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Determination of a novel thrombin inhibitor in human plasma and urine utilizing liquid chromatography with tandem mass spectrometric and ultraviolet detection

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Abstract

An LC–MS–MS method and an HPLC–UV method have been developed for the assay of a novel thrombin inhibitor in human fluids. The LC–MS–MS method is developed for plasma, which usually requires maximum sensitivity. The HPLC–UV method is for urine. In both methods, analytes are extracted using liquid–liquid extraction, and analyzed by reversed-phase high-performance liquid chromatography. A tandem mass spectrometer in the multiple reaction monitoring (MRM) mode is used for detection of the analytes in the plasma method. UV is the detector for the urine method. The plasma method has a lower limit of quantitation (LOQ) of 0.1 ng/ml with a linearity range of 0.1–100 ng/ml. The urine method has an LOQ of 8.12 ng/ml (20 n*M*) and the linear dynamic range is 8.12–8120 ng/ml (20–20 000 n*M*). Both methods are fast, specific and sensitive. Various validation procedures have proven that both methods are rugged, robust and reproducible. The research also suggested that, while LC–MS–MS provides superior sensitivity and selectivity for the determination of drugs and their metabolites at very low concentrations, HPLC with a conventional detector such as UV is still useful in the analysis when the sensitivity requirement is not crucial.

Keywords: Thrombin inhibitor

1. Introduction

3-(2-Phenethylamino)-6-methyl-1-(2-amino-6-methyl - 5 - methylene - carboxamidomethyl - pyridinyl)pyrazinone dihydrochloride monohydrate (**I**, Fig. 1) is a potent thrombin inhibitor [1–4]. No analytical method has been published for the quantification of this compound in biological fluids. The purpose of this research is to develop analytical methods with high sensitivity and a wide dynamic range to quantify **I** in human plasma and urine.

High-performance liquid chromatography (HPLC) has been the most frequently used technique in the quantitative analysis of drugs and their metabolites in biological fluids. However, HPLC with conventional detectors such as UV and fluorescence is being gradually replaced by LC–MS–MS, a combination of HPLC and tandem mass spectrometry (MS–MS). Despite its requirements of personal skill and high operational cost, the tandem mass spectrometer is an ideal detector that has superb selectivity and sensitivity compared to other detection techniques. For

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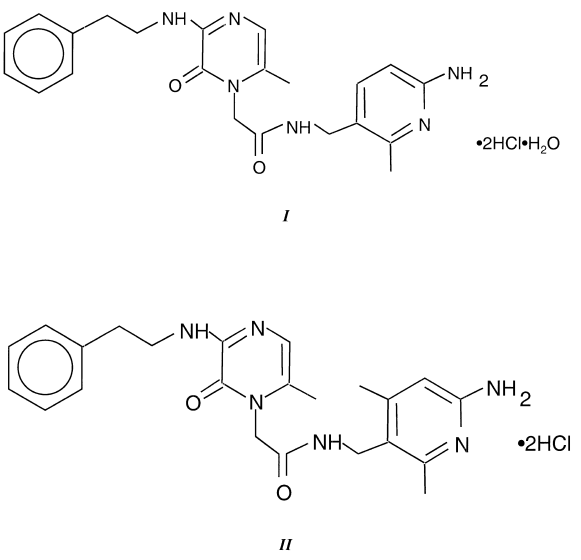

Fig. 1. Chemical structures of compounds **I** and **II**.

this research, we developed two analytical methods, one using HPLC–UV and the other using LC–MS–MS, to quantify **I** in different human fluids. We took advantage of the low operation cost of HPLC–UV for the determination of **I** concentration in human urine, which was in the range of 8 ng/ml to 8 μg/ml. When increased sensitivity was needed for human plasma, we developed the LC–MS–MS method so that the lower limit of quantitation (LOQ) was 0.1 ng/ml, while maintaining the same linearity range (three orders of magnitude).

