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Determination of *para*-aminohippuric acid (PAH) in human plasma and urine by liquid chromatography–tandem mass spectrometry

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ABSTRACT

In this manuscript, we present a simple and reliable method for the quantitation of *para*-aminohippuric acid (PAH; 2-(4-aminobenzamido)acetic acid) in human plasma and urine using liquid chromatography coupled to electrospray ionisation low-energy collision-induced dissociation tandem mass spectrometry (HPLC–ESI-CID-MS/MS) analysis (negative ion mode) via multiple reaction monitoring (MRM). Sample preparation involved protein precipitation of plasma samples with acetonitrile and subsequent dilution of urine samples with the mobile phase. The internal standard (IS) adopted was *para*-aminosalicylic acid (PAS; 4-amino-2-hydroxybenzoic acid). Chromatographic separation was achieved on a Cosmosil HILIC column using an isocratic mobile phase consisting of ammonium acetate buffer (20 mM) and acetonitrile (45:55, v/v) at a flow rate of 200 μl/min. The transactions monitored were m/z 192.9 → 149.1 for PAH and m/z 152.1 → 108.1 for IS. Linear calibration curves were generated over the PAH concentration range of 0.2–100 mg/L in human plasma and urine. The method was validated for selectivity, accuracy, precision, recovery and stability according to USFDA criteria, and has been successfully applied to a pharmacokinetic study in healthy volunteers administered an intravenous dose of 440 mg PAH.

1. Introduction

The accurate measurement of renal plasma flow (RPF) is essential in the assessment of renal function in clinical or research settings. A commonly used marker for estimating RPF is *para*-aminohippuric acid (PAH), since it is freely filtered at the glomerulus and undergoes extensive secretion and negligible reabsorption within renal tubules when its plasma concentration is low [1]. Analytical methods have been developed to measure PAH in human plasma and urine, such as liquid chromatography with electrochemical detection (LCEC) [2] and high-performance liquid chromatography with UV detection (HPLC–UV) [3–10]. In recent years, liquid chromatography coupled to electrospray ionisation tandem mass spectrometry (HPLC–ESI-MS/MS) has increasingly been used as a tool for drug assays in biological fluids due to the higher sensitivity and specificity of MS detection [11]. However, to the best of our knowledge, quantitation of PAH using ESI low-energy collision-induced dissociation tandem mass spectrometry (ESI-CID-MS/MS) has not been performed. This could be because PAH is an ionic compound, and is therefore weakly retained, if at all, in reversed-phase HPLC when run in common aqueous-organic mobile phases without ionic additives. Unfortunately, the use of ion-pairing reagents in the electrospray can influence the MS signal of the analyte due to ion suppression effects [12]. To overcome this problem, we have used hydrophilic interaction liquid chromatography (HILIC) as an alternative to reversed-phase HPLC to increase the chromatographic retention of PAH without compromising its MS signal. The HILIC column selected for this method had a novel triazole stationary phase which exhibited an anion-exchange mechanism, thus improving the retention of acidic compounds such as PAH.

This paper describes a simple, accurate and reliable HPLC–ESI-CID-MS/MS method for the quantitation of PAH in human plasma and urine. Sufficient chromatographic retention of PAH was achieved using a HILIC analytical column, which resulted in a retention time in the region of little or no matrix ion suppression. An added advantage of this method is the ease of sample preparation, involving a protein precipitation step for plasma samples and the simple dilution of urine samples.

2. Experimental

2.1. Chemicals and reagents

para-Aminohippuric acid (PAH; 2-(4-aminobenzamido)acetic acid), *para*-aminosalicylic acid (PAS; 4-amino-2-hydroxybenzoic acid) and ammonium acetate were purchased from Sigma–Aldrich (NSW, Australia). Acetonitrile was of HPLC grade, and all water used was filtered and deionised to a resistivity of 18 MΩ cm. Drug-

