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Simultaneous determination of a novel thrombin inhibitor and its two metabolites in human plasma by liquid chromatography/tandem mass spectrometry

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Abstract

I, 3-(2-phenethylamino)-6-methyl-1-(2-amino-6-methyl-5-methylene-carboxamidomethylpyridinyl)-pyrazinone dihydrochloride monohydrate, is a potent, orally active thrombin inhibitor. The compound also has two metabolites which have been shown to have thrombin inhibitory activity: benzylic alcohol M3 metabolite (***II***) and hydroxymethylpyrazinone M7 metabolite (***III***). A liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the simultaneous determination of ***I*** and its two metabolites in human plasma has successfully been developed. The method consists of treating 0.5 ml plasma with 2 N NaOH and extracting ***I***, ***II***, ***III*** and internal standard (***IV***) with ethyl acetate:methyl-*t*-butyl ether (1:3,v/v). The analytes were then back-extracted into 2% formic acid. The analytes were chromatographed by high performance liquid chromatography (HPLC) and detected by MS/MS. Positive ionization was used on a heated nebulizer probe monitoring precursor → product ion combinations in multiple reaction monitoring mode. The linear range is 0.5–1000 nM for ***I*** and ***III*** and 0.75–1000 nM for ***II***. Recoveries were 88, 74, 78 and 102.1% for ***I***, ***II*** and ***III*** and the internal standard, respectively in human plasma. Intraday variation using this method was $\leq 7.9\%$ for ***I***, $\leq 9.0\%$ for ***II*** and $\leq 9.5\%$ for ***III*** across the standard curve range. This method exhibits good linearity and reproducibility for the three analytes.

Keywords: Thrombin inhibitor; LC/MS/MS; Liquid–liquid extraction

1. Introduction

Thrombin is a serine protease that plays a role in blood coagulation. The role of thrombin includes the cleavage of fibrinogen to release fibrin and the activation of platelets through the thrombin receptor. Fibrin and platelets are the primary components of blood clots, therefore inhibiting thrombin would effect blood coagulation. The development of an orally active thrombin inhibitor which would act as an anti-coagulant has been researched. [1–6].

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I (3-(2-phenyethylamino)-6-methyl-1-(2-amino-6 - methyl - 5 - methylene - carboxamidomethyl-pyridinyl)pyrazinone dihydrochloride monohydrate) is a potent orally active thrombin inhibitor and ***II*** (a benzylic alcohol metabolite of ***I***) and ***III*** (a hydroxymethyl-pyrazinone metabolite of ***I***), have also been shown to exhibit thrombin inhibitory activity [6, internal communi-

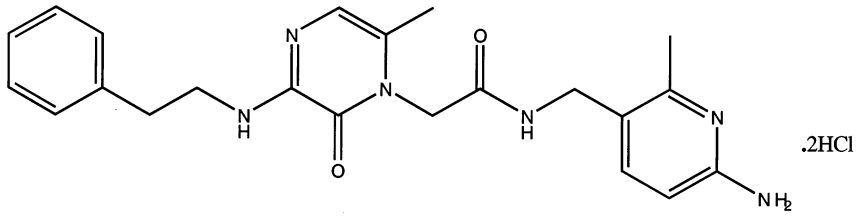
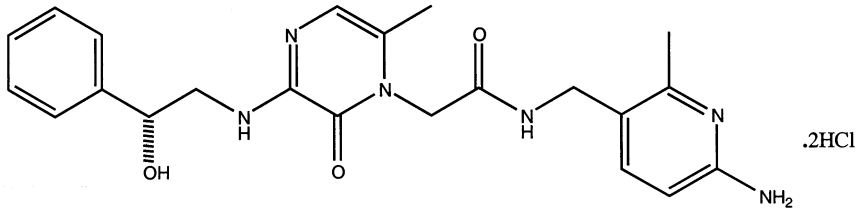
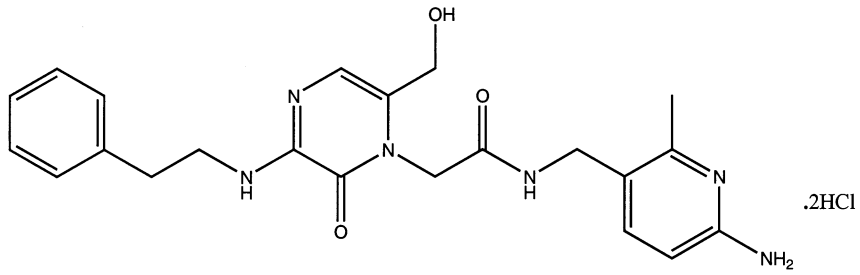


