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Determination of 3′-C-ethynylcytidine in human plasma and urine by liquid chromatographic-electrospray ionization tandem mass spectrometry

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Abstract

A liquid chromatographic-electrospray ionization tandem mass spectrometric (LC-ESI/MS/MS) method was developed for the quantitative analysis of a novel anticancer drug, 3′-C-ethynylcytidine (**I**) in human plasma and urine. **I** and its stable isotope-labeled internal standard (**II**) were extracted from human plasma and urine samples using a polymer-based cation-exchange cartridge, and LC-ESI/MS/MS analysis was performed by monitoring the positive fragment ions of **I** and **II**. The linear ranges are 1–500 ng/ml in plasma and 10–5000 ng/ml in urine. The limits of quantitation for **I** were 1 ng/ml in plasma and 10 ng/ml in urine. The relative errors (RE) for **I** ranged from −8.4 to 3.0% in plasma and from 0.8 to 4.4% in urine. The relative standard deviations (RSD) for **I** ranged from 1.2 to 8.9% in plasma and from 0.7 to 2.8% in urine. This validated analytical method is demonstrated to be useful for the analysis of **I** in human plasma and urine in clinical studies.
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Keywords: 3′-C-ethynylcytidine; LC-ESI/MS/MS

1. Introduction

3′-C-ethynylcytidine (**I**, Fig. 1), a novel cytidine analogue developed as an antitumor agent, was designed to inhibit RNA synthesis by blocking RNA polymerases I, II and III [1–3]. **I** demonstrated cytotoxicity against human lung, colorectal, gastric and other cancer cells in vitro [4]. Furthermore, **I** had an antitumor effect on the nude rat model implanted with human cancer cell lines in vivo [5].

I is currently under clinical studies. In order to evaluate the clinical pharmacokinetic study, in which concentrations of **I** in plasma are anticipated to be low from dog plasma concentration (3 ng/ml) of pre-clinical studies, a highly sensitive and selective analytical method is required for the determination of **I** in plasma and urine. In this respect, it is considered that a liquid chromatographic-electrospray ionization tandem mass spec-

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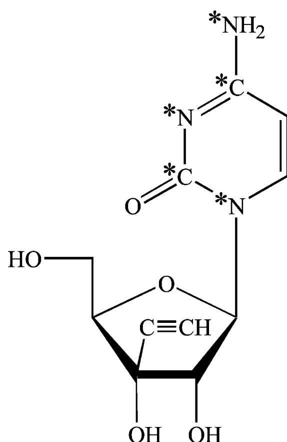

Fig. 1. Chemical structure of 3′-C-ethynylcytidine (**I**). Asterisks denote the labeled positions of the internal standard (**II**).

trometry (LC-ESI/MS/MS) [6,7] is the most suitable technique compared with other detection techniques.

As a sample preparation of hydrophilic compounds such as **I**, an ion-exchange extraction is generally more efficient than reverse-phase and liquid–liquid extractions. Recently a solid-phase extraction cartridge containing a novel polymeric sorbent with cation-exchange functionality has become available [8,9]. The retention behavior of **I** in the polymer-based cartridge is anticipated to be different from that in a conventional silica-based cartridge.

The purpose of this study was to develop and validate a sensitive and selective analytical method for **I** in human plasma and urine. This paper describes the validation of the analytical method for **I** in human plasma and urine by combining LC-ESI/MS/MS and solid-phase extraction using a cation-exchange cartridge.

2. Experimental

2.1. Materials and reagents

I and **II** (Fig. 1) were synthesized by Taiho Pharmaceuticals (Tokyo, Japan). The polymer-based Oasis MCX cartridge (the cation-exchange cartridge) (1 cc/30 mg) and the silica-based Bond Elut SCX (1 cc/100 mg) were supplied by Nihon Waters Corporation (Tokyo, Japan) and GL Sciences Inc. (Tokyo, Japan), respectively. Methanol (HPLC grade), 0.1 M HCl (volumetric analysis grade), ammonium acetate (special grade) and 25% ammonia solution (special grade) were supplied by Wako Pure Chemicals (Osaka, Japan). All water used was purified with a Milli-Q system (Nihon Millipore, Tokyo, Japan). Human blank plasma and urine were supplied from healthy male volunteers.