2. Experimental

2.1. Materials

Compound **I** and the internal standard (**II**, Fig. 1), a chemical analogue with an additional methyl group on the pyridine ring, were obtained from Merck Research Labs. (West Point, PA, USA). HPLC grade methyl-*tert*.-butyl ether (HPLC grade) was obtained from Burdick and Jackson (Muskegon, MI, USA). Control human plasma was purchased from Sera-Tec Biologicals (New Brunswick, NJ, USA). Other chemicals and solvents were from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Apparatus

The LC–MS–MS system for the plasma method (method A) consists of a PE Sciex (Thornhill, Canada) API 300 with a turbo ionspray interface, a Perkin-Elmer (Norwalk, CT, USA) Series 200 autosampler, a Perkin-Elmer Series 200 quarternary qump, and a Jones Chromatography (Lakewood, CO, USA) column heater. The data were processed using MacQuan software (Version 1.4, PE Sciex) on a MacIntosh PowerPC 9500 computer.

A stand-alone HPLC system was used for the urine method (method B). It consisted of a Perkin-Elmer Series 200 autosampler, a Perkin-Elmer Series 200 quarternary pump, a Jones Chromatography column heater, and an Applied Biosystems (Foster City, CA, USA) 785A UV detector. PE Nelson TurboChrom (version 4.1) was used to process chromatographic data.

2.3. Chromatographic conditions

For method A, the separation of analytes was performed on a Higgins (Mountain View, CA, USA) Haisil BD120 C_8 column (50×2.1 mm, 5 μm). The mobile phase was methanol–ammonium acetate (1 m*M*, pH 4.2) (50:50) with a flow-rate of 0.3 ml/min. The HPLC column temperature was fixed at 40°C to avoid retention time change with fluctuation in room temperature.

For method B, a Keystone (Bellefonte, PA, USA) Betabasic C_8 column (150×3.9 mm, 5 μm) was used for analyte separation. The mobile phase was a mixture of acetonitrile–0.1% trifluoroacetic acid (88:12, v/v) with a flow-rate of 0.6 ml/min. The UV detector was set at a wavelength of 295 nm. The HPLC column temperature was 40°C.

2.4. Standard solutions

For method A, a stock standard solution of **I** was prepared at 1 mg/ml (free base) in methanol–water (50:50, v/v). This solution was further diluted to give a series of working standards with concentrations of 1, 2, 5, 20, 50, 200, 500 and 1000 ng/ml. Internal standard (**II**) working solution was prepared at 100 ng/ml in methanol–water (50:50, v/v). All the standard solutions were stored at 4°C. Plasma standards were prepared by adding 50 μl of each

working standard to 0.5 ml of drug-free control plasma. The resulting plasma standard concentrations ranged from 0.1 to 100 ng/ml.

Quality control (QC) stock solution of **I** was prepared separately at 1 mg/ml (free base) in methanol–water (50:50, v/v). This solution was further diluted to give QC working solutions of 30, 600 and 9000 ng/ml. QC samples were prepared by adding 500 μl of appropriate QC working standard to a 50-ml volumetric flask and filling to the mark with control human plasma. The concentration levels were 0.3, 6 and 90 ng/ml, representing low, medium and high QCs, respectively. All QC samples were stored at −20°C until assayed.

For method B, concentrations in urine were reported in molar units instead of ng/ml. A stock standard solution of **I** was prepared at 2.50 m*M* (1.02 mg/ml) in methanol–water (50:50, v/v). This solution was further diluted to give a series of working standards with concentrations ranging from 80, 200, 400, 2000, 4000, 20 000 to 80 000 n*M*. Internal standard (**II**) working solution was prepared at 1000 ng/ml (2380 n*M*) in methanol–water (50:50, v/v). All the standard solutions were stored at 4°C. Urine standards were prepared by adding 100 μl of each working standard to 0.4 ml of drug-free control urine. The resulting standard concentrations ranged from 20 to 20 000 n*M* (8.12 to 8120 ng/ml).