Fig. 1. Chemical structure for compound ***I.***



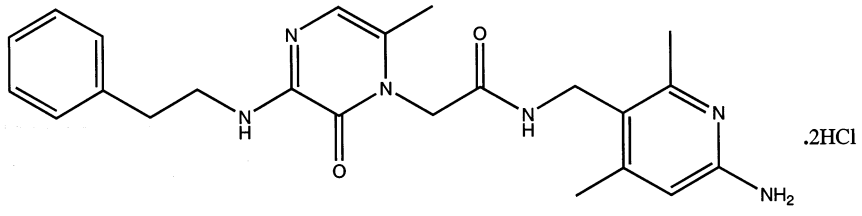
Compound ***II***

Fig. 2. Chemical structure for compound ***II.***



Compound ***III***

Fig. 3. Chemical structure for compound ***III.***



Compound ***IV***

Fig. 4. Chemical structure for compound ***IV***.

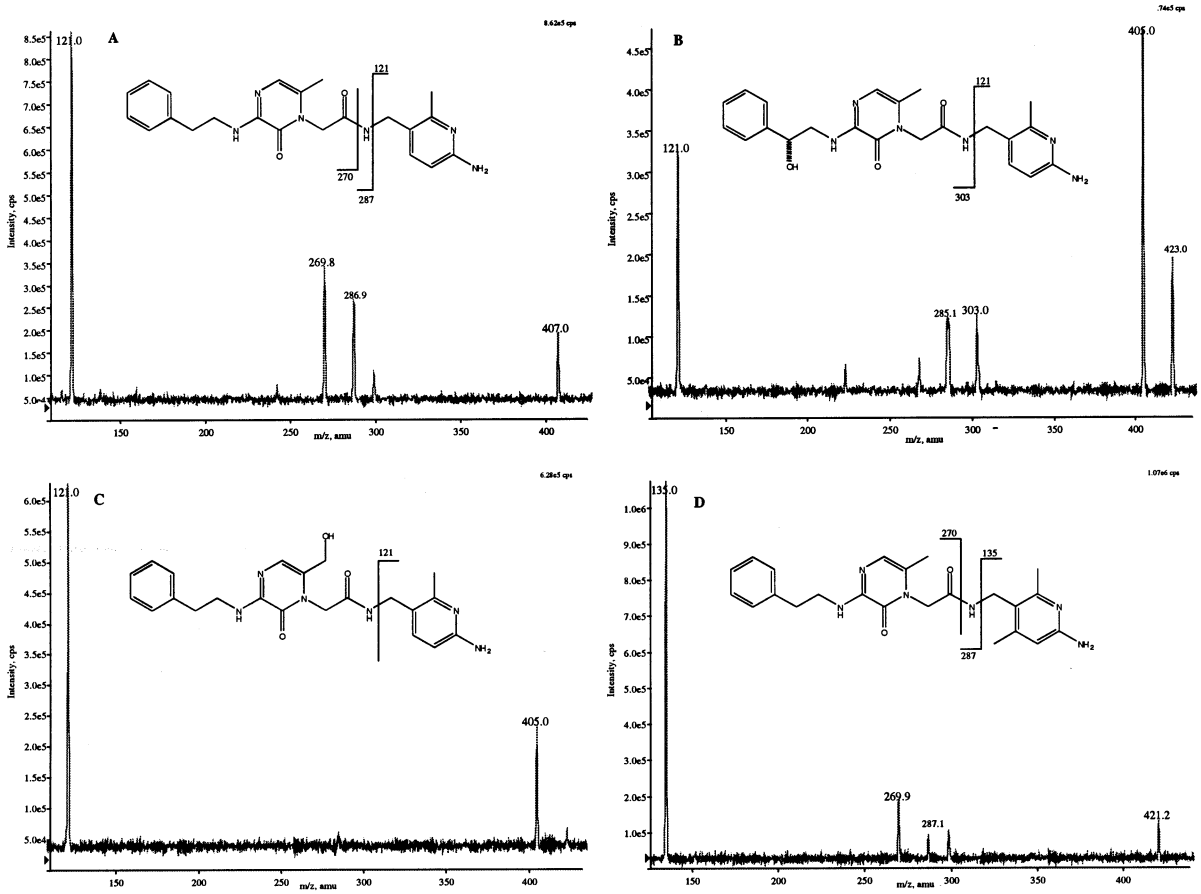


Fig. 5. Product ion spectra of compounds ***I*** (A), ***II*** (B), ***III*** (C) and ***IV*** (D).

Table 1
Representative calibration curve parameters

	Compound ***I***			Compound ***II***			Compound ***III***		
	Slope	Intercept	Corr. coeff.	Slope	Intercept	Corr. coeff.	Slope	Intercept	Corr. coeff.
	0.034	−0.002	0.9998	0.012	0.002	0.9993	0.018	0.003	0.9990
	0.032	−0.003	0.9994	0.011	−0.003	0.9995	0.016	−0.001	0.9996
	0.036	−0.002	0.9999	0.013	0.000	1.0000	0.022	0.000	0.9996
	0.039	−0.005	0.9995	0.013	0.004	0.9997	0.022	0.008	0.9994
Mean	0.035	–	0.9997	0.012	–	0.9996	0.020	–	0.9994
SD	0.003	–	0.0002	0.001	–	0.0003	0.003	–	0.0003
%CV	8.5	–	0.02	7.8	–	0.03	15.4	–	0.03

cation]. The structures for ***I***, ***II***, ***III*** and the internal standard (***IV***) are shown in Figs. 1–4.