2.2. Instruments

The LC/MS/MS system consisted of a Waters 600s controller, 616 pump, and a 717plus autosampler (Waters, Milford, MA, USA) and a Finnigan Mat TSQ 7000 (API 2) triple-stage quadrupole tandem mass spectrometer (San Jose, CA, USA), with ESI source.

An Inertsil ODS-3 column (5 μm, 150 × 2.1 mm I.D., GL Sciences Inc., Tokyo, Japan) at 40 °C was used for the chromatographic separation. The mobile phase was 10 mM ammonium acetate–methanol (95:5, v/v) at a flow-rate of 0.2 ml/min.

2.3. Mass spectrometric conditions

The ESI was performed in the positive-ion mode with a probe capillary potential of 4.5 kV, a sheath gas (N_2) pressure of 70 p.s.i., and an auxiliary gas (N_2) flow of 10 units. The heated capillary was maintained at 350°C. The selected reaction monitoring (SRM) was at a collision offset voltage of −20 V and the collision gas (Ar) pressure was 2.2–2.5 mTorr. The monitored quasi-molecular ions transitions $[M+H]^+$ were m/z 268 → 112 for **I** and m/z 273 → 117 for **II**. The injection volume was 10 μl and the switching valve was used to divert the first 4 min of each chromatographic run to waste. Data acquisition, peak integration and calculation were performed using LC Quan software. Peak area ratios of **I** to **II** were utilized for the construction of calibration curves, using $1/x^2$ weighted linear least-square regression of plasma or urine concentrations and the measured peak area ratios. Concentrations of **I** in quality control (QC) samples were calculated by interpolation from the calibration curves.

2.4. Preparation of standard solutions

Stock standard solutions of **I** and **II** were prepared in water at concentrations of 50 and 20 μg/ml, respectively. Aliquots of the stock standard solutions were diluted with water to provide working solutions with concentrations of 4–10 000 ng/ml. These standard solutions were stored at 5 °C.

2.5. Preparation of quality control samples

Plasma QC samples with concentrations of 400, 40, 4 and 1 ng/ml were prepared by mixing blank plasma and the solutions of 8000, 800, 80 and 20 ng/ml (19:1), respectively. These were stored at −30 °C.

Urine QC samples with concentrations of 4000, 400, 40 and 10 ng/ml were prepared as follows. The residues from desiccating the solutions of 50 μg/ml, 10 000, 400 and 100 ng/ml were dissolved in blank urine. These were stored at −30 °C.

2.6. Sample preparations

Solid phase extraction was performed manually using a vacuum manifold device for sample loading, washing and elution.

To a 0.2-ml aliquot of plasma QC sample, 0.05 ml of water, 0.8 ml of 0.1 M hydrochloric acid and 0.05 ml of internal standard working solution (500 ng/ml) were added. The mixture was loaded onto the Oasis MCX extraction cartridge previously conditioned with 1 ml of methanol, followed by 1 ml of water. After washing with 1 ml of 0.1 M hydrochloric acid and 1 ml of methanol, **I** and **II** were eluted from the cartridge with 1.5 ml of 5% ammonia solution–methanol (1:4, v/v). The eluate was dried under nitrogen at 40 °C and the residue was reconstituted with 0.1 ml of water. A 10-μl aliquot of reconstituted sample was injected into the LC/MS/MS system.

To a 0.2-ml aliquot of blank plasma, 0.05 ml of the working standard solutions (2000, 400, 100, 20 and 4 ng/ml), 0.8 ml of 0.1 M hydrochloric acid and 0.05 ml of internal standard working solution (500 ng/ml) were added. The mixture was processed in the above manner. The resulting plasma standard concentrations ranged from 1 to 500 ng/ml.

