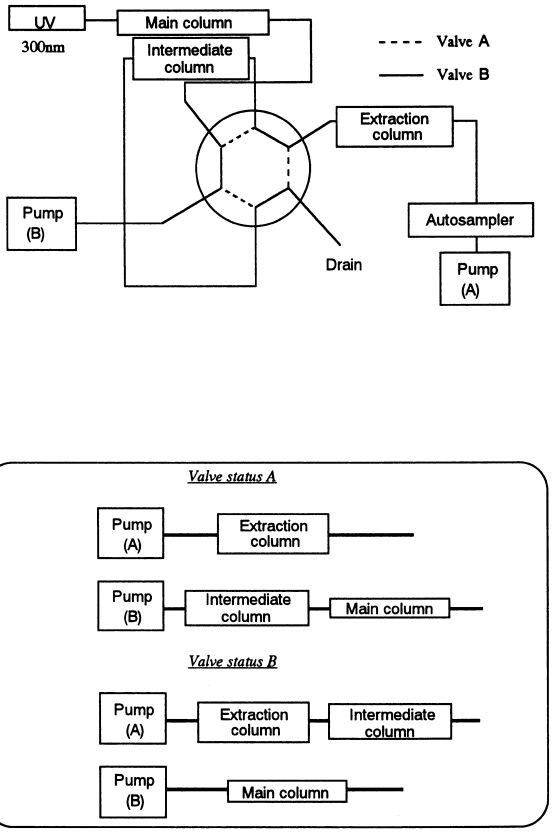


Fig. 2. Schematic diagram of column-switching system.

2.3. Instruments and chromatographic conditions for urine assay

The HPLC system was the same as that for plasma assay except that LiChrosorb NH_2 (10 mm×4.0 mm I.D., 5 μm particle size) (E. Merk, Darmstadt, Germany) was used as an extraction column.

2.4. Column-switching procedure for plasma sample

Step 1 (0–2 min). The valve is in position A. The plasma samples were injected onto the extraction column. One hundred m*M* phosphate buffer (pH 6.5) is used as an eluent for the extraction column at a flow rate of 0.5 ml/min (pump A).

Step 2 (2–8 min). The valve is switched to position B. One hundred m*M* phosphate buffer (pH 6.5) is passed through the extraction column to the intermediate column at a flow rate of 0.2 ml/min for 6 min. The flow rate of the eluent for the extraction column is decreased from 0.5 ml/min to 0.2 ml/min at 2 min to avoid increase in column pressure. The

analyte eluted from the extraction column is trapped on the intermediate column.

Step 3 (8–20 min). The valve is switched to position A and the analytical mobile phase was passed through the intermediate column in the backflush mode and the retained components are eluted onto the analytical column, where the analyte is separated at a flow rate of 0.1 ml/min for ca. 12 min. The flow rate of the eluent for the extraction column is increased from 0.2 ml/min to 0.5 ml/min at 8.5 min. The extraction column is allowed to be stabilized for ca. 11.5 min.

Step 4 (0–2 min). The next sample is injected onto the extraction column.

2.5. Column-switching procedure for urine sample

Step 1 (0–2 min). The valve is in position A. The urine samples are injected onto the extraction column. One hundred m*M* phosphate buffer (pH 6.5) is used as an eluent for the extraction column at a flow rate of 0.2 ml/min (pump A).

Step 2 (2–6 min). The valve is switched to position B. One hundred m*M* phosphate buffer (pH 6.5) was passed through the extraction column to the intermediate column at a flow rate of 0.2 ml/min for 4 min. The analyte eluted from the extraction column was trapped on the intermediate column.

Step 3 (6–18 min). The valve is switched to position A and the analytical mobile phase was passed through the intermediate column in the backflush mode and the retained components are eluted onto the analytical column, where the analyte is separated at a flow rate of 0.1 ml/min for ca. 12 min (pump B). The extraction column is allowed to be stabilized for ca. 12 min.

Step 4 (0–2 min). The next sample is injected onto the extraction column.