No method has been reported for the simultaneous analysis of ***I***, ***II*** and ***III*** in biological samples. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) has not been used ex-

Table 2
Intraday validation precision and accuracy

	Compound *I*			Compound *II*			Compound *III*		
Nominal conc. (nM)	Mean conc. (nM)	Precision[a]	Accuracy[b]	Mean conc. (nM)	Precision[a]	Accuracy[b]	Mean conc. (nM)	Precision[a]	Accuracy[b]
0.5	0.56	4.74	111.2	N/A[c]	N/A[c]	N/A[c]	0.49	9.50	98.2
0.75	0.83	5.60	110.2	0.78	4.78	103.7	0.73	6.37	96.1
1.0	0.99	7.93	99.2	0.91	8.99	94.8	0.80	7.86	93.0
5.0	4.56	3.30	91.1	5.13	3.66	96.6	4.96	2.40	97.2
20	18.13	3.25	90.7	22.10	6.72	101.6	24.34	7.88	107.2
100	98.49	2.51	98.5	111.79	4.03	104.5	116.49	4.76	107.5
500	492.50	3.50	98.5	543.33	7.98	99.7	553.69	5.10	104.9
750	736.40	5.20	98.2	687.46	4.83	97.9	676.17	4.97	96.8
1000	1024.79	3.10	102.5	1023.47	1.70	101.2	1053.79	4.21	99.1

[a] Expressed as CV%.
[b] Calculated as (mean concentration/nominal concentration) × 100.
[c] LOQ for compound *II* is 0.75 nM.