To a 0.05-ml aliquot of urine QC sample, 0.025 ml of water, 0.8 ml of 0.1 M hydrochloric acid and 0.05 ml of internal standard working solution (500 ng/ml) were added. The mixture was processed in the manner mentioned in regard to plasma.

To a 0.05-ml aliquot of blank urine, 0.025 ml of the working standard solutions (10 000, 2000, 400, 100 and 20 ng/ml), 0.8 ml of 0.1 M hydrochloric acid and 0.05 ml of internal standard working solution (500 ng/ml) were added. The mixture was processed in the manner mentioned in regard to plasma. The resulting urine standard concentrations ranged from 10 to 5000 ng/ml.

For sample preparation using the silica-based cartridge, the Bond Elut SCX was processed in the manner mentioned in regard to plasma except with 0.1 M hydrochloric acid instead of water in the column conditioning, and water instead of 0.1 M hydrochloric acid in the column washing. The elute volume was 2.0 ml rather than 1.5 ml.

3. Results and discussion

3.1. MS–MS

In the electrospray spectra, characteristic ions at *m/z* 268.1 and 290.1 for **I**, and, 273.1 and 295.1 for **II** were considered as $[M+H]^+$ and $[M+Na]^+$, respectively. Fig. 2A and B show the product ions spectra of $[M+H]^+$. Intense product ions at *m/z* 112.1 for **I** and 117.1 for **II** were obtained. These ions were considered as $[M-\text{ribose}]^+$ for both (Fig. 2C).

3.2. Recovery

Table 1 shows the recoveries of **I** in the standard solution or human plasma using polymer-based and silica-based cartridges. The recovery of **I** in both standard solution and plasma using the polymer-based cartridge was 1.5–1.7-fold that using the silica-based cartridge. Furthermore, the recovery from plasma was higher than that from the standard solution for both cartridges. As **I** was completely retained in both cartridges, the differ-