2.6. Preparation of standard solutions

Stock solutions of standards were prepared by dissolving accurately weighed DU-6681a (approximately 10 mg) in 50 m*M* MOPS buffer (pH 7.0) in a volumetric flask. The solutions were stored at −20°C in freezer. The working standard solutions were prepared just before use by diluting the stock solution with 1 *M* MOPS buffer (pH 7.0). Plasma standards were prepared at concentrations of 0.008, 0.024, 0.072, 0.217, 0.650, 1.95 and 5.85 μg/ml of the free acid of DU-6681a by adding working standard solutions of DU-6681 (0.1 ml) to the control human plasma (0.1 ml). Urine standards were prepared at concentrations of 0.32, 0.97, 2.90, 8.70, 26.1, 52.2 and 104.4 μg/ml by adding the working standard solutions of DU-6681 (0.1 ml) to the control human urine (0.1 ml).

2.7. Assay procedures for plasma

Human plasma (0.1 ml) was diluted with 1 *M* MOPS buffer (pH 7.0) (0.1 ml) and the mixture was filtered through an Ultrafree (C3GV, 0.22 μm, Millipore). The resulting filtrate was injected without further cleanup to a column-switching semi-microcolumn HPLC system described above. The injection volume of plasma sample was 100 μl.

2.8. Assay procedures for urine

Human urine (0.1 ml) was diluted with 1 *M* MOPS buffer (pH 7.0) (0.1 ml) and the mixture was injected without further cleanup to a column-switching semi-microcolumn HPLC system described above. The injection volume of urine sample was 2 μl.

2.9. Calibration curves

The calibration curve for DU-6681 was constructed from a least-square linear regression of the peak area of the standards versus the drug concentrations. The drug concentrations in human plasma and urine were expressed as equivalents of DU-6681 (the free acid of DU-6681a).

2.10. Recovery

The absolute recovery of DU-6681 from human plasma and urine was estimated by comparing the peak area obtained from injections of standards with those obtained from the injection of plasma and urine samples spiked with known concentrations of DU-6681.

2.11. Selectivity

Control plasma and urine from six healthy male volunteers were assayed by the procedures described above to evaluate the selectivity of the method.

2.12. Stability

The stability of DU-6681 in human plasma and urine, and in human plasma and urine diluted with 1 *M* MOPS buffer (pH 7.0) was investigated by preparing pooled plasma or urine spiked with known amounts of DU-6681. Samples (0.5–1.0 ml) in capped plastic tubes (1.5 ml) were stored at −20 and −80°C for 4 weeks. These samples were assayed periodically (0, 1, 2 and 4 week) by the method described above. The stability of DU-6681 in plasma and urine diluted with 1 *M* MOPS buffer was also investigated in autosampler. The plasma and urine spiked with DU-6681 were placed in autosampler at 4°C and 0.1 ml aliquots were injected into the HPLC system at 0, 6, 12 and 24 h.

2.13. Precision and accuracy

Intra-day precision and accuracy of the methods were evaluated by replicate analyses ($n=6$) of the plasma and urine calibration standards. Inter-day precision and accuracy were determined by assaying the plasma and urine calibration standards on four separate days. 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 to theoretical one.

2.14. Freeze–thaw stability of DU-6681

Stability of DU-6681 in plasma and urine diluted with 1 *M* MOPS buffer (pH 7.0) to three freeze–thaw cycles was investigated at each quality control (QC) concentration.

2.15. Sample dilution

The accuracy and precision of QC samples at concentration of 10.97 μg/ml for plasma and 471.3 μg/ml for urine after 10-fold dilution was investigated.

3. Results and discussion

3.1. Stability

In the initial study, DU-6681 in 50 m*M* MOPS buffer (pH 7.0) (20.6 μg/ml) was found to be stable at −20°C and −80°C with no significant loss of the drug over 28 days. Stability of DU-6681 in 50 m*M* MOPS buffer (pH 7.0) to freeze–thaw cycles was also investigated at the concentration of 20.6 μg/ml. No loss of the drug was observed when exposed to up to ten freeze–thaw cycles. When the same solution (18.0 μg/ml) was allowed to stand at room temperature, DU-6681 was found to be stable up to 8 h with only 2% loss of the drug, and about 4% and 8% of the drug were lost over 24 h and 48 h.