Table 3
Intraday precision and accuracy for quality control samples

	Compound *I*	Compound *I*	Compound *III*
High QC—Nominal concentration 800 nM			
Mean found conc. (nM)	849.54	805.42	778.83
Precision%	5.60	4.61	7.65
Accuracy%	106.2	100.7	97.4
Medium QC—Nominal concentration 160 nM			
Mean found conc. (nM)	161.86	174.74	168.35
Precision%	5.12	3.22	4.63
Accuracy%	101.2	109.2	105.2
Low QC—Nominal concentration 1.6 nM			
Mean found conc. (nM)	1.66	1.57	1.57
Precision%	4.85	6.47	8.78
Accuracy%	103.6	98.3	98.3

tensively for the quantitative analysis of thrombin inhibitors in biological samples. A method was reported by Song et al. for the determination of ***I*** in human urine by high performance liquid chromatography with ultraviolet detection (HPLC–UV) and in human plasma by LC/MS/MS [7]. The LC/MS/MS method utilized Turbo Ionspray as the MS source, where heated nebulizer is the source for the present work. LC/MS and LC/MS/MS were used to identify the oxidative degradates of **I** formed in tablets and i.v. solutions [8]. Lee et al. report the use of HPLC–UV for the determination of a new thrombin inhibitor in the blood of rats and dogs and Mendoza et al. used HPLC with fluorescence detection for the quantitation of a novel thrombin inhibitor in human blood and urine [9,10].

The purpose of this research was to develop a sensitive and reliable method for the analysis of ***I***, and its two metabolites, ***II*** and ***III*** in human plasma. In order to meet the requirements of sensitivity, selectivity and a wide dynamic range for all three compounds, heated nebulizer LC/MS/MS was used for analysis.

2. Experimental

2.1. Materials

Compounds ***I***, ***II*** and ***III*** and the internal standard (***IV***) were obtained from Merck Research Laboratories (West Point, PA). Methyl-tert-butyl ether (MTBE) was obtained from Burdick & Jackson (Muskegon, MI). HPLC grade ethyl acetate was purchased from EM Science (Gibbstown, NJ). Control human plasma was purchased from Sera-Tec Biologicals (New Brunswick, NJ). All other solvents and chemicals were obtained from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical (St. Louis, MO).

Table 4
Stability of ***I***, ***II***, and ***III*** under various conditions in human plasma

QC level	Compound *I*			Compound *II*			Compound *III*		
	High[a]	Med[a]	Low[a]	High[a]	Med[a]	Low[a]	High[a]	Med[a]	Low[a]
	800 nM	160 nM	1.6 nM	800 nM	160 nM	1.6 nM	800 nM	160 nM	1.6 nM
3 Freeze–Thaw cycles	101.7	102.8	110.8	106.3	93.4	100.6	108.6	98.8	105.1
4°C extract	90.7	99.7	94.6	95.1	92.6	105.7	96.1	96.1	105.1
Room temp ext.	97.2	103.8	105.4	93.5	98.0	94.3[b]	98.1	102.4	99.4
10 day plasma at −20°C	97.4	97.3	101.5	93.3	88.8	110.2	98.7	93.0	104.3
35 day plasma at −20°C	97.7	100.8	94.6	95.6	98.0	97.1	97.9	101.9	97.5

[a] Nominal QC values are shown. Stability was determined by accuracy of QC values under experimental conditions as compared to the mean found QC values.

[b] Due to an sample processing error, $n = 2$ samples were used; $n = 3$ values were used for all other samples.