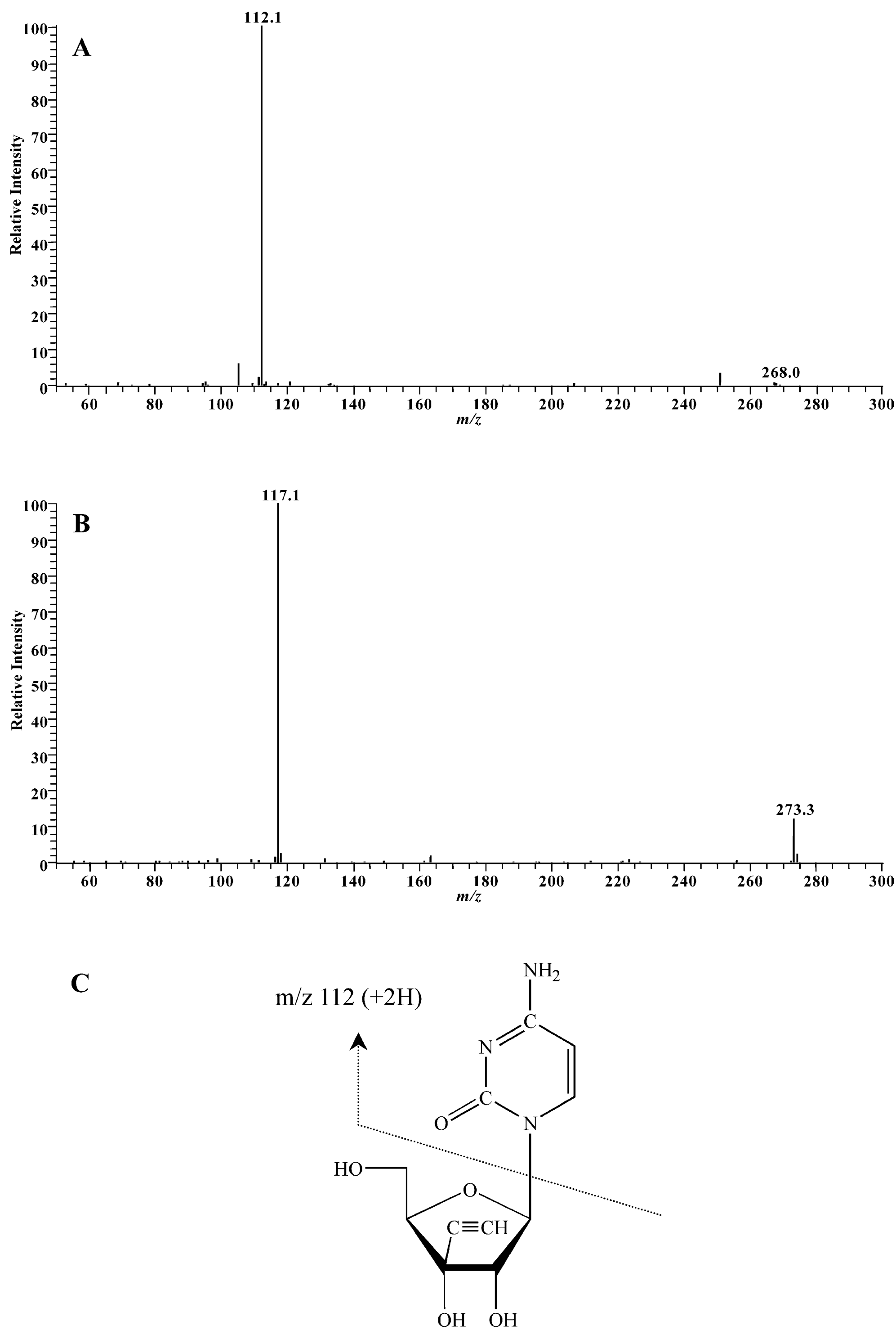

Fig. 2. Product ion spectra of **I** (A) and **II** (B), and proposed fragmentation pattern of **I** (C).

Table 1
Recovery of **I** in standard solution and human plasma using silica-based or polymer-based cartridges

Nominal concentration (ng/ml)	Recovery (%) (mean ± S.D.)			
	Silica-based cartridge, ($n=3$)		Polymer-based cartridge, ($n=3$)	
	Standard solution	Human plasma	Standard solution	Human plasma
4	33.4 ± 3.0	58.6 ± 7.0	57.2 ± 3.4	85.9 ± 3.5
40	31.8 ± 1.2	54.6 ± 3.0	56.9 ± 2.7	79.5 ± 3.3
400	35.0 ± 1.2	50.8 ± 4.9	59.6 ± 2.7	82.4 ± 3.2

ence in the recoveries between the two cartridges was attributed to the difference in the elution of **I** from the cartridges. The reason why both cartridges, which contain common alkyl sulfonic groups, were different in the elution has not been clarified. However, it can be considered that besides interacting ion-exchange groups, **I** binds more strongly to the silicic sorbent via undesirable interactions such as a remained silanol effect, which may cause lower recoveries compared with the polymeric sorbents. The plasma components may perform as competitors to the undesirable interactions of **I** with the two sorbents, resulting in the improved recoveries.

In validation study using the polymer-based cartridge, the mean recoveries ($n=5$) of **I** were >80% for plasma and >74% for urine.

3.3. Selectivity

Fig. 3 shows representative chromatograms of extracts obtained from blank plasma and plasma spiked with 1 ng/ml of **I**. Fig. 4 shows representative chromatograms of extracts obtained from blank urine and urine spiked with 10 ng/ml of **I**. There was no interference peak from endogenous components at the retention times for the analytes in the ion chromatograms obtained from plasma and urine of the six individuals.