Carbapenems exhibit limited stability in biological fluids and special precautions are necessary to minimize artificial degradation during analytical procedures [6–10]. For the quantitative determination of such unstable compounds by HPLC, stabilization of the compounds in biological fluids by adding MOPS or MES buffer as stabilizers and quick sample-processing procedures are required.

In the present study, the stability of DU-6681 in plasma and urine diluted with 1 *M* MOPS buffer (pH 7.0) was investigated in autosampler at 4°C (Table 1). DU-6681 showed acceptable stability for up to 24 h with loss of the drug of <3.9% in plasma sample and <12.2% in urine sample. However, the drug stability was still limited, it was recommendable to avoid sample clean-up procedures such as liquid–liquid extraction, solid-phase extraction and deproteination prior to chromatography.

The stability of DU-6681 not only in plasma and urine, but also in plasma and urine diluted 1:1 with 1 *M* MOPS buffer (pH 7.0) was also assessed at −20°C and −80°C (Tables 2 and 3). About 20% and 10% of the drug were lost over 4 weeks at −20°C in plasma and urine, respectively. In 1:1 mixture of the buffer and plasma or urine, the drug stability was enhanced such that much less loss of the compound occurred at −20°C for 4 weeks (Table 3). MOPS

Table 1
Stability of DU-6681 in human plasma and urine at 4°C in autosampler[a]

Initial concentration (μg/ml)	Percent difference from the initial concentration at indicated time			
	0 h	6 h	12 h	24 h
Plasma				
0.059	0.0	0.5	−2.2	−3.8
0.585	0.0	−0.8	−1.9	−3.9
2.93	0.0	−1.5	−1.8	−3.0
Urine				
2.78	0.0	1.0	−3.5	−10.6
13.9	0.0	−1.1	−2.7	−6.9
69.6	0.0	−2.3	−6.8	−12.2

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

buffer was found to act as a stabilizer. At −80°C, DU-6681 was found to be stable in plasma and urine with and without addition of 1*M* MOPS buffer for up to 4 weeks. However, it was advisable to add 1 *M* MOPS buffer to plasma and urine as a stabilizer and store the samples frozen at at −80°C to avoid possible loss of the compound during storage.

Table 2
Stability of DU-6681 in human plasma and urine at −20°C and −80°C[a]

Initial concentration (μg/ml)	Percent difference from the initial concentration at indicated time			
	0 h	1 w	2 w	4 w
Plasma				
−20°C				
0.232	0.0	−6.5	−9.8	−17.7
2.32	0.0	−9.1	−9.6	−18.9
−80°C				
0.232	0.0	0.9	8.8	2.0
2.32	0.0	1.1	2.5	−2.0
Urine				
−20°C				
5.23	0.0	−12.9	−8.9	−11.8
52.3	0.0	−8.6	−5.1	−7.1
−80°C				
5.23	0.0	−9.5	−6.2	−6.7
52.3	0.0	−5.2	−2.4	−0.8

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

Table 3
Stability of DU-6681 in human plasma and urine diluted 1:1 with 1 *M* MOPS buffer (pH 7.0) at −20°C and −80°C[a]

Initial concentration (μg/ml)	Percent difference from the initial concentration at indicated time			
	0 h	1 w	2 w	4 w
Plasma				
−20°C				
0.232	0.0	−4.2	−7.1	−6.9
2.32	0.0	−8.1	−14.0	−11.1
−80°C				
0.232	0.0	−0.3	6.3	2.2
2.32	0.0	−3.5	1.9	−1.3
Urine				
−20°C				
5.23	0.0	−7.5	−8.3	−9.8
52.3	0.0	−4.1	−0.8	−6.5
−80°C				
5.23	0.0	−2.9	−3.5	0.2
52.3	0.0	−1.3	3.5	3.3

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

3.2. Sample extraction by column-switching system

The column-switching techniques have been increasingly used for on-line sample clean-up and/or enrichment of analytes and is widely adopted for the analysis of unstable antibiotics in biological matrices [8,10,18]. In the present study, we employed a semi-microcolumn HPLC with column-switching system, which consists of extraction column, intermediate column and main (analytical) column [15,16] in order to develop simple, rapid and sensitive analytical methods.