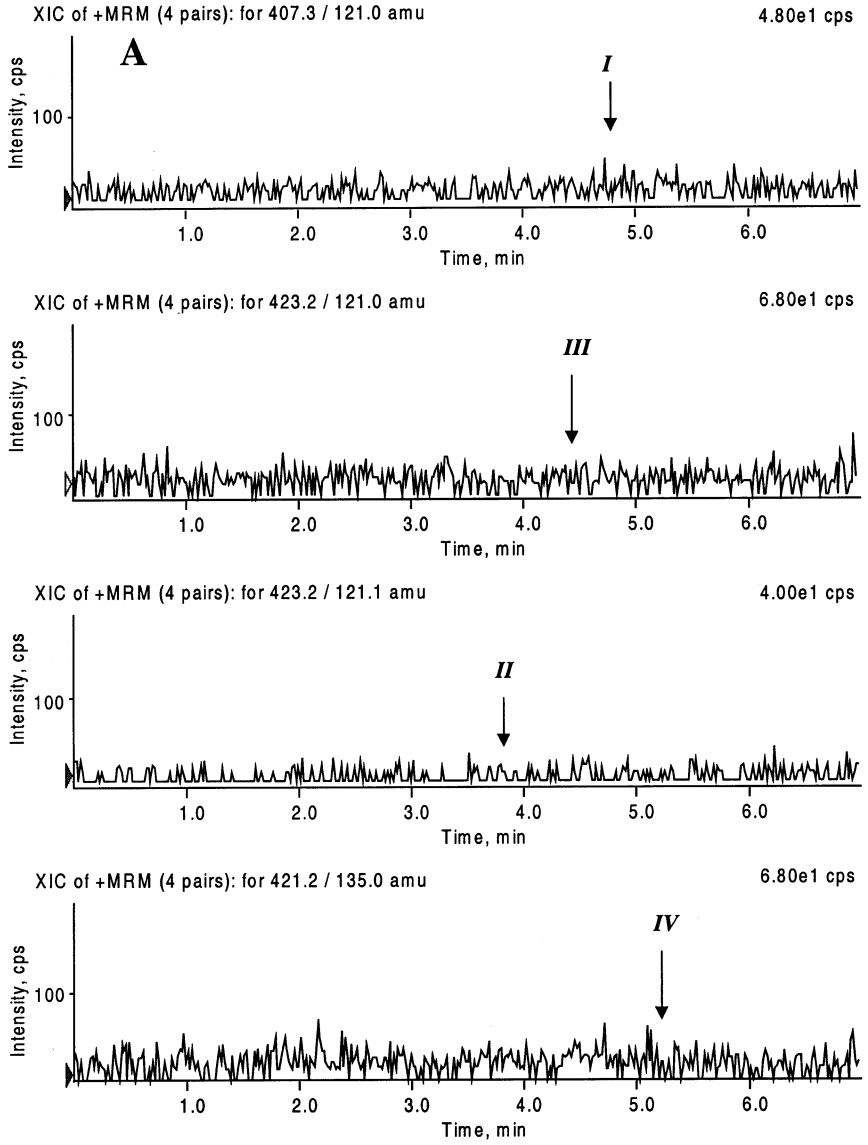


Fig. 6. Representative chromatogram for ***I***, ***II***, ***III*** and ***IV*** in human plasma. (A) Blank control plasma; (B) Control plasma spiked with 0.5 nM ***I*** and ***III***, 0.75 nM ***II*** and 237.8 nM ***IV***.

2.2. Apparatus

The LC/MS/MS system consisted of a PE Sciex (Thornhill, Ontario, Canada) API 365 mass spectrometer with a heated nebulizer interface, two Perkin-Elmer (Norwalk, CT) Series 200 high pressure mixing pumps and a Perkin-Elmer Series 200 autosampler. Data was processed using MacQuan software (Version 1.4, PE Sciex) on a Macintosh PowerPC 9600 computer.

2.3. Chromatographic conditions

The analytical column used to separate the three analytes and the internal standard (***IV***) was a Thomson BIOAdvantage C18 (100 × 4.6 mm, 5