3.4. Calibration curves

Good linearity of calibration curves were observed over the concentration range of 1–500 ng/ml for plasma and 10–5000 ng/ml for urine. The correlation coefficients (r^2, $n=5$) were above 0.9995 for both.

3.5. Accuracy and precision

The accuracy and precision for plasma are shown in Table 2. The intra-day relative error (RE) ranged from −8.4 to 3.0%, and the relative standard deviation (RSD) ranged from 1.2 to 5.3%. The inter-day RE ranged from 0.2 to 1.3%, with the RSD ranging from 6.8 to 8.9%. The RE at the limit of quantitation (LOQ) (1 ng/ml) was 3.0% and the RSD was 5.3%.

The accuracy and precision for urine are shown in Table 3. The intra-day RE ranged from 0.8 to 4.2%, and the RSD ranged from 0.7 to 2.5%. The inter-day RE ranged from 2.5 to 4.4%, with the RSD ranging from 1.0 to 2.8%. The RE at LOQ (10 ng/ml) was 4.2% and the RSD was 2.5%. These results indicate the analytical method allows reliable accuracy and precision over the concentration range of 1–500 ng/ml for plasma and 10–5000 ng/ml for urine [10].

3.6. Stability

Standard solutions of **I** and **II** were stable at 5 °C for at least 6 months. **I** and **II** extracted from plasma and urine were stable in the autosampler for at least 48 h. **I** in plasma and urine after three cycles of freeze-thaw was found to be stable. **I** in plasma and urine was found to be stable at −30 °C for about 12 months.

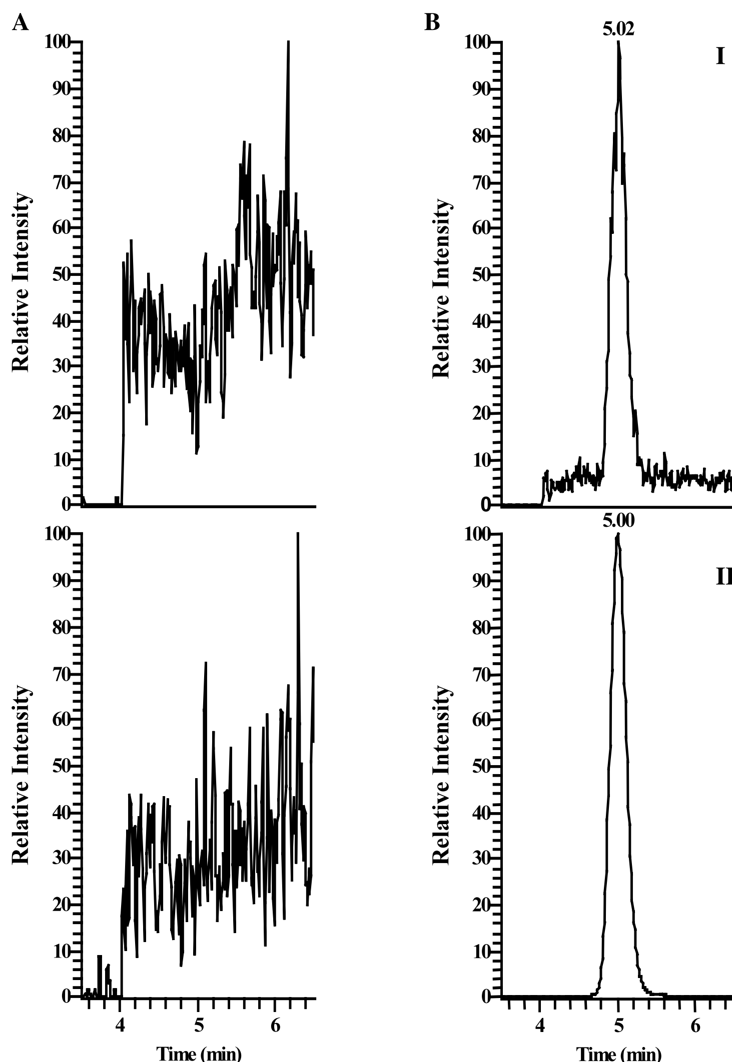

Fig. 3. Chromatograms of extracted human blank plasma (A) and plasma spiked with 1 ng/ml **I** and 125 ng/ml **II** (B).