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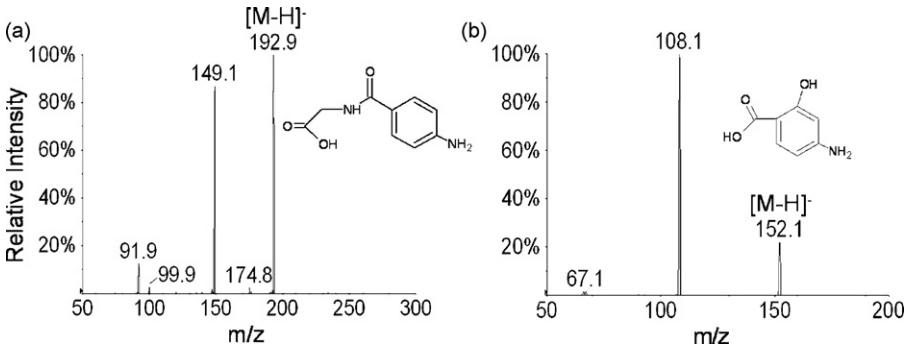

Fig. 1. Structure and product ion spectra of the $[M-H]^-$ ions of (a) PAH and (b) PAS.

free (blank) human plasma and urine were obtained from healthy volunteers participating in a pharmacokinetic study (described in Section 2.6).

2.2. Preparation of standards and quality control samples

The primary stock solution of PAH was prepared by dissolving PAH in deionised water at a concentration of 5000 mg/L. Working solutions of PAH were prepared fresh on each day of analysis by serial dilution with deionised water. The primary stock solution of the internal standard (IS) was prepared by dissolving PAS in acetonitrile at a concentration of 1000 mg/L, which was further diluted to produce appropriate concentrations of the IS working solution. The calibration curve for plasma was constructed by spiking PAH in blank plasma at concentrations of 0.2 mg/L, 0.5 mg/L, 1 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L. Quality control (QC) samples were prepared as a batch in blank plasma at PAH concentrations of 0.4 mg/L (low), 20 mg/L (medium) and