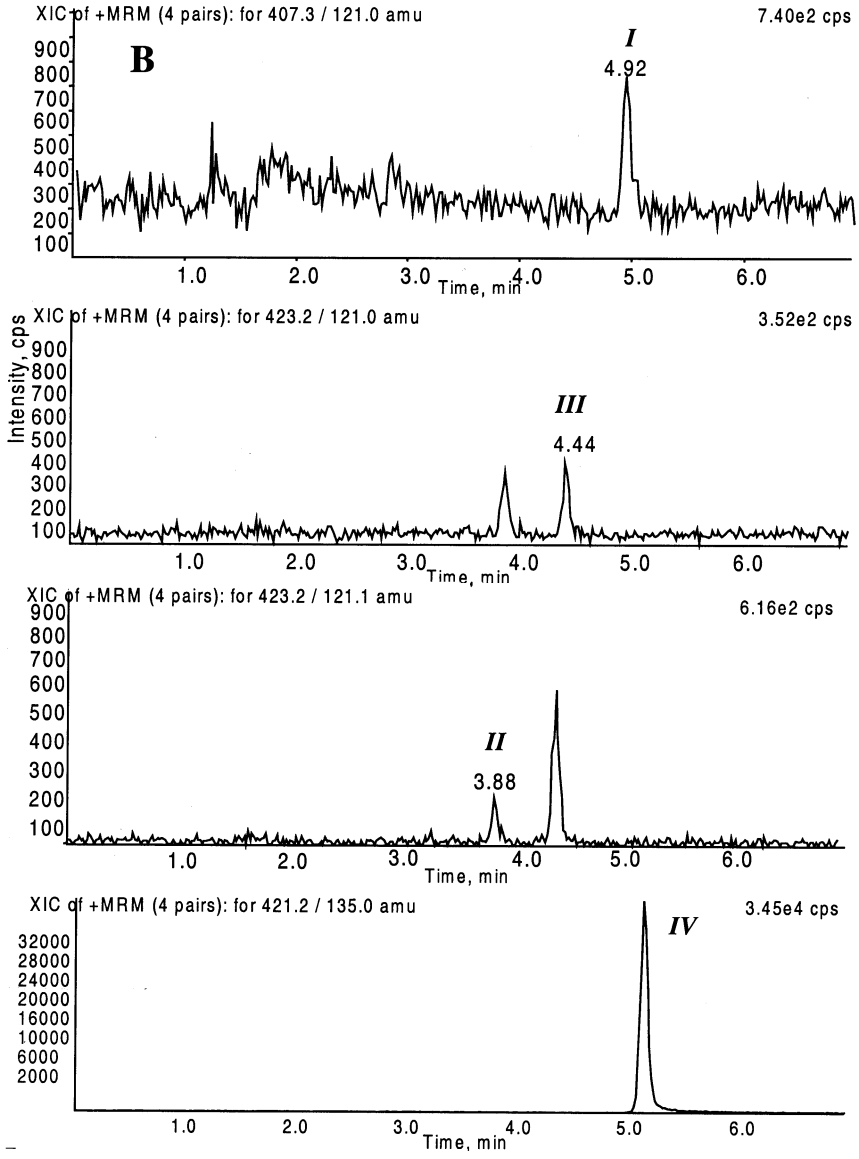


Fig. 6. (*Continued*)

μm) from Thomson Instrument Company (Chantilly, VA). Mobile phase A consisted of ammonium acetate (10 mM)-formic acid (99.9:0.1 v/v) and mobile phase B was methanol. The analytes were separated using a gradient of A/B (65:35, v/v) at time = 0 min changing linearly to A/B (10:90, v/v) at time = 7 min. The column was equilibrated in the initial conditions for 5 min prior to the introduction of the next sample. The flow rate was 0.8 ml min^{-1} and the column was at ambient temperature.

2.4. Sample preparation

A 2.0 mM stock solution of ***I*** was prepared in methanol/water (50:50, v/v). Stock solutions of ***II*** and ***III*** were prepared at 100 M in methanol/water (50:50, v/v). All of the stock solutions were

further diluted to make a series of working standard solutions with concentrations of 10000, 7500, 5000, 1000, 200, 50, 10, 7.5 and 5 nM. Due to sensitivity limitations, the 5 nM standard was not prepared for ***II***. Internal standard (***IV***) working solution was prepared at 100 ng ml^{-1} (237.8 nM). All of the standard solutions were stored at 4°C. Plasma standards were prepared daily by adding 50 μl of each working standard to 0.5 ml of drug-free control human plasma. The resulting plasma concentrations ranged from 0.5 to 1000 nM for ***I*** and ***III***. and from 0.75 to 1000 nM for ***II***.