QC stock solution of **I** was prepared separately at 2.50 m*M* (1.02 mg/ml) in methanol–water (50:50, v/v). This solution was further diluted to give QC working solutions of 3, 60 and 900 μ*M*. Urine QC samples were prepared by adding 1 ml of appropriate QC working standard to a 50-ml volumetric flask and filling to the mark with control human urine. The concentration levels were 60, 1200 and 18 000 n*M* (24.5, 490 and 7350 ng/ml), representing low, medium and high QCs, respectively. All QC samples were stored at −70°C until assayed.

2.5. Sample preparation

For method A, frozen plasma samples were thawed and brought to room temperature. A 0.5-ml aliquot of plasma was pipetted into a 5-ml polypropylene culture tube. a 50-μl volume of 0.1 μg/ml internal standard (equivalent to 10 ng/ml of **II** in plasma) was added, followed by 0.25 ml of 2.0 *M* NaOH. After addition of 3 ml of methyl-*tert*.-butyl ether, the tube was capped and rotated for 15 min at 60 rpm. The lower layer (aqueous) of the mixture was frozen in a dry ice–acetone bath and the organic phase was transferred to a clean 5-ml polypropylene conical tube. The organic extracts were evaporated to dryness under a stream of nitrogen and the residue was reconstituted in 50 μl of HPLC mobile phase. A 20-μl volume of the final solution was injected onto the LC–MS–MS system.

For method B, frozen urine samples were thawed and brought to room temperature. A 0.4-ml aliquot was pipetted into a 15-ml polypropylene culture tube, followed by the addition of 100 μl of 1000 ng/ml internal standard and then 0.25 ml of 2.0 *M* NaOH. After the addition of 5 ml of methyl-*tert*.-butyl ether, the tube was capped and rotated for 15 min at 60 rpm. The lower layer (aqueous) of the mixture was frozen in a dry ice–acetone bath and the organic phase was transferred to a clean 12-ml polypropylene conical tube containing 200 μl of 2% formic acid. The sample was capped and rotated for 15 min at 60 rpm. The lower layer was frozen again via a dry ice–acetone bath and the organic phase was discarded. Residual solvent was evaporated under a stream of nitrogen. An additional 200 μl of 2% formic acid was added, and 20 μl of the final solution was injected onto the HPLC system.

2.6. Quantification

Calibration standards were prepared daily to construct the standard curve. For the plasma method, the concentration of **I** was calculated from the linear least-squares fitted line of peak area ratios of **I** to the internal standard **II** versus the concentration of **I** with reciprocal weighing on the concentration ($1/x$). For the urine method, peak height ratios were used instead of peak area ratios. The standard samples were assayed along with QC and unknown samples.

3. Results and discussion

3.1. LC–MS–MS conditions for method A

3.1.1. Ion source

We have tested both the turbo ionspray (TIS) and atmospheric pressure chemical ionization (APCI) ion sources. The TIS showed at least two-fold better

sensitivity than APCI for the detection of **I**. Since one of the major requirements for method A was sensitivity, turbo ionspray was selected as the ion source for this method.

3.1.2. Solvent effect

Double-charged parent ion and metal adducts of **I** were observed in the Q1 scan spectrum. The intensity and relative abundance of these ions depended on the pH value of the solvent. When the solvent pH decreased, the intensity of double-charged parent ion (m/z 204) increased rapidly, while the intensity of single-charged ion decreased. The same trend was found for metal ion adducts $[M+Na]^+$ and $[M+K]^+$: $[M+Na]^+$ decreased when pH decreased, while $[M+K]^+$ increased. To minimize the formation of double-charged parent ions and metal adducts, a mixture of ammonium acetate buffer (pH 4.2) and methanol was finally chosen as the HPLC mobile phase. A pH of 4.2 was high enough to sufficiently suppress the formation of double-charged parent and $[M+K]^+$ ions, while the $[M+Na]^+$ peak was not significant.

3.1.3. Ion detecting channels

The positive product scan spectra for the protonated molecule $[M+H]^+$ of **I** at m/z 407 and **II** at m/z 421 are shown in Fig. 2. Based on the ionization of these compounds, the channels used for the quantitative determination of these compounds were m/z 407→121 (**I**) and m/z 421→135 (**II**).