3.7. *Application to clinical samples*

This analytical method is currently applied to the determination of **I** in plasma and urine from cancer patients administered **I** intravenously. Representative chromatograms of plasma samples collected prior to administration and at 5 min after a 4.7 mg intravenous dose are shown in Fig. 5A and B. Representative chromatograms of urine samples collected prior to administration and 0–6 h after administration are shown in Fig. 5D and E. There was no interfering peak in the chromatograms of pre-dose plasma and urine samples, and **I** in plasma sample at 24 h (Fig. 5C) and urine sample for 12–24 h (Fig. 5F) could be detected. Thus, this method has been proved to be selective and sensitive enough to be applied to clinical samples.

4. Conclusions

We developed LC-ESI/MS/MS analysis combined with sample preparation using an Oasis

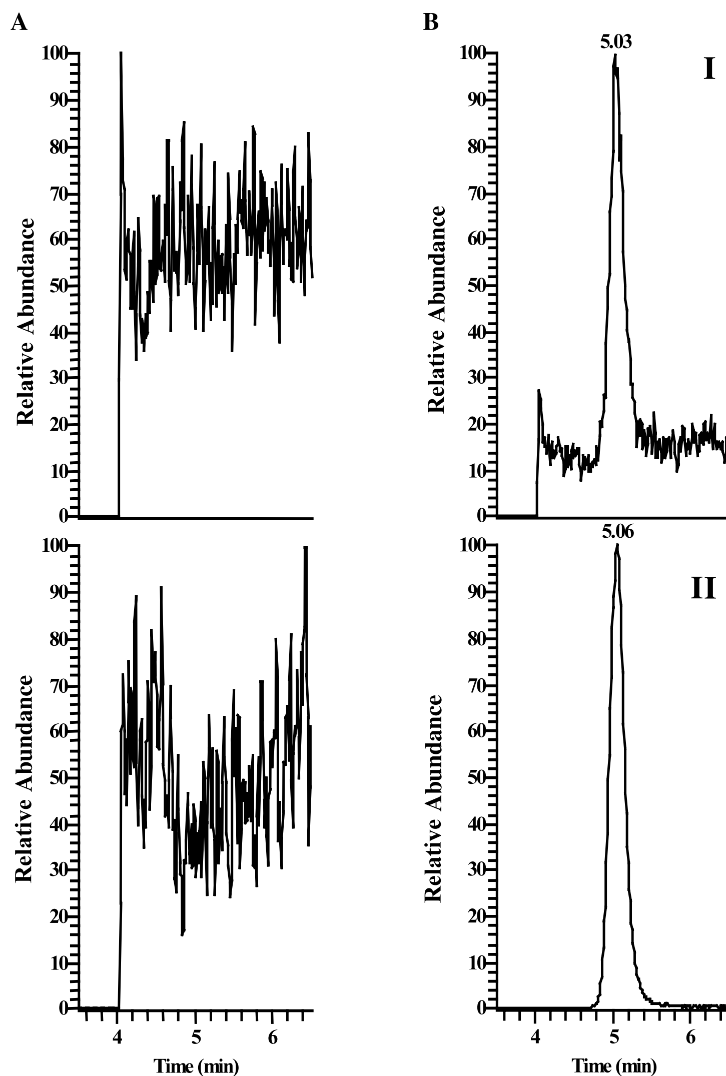

Fig. 4. Chromatograms of extracted human blank urine (A) and urine spiked with 10 ng/ml **I** and 125 ng/ml **II** (B).