Quality control (QC) standard solutions were prepared separately at 800 M for ***I***, ***II*** and ***III*** for the high QC. Each solution was further diluted to make medium (160 μM) and low (1.6 μM) QC solutions. All solutions for ***I***, ***II*** and ***III*** were prepared separately. QC samples were prepared by adding 50 μl of the appropriate QC standard solution for ***I***, ***II*** and ***III*** to a 50 ml volumetric flask containing control, drug-free human plasma. QC plasma concentrations were 800, 160 and 1.6 nM for the high, medium and low QCs, respectively. All QC samples were stored at −20°C until assayed.

2.5. Extraction procedure

Frozen plasma samples were thawed and brought to room temperature. A 0.5 ml aliquot of plasma was added to a 14 ml polypropylene tube. The internal standard was added (50 μl of the 100 ng ml^{-1} solution) followed by the addition of 250 μl of 2 M NaOH and 1 ml of ethyl acetate. Samples were vortexed for 5 min. A total of 3 ml MTBE was added and the samples were rotated for 15 min at 60 rpm and centrifuged for 10 min at $2060 \times g$. The aqueous layer was frozen in a dry/ice acetone bath and the organic layer transferred to a 12 ml conical polypropylene tube containing 150 μl of 2% formic acid. The samples were vortexed and then centrifuged at $2060 \times g$. The samples were frozen in a dry ice/acetone bath and the organic layer discarded. Residual organic solvent was evaporated under a stream of nitrogen. The samples were transferred to 200 μl autosampler vials and 40 μl was injected for analysis.

2.6. Quantitation

Plasma calibration standards were prepared daily to construct the standard curve. The concentration of ***I***, ***II*** or ***III*** was determined from the linear least-squares fitted line of the peak area ratios of ***I***, ***II*** or ***III*** to the internal standard (***IV***) versus the concentration of ***I***, ***II*** or ***III*** with reciprocal weighting $(1/x)$ on the concentration. Standards were assayed with quality control and unknown samples.

3. Results and discussion

3.1. LC/MS/MS conditions

The benzylic metabolite (***II***) is a chiral compound. The mass spectral conditions and response for both the R and S isomers were determined to be the same, hence total benzylic metabolite concentration is determined. The R isomer of ***II*** was used as the standard for this research.

Heated nebulizer was used as the ion source because it allowed for a wide dynamic range and good sensitivity for all three analytes. The positive product ion scan spectra for the protonated molecules $[M+H]^+$ of ***I*** at m/z 407.3, ***II*** at m/z 423.2, ***III*** at m/z 423.2 and ***IV*** at m/z 421.2 are shown in Fig. 5. The ionization of each molecule led to the use of the following channels for quantitative determination: m/z $407.3 \rightarrow 121.0$ for ***I***, m/z $423.2 \rightarrow 121.0$ for ***II***, m/z $423.2 \rightarrow 121.1$ for ***III*** and m/z $421.2 \rightarrow 135.0$ for ***IV***. Compounds ***II*** and ***III*** have the same precursor ion and each exhibited a product ion at m/z 121. The two metabolites were monitored using the same ion pairs, while optimizing the mass spectrometric parameters for each compound to achieve the best sensitivity. Compounds ***II*** and ***III*** were separated chromatographically with no interference from each other. Furthermore, liquid–liquid extraction provided for clean extracts with no matrix effects present on the LC/MS/MS chromatograms.

3.2. Sensitivity and linearity

Sensitivity was determined by the lowest limit

of reliable quantitation for the compounds. The limit of quantitation (LOQ) was 0.5 nM for ***I*** and ***III*** and 0.75 nM for ***II*** using 0.5 ml plasma. The linear dynamic range was from LOQ to 1000 nM for all three analytes. For all standard curves analyzed, the correlation coefficient using weighted $(1/x)$ linear least-squares regression was >0.999 for the three analytes. Calibration curve parameters are shown in Table 1.

3.3. Accuracy and precision

Intraday accuracy and precision for the methods were determined from the analysis of five standard curves at all concentrations. Over the range of the curves, accuracy was 91–111% for ***I***, 95–105% for ***II***, and 93–108% for ***III***. Accuracy was determined as the percentage of calculated concentration compared to the nominal concentration. Precision (CV%) was $\leq 7.9\%$ for ***I***, $\leq 9.0\%$ for ***II*** and $\leq 9.5\%$ for ***III***. The data are summarized in Table 2.