Bioptic AV-2 and LiChrosorb NH_2 were employed as stationary phase in the extraction columns for the analyses of plasma and urine samples, respectively. The retention of DU-6681 on the column was investigated using 100 m*M* phosphate buffer (pH 6.5) as an eluent. Elution of the drug was not observed when the extraction column was flushed for 2 min. When plasma and urine samples were injected

onto the extraction column, the most polar matrix components were eluted from the column during the flushing time of 2 min.

3.3. Chromatography and selectivity

The column-switching system described provided adequate clean-up of plasma and urine samples as shown by the absence of endogeneous peaks which interfered with the detection of DU-6681 at its retention time in control plasma and urine (Figs. 3A and 4A). Well-defined chromatographic peak of DU-6681 was achieved using Inertsil ODS-2, where the free silanol groups were almost completely end-capped, as analytical stationary phase and a mixture of 100 m*M* phosphate buffer (pH 6.5)/acetonitrile (95:5, v/v) as a mobile phase at a flow rate of 0.1 ml/min. A typical chromatograms of control plasma and urine spiked with DU-6681 are shown in Figs. 3B and 4B. The retention times of DU-6681 for plasma and urine samples were ca. 17 min and 15 min, respectively. Plasma and urine samples collected from six healthy volunteers also showed no interference with the assay, indicating that the present method had a high selectivity. The extraction, intermediate and analytical columns had acceptable column life and at least 100 plasma or urine samples were analyzed without no significant sign of deterioration of the columns.

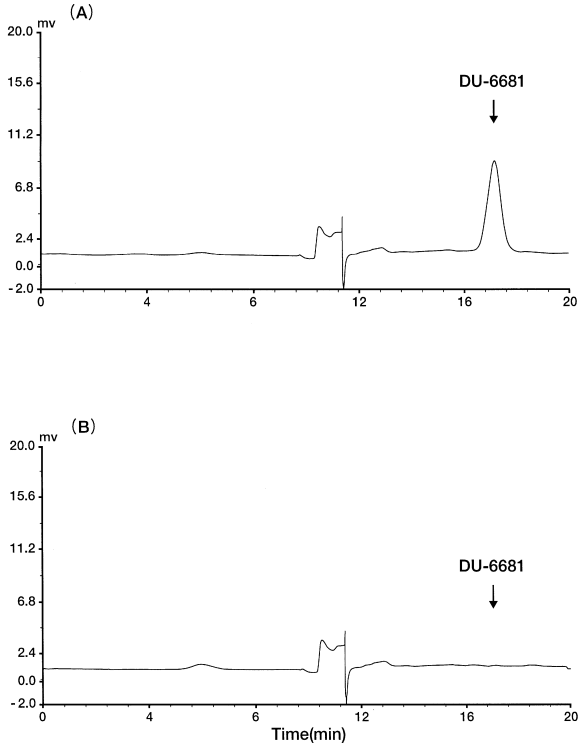


Fig. 3. Representative chromatograms obtained with (A) control human plasma spiked with DU-6681 (0.64 μg/ml) and (B) control human plasma. The chromatograms are shown with a full scale of 20 mV.

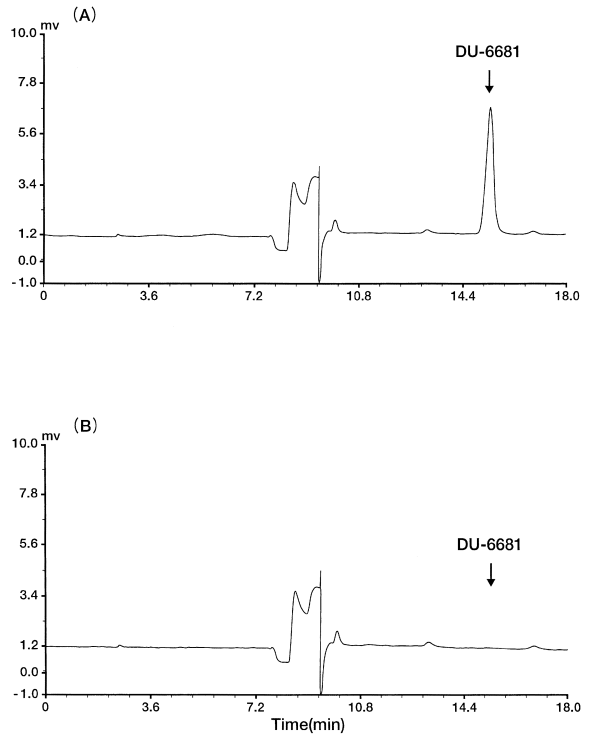


Fig. 4. Representative chromatograms obtained with (A) control human urine spiked with DU-6681 (8.70 μg/ml) and (B) control human urine. The chromatograms are shown with a full scale of 10 mV.