Table 2
Accuracy and precision of the analysis of **I** in human plasma

Nominal Concentration (ng/ml)	Intra-day ($n = 5$)			Inter-day ($n = 5$, 5 days)		
	Found (mean) (ng/ml)	RE (%)	RSD (%)	Found (mean) (ng/ml)	RE (%)	RSD (%)
1[a]	1.030	3.0	5.3	–	–	–
4	3.990	−0.3	5.0	4.017	0.4	6.8
40	36.654	−8.4	3.6	40.517	1.3	8.2
400	383.320	−4.2	1.2	400.799	0.2	8.9

[a] Concentration of LOQ.

Table 3
Accuracy and precision of the analysis of **I** in human urine

Nominal Concentration (ng/ml)	Intra-day ($n = 5$)			Inter-day ($n = 5$, 5 days)		
	Found (mean) (ng/ml)	RE (%)	RSD (%)	Found (mean) (ng/ml)	RE (%)	RSD (%)
10[a]	10.424	4.2	2.5	–	–	–
40	40.665	1.7	1.7	41.751	4.4	1.9
400	404.289	1.1	0.7	411.633	2.9	2.8
4000	4030.611	0.8	1.4	4100.103	2.5	1.0

[a] Concentration of LOQ.

MCX cartridge. This analytical method for **I** in human plasma and urine was validated in the concentration range of 1–500 ng/ml and 10–5000 ng/ml, respectively. This analytical method has been proved to be useful for clinical pharmacokinetic studies.

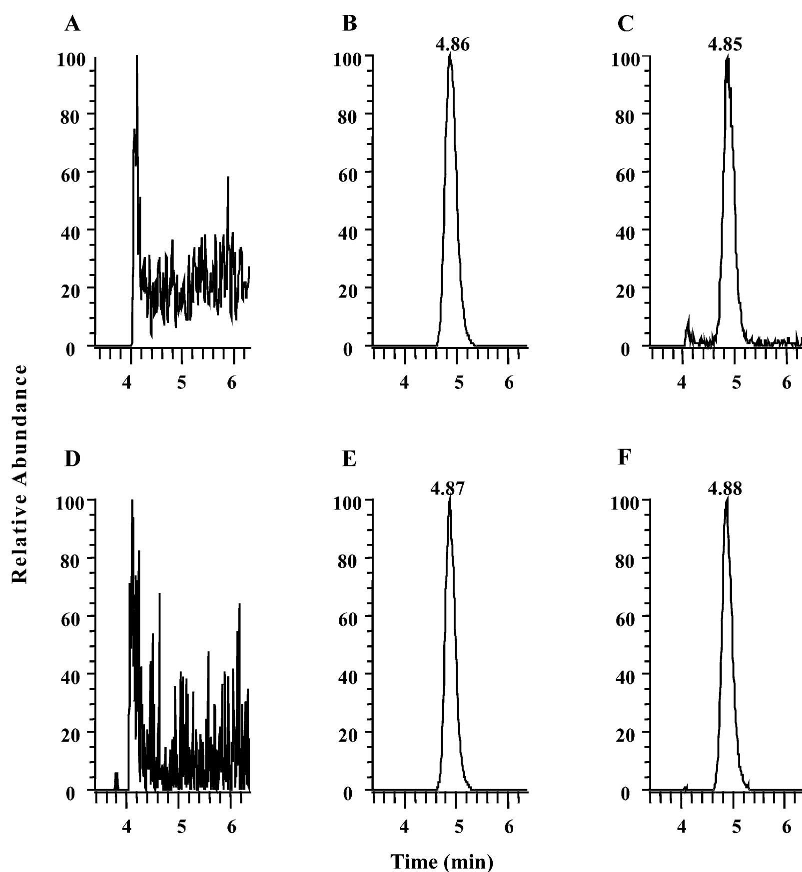

Fig. 5. Chromatograms of **I** in plasma (A) prior to administration and at 5 min (B), 24 h (C) after 4.7 mg intravenous dose. Chromatograms of **I** in urine (D) prior to administration and 0–6 h (E), 12–24 h (F) after administration.

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