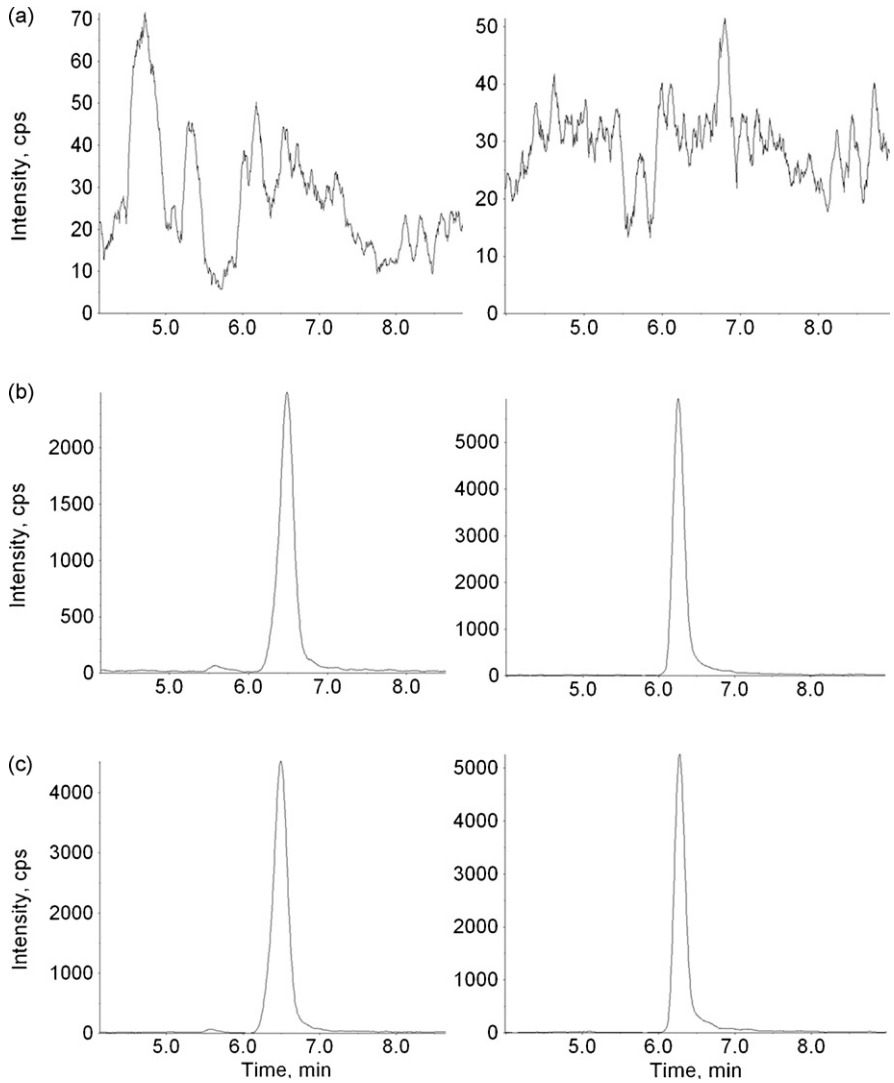

Fig. 2. Representative chromatograms of PAH (left panel) and IS (right panel) obtained from (a) double blank plasma, (b) plasma spiked with PAH (10 mg/L) and the IS, and (c) a volunteer plasma sample 12 min post-dose.

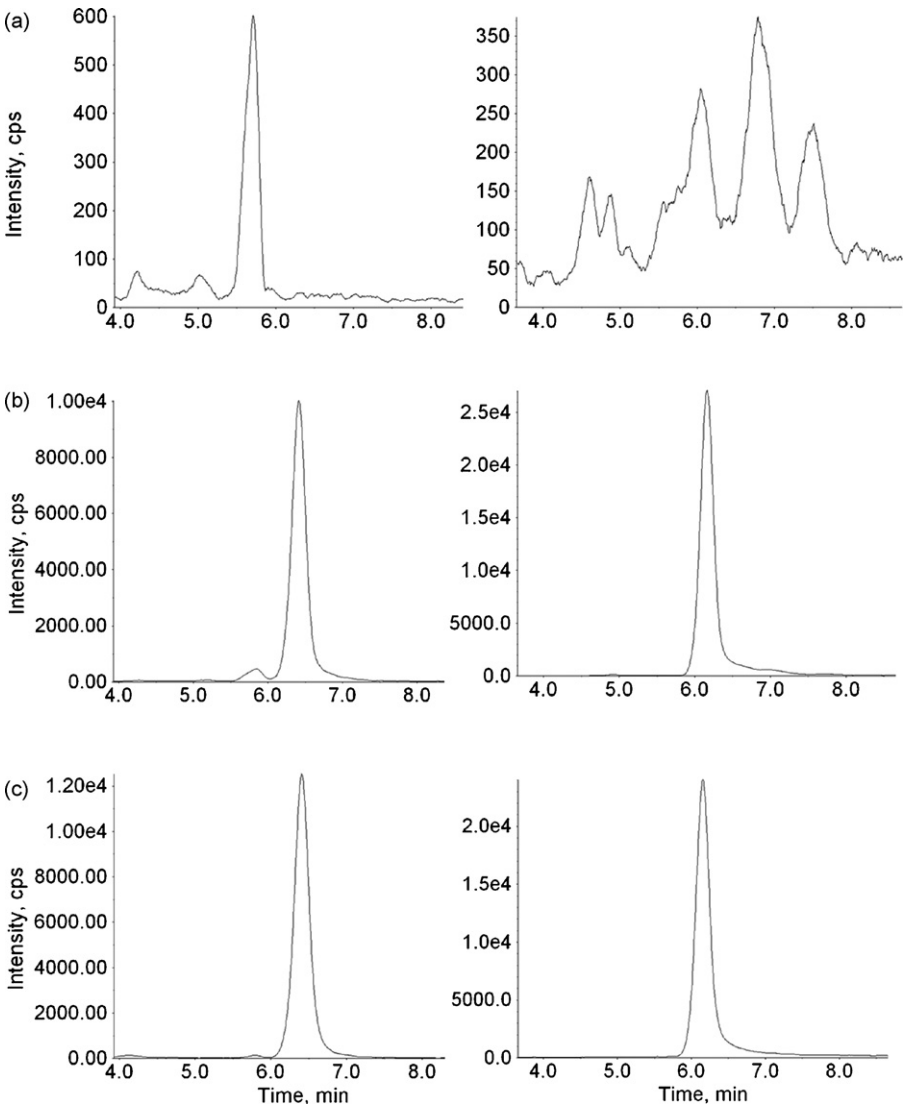

Fig. 3. Representative chromatograms of PAH (left panel) and IS (right panel) obtained from (a) double blank urine, (b) urine spiked with PAH (10 mg/L) and the IS, and (c) a volunteer urine sample pooled over 0–3 h post-dose.