3.4. Analyte recovery

The absolute recoveries of DU-6681 from human plasma and urine were determined by comparing the

peak area obtained after injection of the fluids spiked with known concentrations of the drug to those produced by the same concentrations of the drug dissolved in 1 *M* MOPS buffer (pH 7.0) (Table 4). The recoveries of DU-6681 from spiked human plasma were evaluated at the concentrations of 0.059, 0.585 and 2.93 μg/ml in replicates of six. The recoveries from spiked human urine were determined at the concentrations of 2.78, 13.9 and 69.6 μg/ml in replicates of six. The recoveries ranged from 97.3 to 99.3% in plasma and from 95.9 to 98.5% in urine. Column-switching system has been successfully applied to the extraction of DU-6681 from human plasma and urine.

3.5. Calibration curves

Calibration curves for plasma obtained in four separate days were linear and reproducible with mean±standard deviation values for the constants in the regression equation of $y=[(6.552\pm0.113)\times 10^{-9}]x+(-0.0007\pm0.0014)$. Correlation coefficients were always greater than 0.999. The inter-day coefficient of variation of the slope of the calibration curve was 1.7%. The least-square linear regression equation obtained for human urine ($n=4$) was $y=[(2.872\pm0.114)\times10^{-7}]x-(0.0004\pm0.0973)$ with correlation coefficients greater than 0.999. The inter-day coefficient of variation of the slope of the calibration curve was 4.0%. The curve for plasma was linear in the concentration range of 0.008–5.85 μg/ml and for urine 0.32–104.4 μg/ml, respectively.

Table 4
Recoveries of DU-6681 from human plasma and urine

Concentration (μg/ml)	Recovery (%)	C.V.[a] (%)	*n*
Plasma			
0.059	98.7	1.3	6
0.585	97.3	0.7	6
2.93	99.3	1.1	6
Urine			
2.78	95.9	2.4	6
13.9	96.0	2.4	6
69.6	98.5	4.6	6

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

3.6. Precision and accuracy

The intra-day precision and accuracy of the method for plasma were evaluated by analyzing human plasma spiked with DU-6681 at concentrations of 0.008, 0.024, 0.072, 0.217, 0.650, 1.95 and 5.85 μg/ml in replicates of six (Table 5). 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 to the theoretical value. The C.V. ranged from 0.6 to 5.0% and the R.E. ranged from −6.1 to 2.4% of the theoretical values at concentrations above 0.024 μg/ml. The limit of quantitation using a 0.1 ml sample was set at the concentration of the lowest calibration standard or 0.008 μg/ml of plasma with C.V. of 8.2%, R.E. of 14.2% and the signal-to-noise ratio of 5.0. The present method showed at least six times higher sensitivity than other reported methods [6–10], where conventional HPLC systems were used.

Intra-day precision and accuracy of the method for urine were evaluated by analyzing human urine

Table 5
Intra-day precision and accuracy for analysis of DU-6681 in human plasma and urine

Theoretical concentration (μg/ml)	Mean found concentration (μg/ml)	C.V.[a] (%)	R.E.[b] (%)	*n*
Plasma				
0.008	0.009	8.2	14.2	6
0.024	0.023	5.0	−3.8	6
0.072	0.073	2.7	1.5	6
0.217	0.208	1.5	−4.1	6
0.650	0.611	1.6	−6.1	6
1.95	1.88	1.2	−3.8	6
5.85	5.99	0.6	2.4	6
Urine				
0.32	0.29	8.7	−10.1	6
0.97	0.96	5.9	−0.5	6
2.90	3.13	6.2	8.0	6
8.70	9.10	1.2	4.5	6
26.1	25.4	2.7	−2.7	6
52.2	52.7	0.9	0.9	6
104.4	103.9	0.5	−0.5	6