3.1.4. Matrix effect

Matuszewski and co-workers have discussed adverse matrix effects in quantitative LC–MS–MS analysis thoroughly [5,6]. When an analyte co-elutes with other compounds from an HPLC column, the ionization efficiency of the analyte might be affected if electrospray or ionspray is used. This is caused by the charge competition between the analyte and the co-eluents. This ion suppression effect makes the quantification of the analyte inaccurate, especially from a biological fluid sample which usually contains a complex background. To evaluate possible ion suppression in our assay, two groups of samples were prepared by spiking the same amounts of **I** and internal standard into: (1) control plasma extracts and (2) HPLC mobile phase. The control plasma extract was obtained by processing drug-free control plasma through the sample preparation procedure. Chromatographic peak areas of **I** from these two groups of samples were compared and no quantitatively significant difference was found after evaluating as many as five different lots of plasma. The same evaluation was performed on internal standard and no significant peak area differences were observed. Thus, we concluded that ion suppression from plasma matrix was not significant for this method.

3.2. Sensitivity and linearity

For method A, the LOQ is 0.1 ng/ml **I** using 0.5 ml of human plasma. The linear dynamic range is three orders of magnitude from 0.1 ng/ml to 100 ng/ml. For all completed experiments to this point, the coefficient of determination (r^2) for the calibration curves was greater than 0.999.

For method B, the LOQ is 20 n*M* (8.12 ng/ml) using 0.4 ml of urine. The linearity range is 20–20 000 n*M* (8.12–8120 ng/ml). If necessary, an LOQ as low as 2.5 n*M* (~1 ng/ml) could be achieved by reducing the final reconstitution volume to 100 μl and increasing the injection volume to 80 μl. However, the highest concentration in the standard curve should be lowered to 2500 n*M* in order to maintain the linearity range of three orders of magnitude. The responses of the UV detector at concentrations beyond the calibration curve are not proportional to the drug concentrations under the experimental conditions. The current linearity range of 20–20 000 n*M* for this method reflects the best estimated operational range to analyze **I** in human urine.

3.3. Accuracy and precision

Intra-day accuracy and precision of the methods was determined by analyzing five replicates of calibration standards at all concentrations. In method A, the precision (RSD, $n=5$) at LOQ (0.1 ng/ml) was 6.2% and the accuracy (percentage of nominal value) was 101.0%. The precision for all concentrations ranged from 2.7 to 6.6%, while the accuracy ranged from 97.8 to 103.0%. In method B, the