Quality control samples were prepared for use in the determination of inter-assay accuracy and precision of the method and to assess the stability of ***I***, ***II*** and ***III*** in human plasma. Accuracy and precision were investigated by the replicate $(n=5)$ analysis of high, medium and low QC samples. Precision and accuracy for the intraday analysis of QC samples was assessed by the determination of the of the mean calculated concentrations as compared to nominal concentrations. The coefficient of variation (CV%) was also determined for the calculated concentrations. Accuracy ranged from 101 to 106% for ***I***, 98 to 109% for ***II*** and 97 to 105% for ***III***. Precision was $\leq 5.6\%$, $\leq 6.5\%$ and $\leq 8.8\%$ for ***I***, ***II*** and ***III***, respectively. Data obtained from the replicate analysis of QC samples indicate that the method is accurate and reliable. Data are summarized in Table 3.

3.4. Sample stability

Storage stability of ***I***, ***II*** and ***III*** was evaluated to determine whether degradation may occur during long-term storage. Stability was determined by analyzing QC samples stored at -20°C over a period of 10 and 35 days. The data indicate that ***I***, ***II*** and ***III*** were stable at -20°C for at least 1 month.

Due to the need for occasional delayed injection or re-injection, extract stability was studied. Six replicates of each QC level were extracted. Of the six replicates, three replicates for each level remained at 4°C for 24 h and the other three replicates remained at room temperature for 24 h prior to analysis. The results showed that all three analytes were stable in the extract solution at both temperatures for 24 h.

The analytes were also tested for freeze–thaw stability. Three freeze–thaw cycles were tested where one cycle consisted of thawing replicate $(n=3)$ QCs at each level at room temperature for 4 h and re-freezing at -20°C. The freeze–thaw samples were assayed and mean concentrations were compared to those values obtained for the QC intraday analysis. The results indicate that ***I***, ***II*** and ***III*** were stable after three freeze–thaw cycles. Stability data is summarized in Table 4.

3.5. Recovery

Recovery was determined by adding ***I***, ***II*** and ***III*** and internal standard to control human plasma. Five replicates at three concentrations (1, 100 and 1000 nM ***I***, ***II*** and ***III*** and 23.78 nM ***IV***) were prepared. The plasma was extracted under the conditions noted in section 2.5. Prior to transferring the samples to autosampler vials, the final volume of each extract was recorded. Neat standards were prepared at the same concentrations. Recovery was determined by comparison of the integrated peak area response for the extracted samples to the response for the neat solutions adjusted by the difference in volume between extracted samples and neat standards. Average recoveries were 87.9, 74.2, 78.1 and 102.1% for ***I***, ***II***, ***III*** and ***IV***, respectively.

3.6. Specificity

The specificity of the method was determined by analyzing six different lots of blank control

both with and without the internal standard. The chromatograms indicate that liquid–liquid extraction provided for clean extracts with no interference from endogenous compounds at the retention times for ***I***, ***II*** and ***III*** and the internal standard (***IV***). Representative chromatograms of plasma spiked with and without the analytes and internal standard are shown in Fig. 6.

3.7. Use of the method

Overall, the method, including sample preparation and analysis, was relatively fast. Sample preparation could be complete in a few hours, depending on the number of samples being analyzed. The LC/MS/MS run time was relatively short at 8 min. Data was generally obtained within 24 h of beginning the extraction procedure.

4. Conclusions

Reproducibility, specificity and sample stability were assessed under various conditions using a heated nebulizer LC/MS/MS procedure for ***I*** and its two metabolites, ***II*** and ***III***, in human plasma. Experimental results show that liquid–liquid extraction with LC/MS/MS analysis is sensitive, accurate, selective and reproducible within the calibration range 0.5–1000 nM for ***I*** and ***III*** and 0.75–1000 nM for ***II***. This LC/MS/MS method provides good sensitivity and selectivity for the three analytes while utilizing a relatively short run-time.

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