[a] C.V.=coefficient of variation.
[b] R.E.=relative error.

spiked with DU-6681 at concentrations of 0.32, 0.97, 2.90, 8.70, 26.1, 52.2 and 104.4 μg/ml in replicates of six (Table 5). C.V.s were ≦6.2% and the R.E. ranged from −2.7 to 8.0% of the theoretical values at concentrations above 0.97 μg/ml. The limit of quantitation using a 0.1 ml sample was set at the concentration of the lowest calibration standard, or 0.32 g/ml of urine with C.V. of 8.7%, R.E. of −10.1% and the signal-to-noise ratio of at least 11.

The inter-day precision and accuracy for plasma was assessed by the analysis of calibration standard samples at concentrations of 0.008 to 5.85 μg/ml of plasma on four separate days (Table 6). The C.V. was ≦2.1% and the R.E. ranged from −6.2 to 1.5% at concentrations above 0.024 μg/ml. At the quantitation limit of 0.008 μg/ml, the method showed acceptable precision and accuracy with C.V. of 5.4% and R.E. of 15.2%. The inter-day precision and accuracy for urine was assessed by the analysis of calibration standard samples at concentrations of 0.32–104.4 μg/ml of urine on four separate days (Table 6). The C.V. was ≦11.3% and the R.E. ranged from −1.3 to 5.7%. At the quantitation limit of 0.32 μg/ml, the inter-day precision and accuracy would be satisfactory with C.V. of 21.3% and R.E. of −3.1%.

Table 6
Inter-day precision and accuracy for analysis of DU-6681 in human plasma and urine

Theoretical concentration (μg/ml)	Mean found concentration (μg/ml)	C.V.[a] (%)	R.E.[b] (%)	*n*
Plasma				
0.008	0.009	5.4	15.2	4
0.024	0.024	2.1	−1.4	4
0.072	0.072	0.7	−0.7	4
0.217	0.205	1.5	−5.4	4
0.650	0.610	1.0	−6.2	4
1.95	1.906	1.4	−2.3	4
5.85	5.943	0.5	1.5	4
Urine				
0.32	0.31	21.3	−3.1	4
0.97	0.96	7.2	−0.7	4
2.90	3.07	11.3	5.7	4
8.70	8.65	5.2	−0.6	4
26.1	25.9	2.2	−0.7	4
52.2	51.5	2.9	−1.3	4
104.4	105.0	0.9	0.6	4

[a] C.V.=coefficient of variation.
[b] R.E.=relative error.

Table 7
Freeze–thaw stability of DU-6681 in human plasma and urine[a]

Concentration (μg/ml)	Percent difference from the baseline Freeze–thaw cycle			
	0	1	2	3
Plasma				
0.059	0.0	1.4	4.3	5.8
0.585	0.0	−2.1	−1.8	−2.8
2.93	0.0	−0.3	−1.0	−1.8
Urine				
2.78	0.0	−5.1	−5.4	−3.9
13.9	0.0	−0.8	−1.4	−0.7
69.6	0.0	−0.5	−1.2	0.0

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

3.7. Freeze–thaw stability

DU-6681 exhibits acceptable stability in human plasma and urine, when exposed to up to three freeze–thaw cycles with the drug loss of ≦5.8% (Table 7).

3.8. Sample dilution

The intra-batch precision and accuracy in the quality control plasma samples at concentration of 10.97 μg/ml were 1.3% and −1.5%, respectively, after ten-fold dilution.

The intra-batch precision and accuracy in the quality control urine samples at concentration of 471.3 μg/ml were 3.0% and 4.2%, respectively, after ten-fold dilution. There was no effect of sample dilution on the concentrations of DU-6681 in plasma and urine.

4. Conclusions

A sensitive and selective analytical method for the determination of DU-6681 in human plasma and urine was established by combination of semi-micro-column HPLC and column-switching system. The

present method allows direct injection of plasma and urine samples diluted with MOPS buffer to the HPLC, which minimizes sample handling loses, and also reduces human contact with biological samples. This method proved to be suitable for the clinical pharmacokinetic studies of DU-6681. The results of these studies will be reported elsewhere.

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