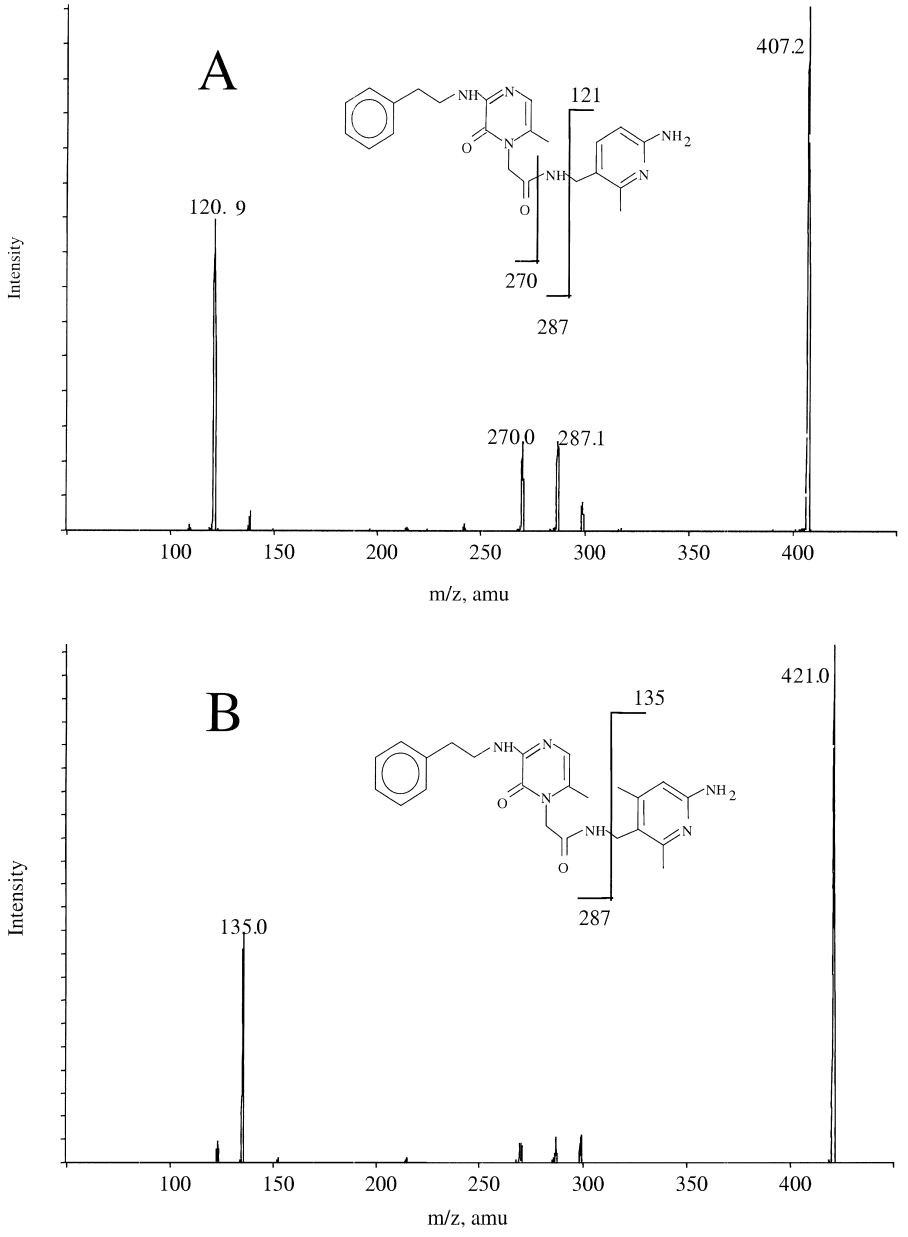

Fig. 2. Product scan spectra of compounds **I** (A) and **II** (B).

precision at LOQ (20 n*M*) was 12.2% with an accuracy of 95%. For all the concentration levels in the standard curve, the precision varied from 1.1 to 12.2%, and the accuracy ranged from 95.0 to 102.8%. The data are summarized in Tables 1 and 2.

Inter-day precision and accuracy were determined by analyzing quality control samples on three different days. For method A, the precision ($n=3$) was 8.5, 1.1 and 4.5% and accuracy was 106.7, 108.3 and 100.6% for low, medium and high QC samples, respectively. For method B, precision ($n=3$) was 8.8, 5.1 and 1.9%, and accuracy was 93.1, 95.6 and

Table 1
Intra-day validation precision and accuracy for method A

Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	Accuracy[a]	Precision[b]
0.1	0.101	101.0	6.2
0.2	0.206	103.0	6.0
0.5	0.489	97.8	5.8
2	1.999	100.0	2.7
5	4.885	99.7	2.7
20	19.94	99.7	3.9
50	50.99	102.0	6.6
100	99.19	99.2	2.7

[a] Calculated as (mean found concentration/nominal concentration)×100.
[b] Expressed as RSD (%).

93.7% for low, medium and high QCs, respectively. The data are summarized in Table 3.

3.4. Sample stability

The long-term stability of **I** at low temperatures was evaluated in order to prevent possible degradation under storage conditions. At −20°C, **I** was found to be stable in the plasma matrix for at least 120 days. However, long-term storage of **I** in urine at −20°C showed slowly decreasing concentrations of **I**. At day 120, the average concentration of urine QC samples was only 84.2% of that from day zero. One possible explanation for the greater stability of **I** in plasma was the binding of **I** to plasma protein resulting in protection from degradation. **I** was found stable in urine at −70°C for at least 3 months.

Autosampler stability was tested to reflect the need of occasional delayed injection or re-injection. This was performed by re-injecting the final extracts 24 h after the first injection. The samples remained in the autosampler tray for this time. The results showed that **I** was stable in the final extraction solutions for more than 24 h at ambient temperature for both methods.

Compound **I** was also tested for freeze–thaw stability in plasma and urine. The compound was stable after three freeze–thaw cycles as required by departmental standard operation procedures. In each cycle, a group of frozen QC samples was thawed to room temperature for more than 4 h and then re-frozen to the storage temperature. These samples were processed along with corresponding QC samples that had not undergone the freeze–thaw cycles. Peak area ratios to internal standard were used for the comparison of these two groups of samples. The

Table 2
Intra-day validation precision and accuracy for method B

Nominal concentration (n*M*)	Mean found concentration (n*M*)	Accuracy[a]	Precision[b]
20	19	95.0	12.2
50	48	96.0	7.8
100	99	99.0	4.2
500	512	102.4	2.9
1000	1022	102.2	1.1
5000	5139	102.8	2.7
10 000	10 217	102.2	1.9
20 000	19 612	98.1	1.7

[a] Calculated as (mean found concentration/nominal concentration)×100.
[b] Expressed as RSD (%).

Table 3
Inter-day validation ($n=3$)

	Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	Precision (%)	Accuracy (%)
Method A				
Low QC	0.30	0.32	8.5	106.7
Medium QC	6.00	6.50	1.1	108.3
High QC	90.00	90.54	4.5	100.6
	Nominal concentration (n*M*)	Mean found concentration (n*M*)	Precision (%)	Accuracy (%)
Method B				
Low QC	60	56	8.8	93.1
Medium QC	1200	1147	5.1	95.6
High QC	18 000	16 870	1.9	93.7

results indicated that compound **I** is stable after three freeze–thaw cycles in both plasma and urine.

3.5. Specificity, reproducibility and ruggedness

Although the superior selectivity of MS–MS detection has been well recognized, sufficient chromatographic separation of an analyte from its metabolites and/or parent drug is always recommended in drug metabolism studies. Due to structural similarity of parent drug and its metabolites, these compounds may share some common mass spectrometric detecting channels. The absolute response of these compounds in these detecting channels might vary, but the quantitative detection will be inaccurate if more than one of these compounds are present in the analysis. To evaluate the possibility of this effect in method A, three known major in vitro metabolites of **I** were collected, mixed with **I** and injected into the LC–MS–MS system. All of the metabolites showed some response in the detecting channel for **I**; however, the chromatographic separation of **I** from these metabolites was sufficient and no metabolite peak appeared to interfere with the detection of the compound.

To further evaluate the specificity of both methods, chromatograms of more than six batches of blank control plasma and urine samples were tested. No peaks were found near the retention times for both the analyte and internal standard, indicating no interference from endogenous compounds. Representative chromatograms of plasma and urine spiked with and without the analyte and internal standard are shown in Figs. 3 and 4.

Both of the methods were separately validated by three chemists and showed excellent robustness and ruggedness.

3.6. Recovery

To obtain the recovery data, a known amount of the compound was spiked into control plasma or urine, extracted and analyzed. Since the matrix effect was insignificant for both methods, the recovery was calculated by comparing peak area to that of the direct injection of neat standard at the same concentration.

For method A, the recoveries were 80.7% for **I** at three concentrations (0.5, 5, 100 ng/ml) and 82.5% for the internal standard. For method B, the average recovery was 99.1% at 50, 1000 and 20 000 n*M*, and 97.5% for the internal standard.

3.7. Comparison of the two methods

The evaluation results indicate that both methods A and B were satisfactory for the determination of **I** in human fluids. Method A is more sensitive, and thus useful for human plasma samples. Due to the high selectivity of LC–MS–MS, the sample preparation procedure for method A requires only a one-step extraction, while method B requires an additional extraction step to ensure interference is effectively removed. The method A assay run-time is only 5

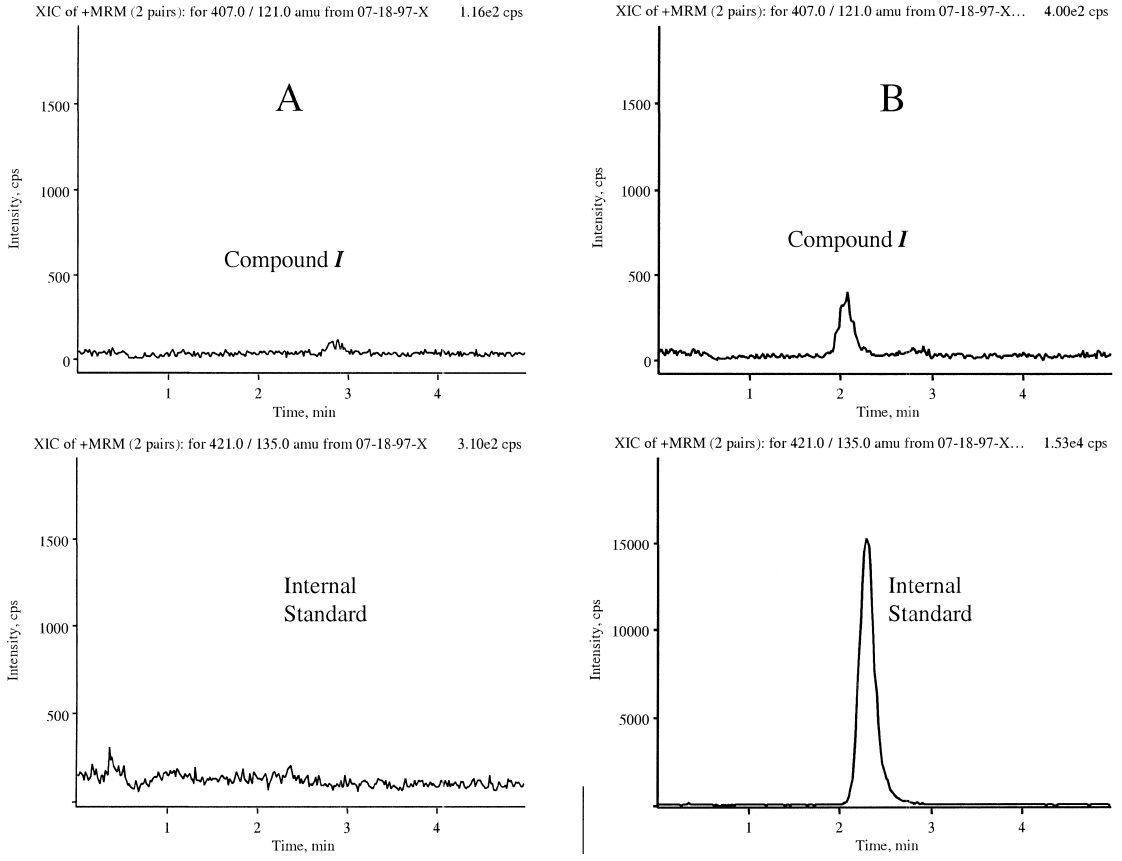

Fig. 3. Representative chromatograms of **I** and **II** in human plasma. (A) Blank control plasma; (B) control plasma spiked with 0.2 ng/ml of **I** and 10 ng/ml of **II** (as internal standard).

min, significantly shorter than the 15-min HPLC–UV method. The advantages of method B are from the low operating cost of HPLC–UV. When the concentration of **I** is high, such as in urine, method B serves very well by providing enough sensitivity. When an expensive LC–MS–MS instrument is not available and/or the sensitivity is not crucial, the HPLC–UV method is a very good alternative for the determination of **I** in human fluids.

4. Conclusions

An LC–MS–MS method and an HPLC–UV method have been developed for the quantitative determination of a novel thrombin inhibitor (**I**) in human fluids. The LC–MS–MS method was evaluated and validated for the determination of **I** in human plasma, while the HPLC–UV method was evaluated and validated in human urine. The LC–MS–MS method has a lower limit of quantitation of 0.1 ng/ml and the HPLC–UV method, 8.12 ng/ml (20 n*M*). Both methods have linear dynamic ranges of three orders of magnitude. The methods have been proven fast, specific and sensitive. Various validation procedures have proven that both methods are rugged, robust and reproducible. The study also suggests that while LC–MS–MS provides superior sensitivity and selectivity, thus an excellent choice for the determination of drugs and their metabolites in plasma at very low concentrations, HPLC–UV is

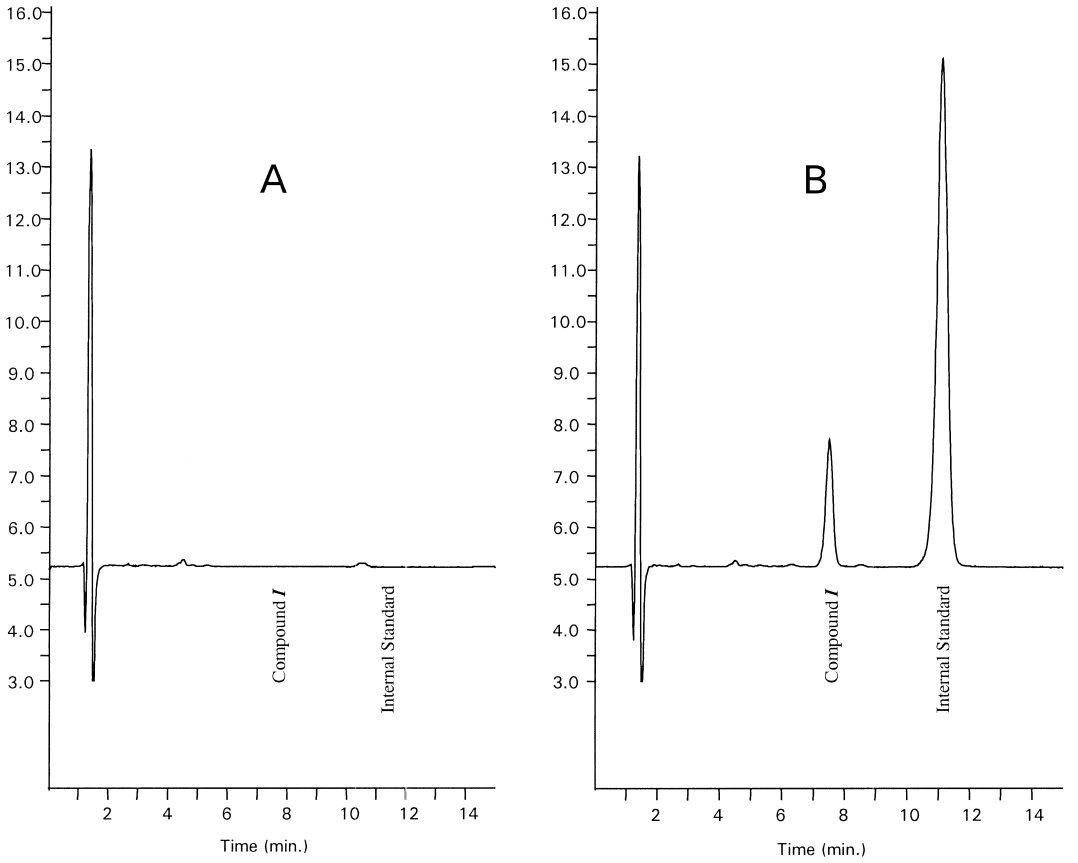

Fig. 4. Representative chromatograms of **I** and **II** in human urine. (A) Blank control urine; (B) control urine spiked with 1000 n*M* (406 ng/ml) of **I** and 2380 n*M* (1000 ng/ml) of **II** (as internal standard).

still useful in the analysis when sensitivity is not crucial, such as in human urine.

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