75 mg/L (high), and stored in aliquots at −80 °C. The calibration curve for diluted urine was prepared in a similar manner to produce a range of 0.2–100 mg/L, with QC samples in diluted urine at concentrations of 0.4 mg/L (low), 20 mg/L (medium) and 75 mg/L (high).

2.3. Sample preparation

After thawing at room temperature, a 100 μL aliquot of the plasma sample was added to a 1.5 ml polypropylene tube followed by the addition of 200 μL of acetonitrile containing IS (1 mg/L). The mixture was vortex-mixed and then centrifuged at 8000 rpm for 5 min at 20 °C. The supernatant was removed and a 5 μL aliquot used for injection onto the HPLC. For the analysis of urine samples, a 50 μL aliquot of the urine sample was added to a 1.5 ml polypropylene tube followed by dilution with 950 μL of deionised water–acetonitrile (45:55, v/v) containing IS (0.05 mg/L). The mixture was vortex-mixed and then centrifuged at 8000 rpm for 5 min at 20 °C. A 5 μL aliquot of the diluted urine mixture was used for injection onto the HPLC.

Table 1
Accuracy of standards used in the calibration curve of PAH.

Theoretical concentration (mg/L)	Plasma		Urine	
	Measured concentration (mg/L)	Accuracy (%)	Measured concentration (mg/L)	Accuracy (%)
0.2 (LLOQ)	0.19 ± 0.03	95.5	0.20 ± 0.01	101.3
0.5	0.54 ± 0.02	107.4	0.48 ± 0.02	96.9
1	0.97 ± 0.08	96.6	1.00 ± 0.03	99.9
5	5.03 ± 0.33	100.6	5.42 ± 0.16	108.5
10	10.46 ± 0.77	104.6	10.39 ± 0.09	103.9
25	23.67 ± 1.96	94.7	24.09 ± 0.32	96.4
50	50.77 ± 1.68	101.5	47.89 ± 0.38	95.8
100	102.79 ± 4.81	102.8	100.3 ± 4.03	100.3

2.4. Chromatographic and mass spectrometric conditions

The HPLC system consisted of an Agilent 1100 LC pump and an Agilent 1100 well plate autosampler maintained at a temperature of 20 °C using an in-built cooler. Chromatographic separations were performed on a Cosmosil HILIC (2.0 mm × 150 mm, 5 μm) analytical column (Nacalai Tesque, Kyoto, Japan). The mobile phase used for analysis consisted of a mixture of 20 mM of ammonium acetate buffer and acetonitrile (45:55, v/v) delivered isocratically at a flow rate of 200 μl/min through the column. An API 3000 tandem mass spectrometer equipped with a turbo ion spray interface and supported by Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA) was used for detection of the separated compounds and processing of data. The ion spray source was operated in negative ion mode with a needle potential of −4200 V and the temperature of the ion spray was maintained at 300 °C. Nitrogen was used as a nebulising gas, with the curtain gas, nebuliser gas, and collision gas flows maintained at 8, 12 and 4 arbitrary units respectively. The MS analyser parameters for PAH and IS were: declustering potential −41 V and −21 V; focusing potential −170 V and −110 V; entrance potential −10 V and −10 V; collision energy −18 V and −20 V; and cell exit potential −25 V and −5 V respectively. A dwell time of 150 ms was used for all transitions for data collection. Detection was achieved in the multiple reaction monitoring (MRM) mode. The $[M-H]^-$ ions of PAH and IS were monitored at the specific transitions of m/z 192.9 → 149.1 and m/z 152.1 → 108.1 respectively.

2.5. Method validation

The method was validated for selectivity, linearity, sensitivity, accuracy, precision, recovery and stability according to the US Food and Drug Administration (USFDA) guidelines [13].

2.5.1. Selectivity

The selectivity of the method was tested by visual inspection of chromatograms of extracted human plasma and urine samples from six different volunteers for the presence of endogenous interfering peaks.

2.5.2. Calibration curves, accuracy and precision

For each biological matrix (plasma or urine), an eight-point calibration curve was constructed using the peak area ratio of PAH against IS. The linearity of the curve was assessed at PAH concentrations ranging from 0.2 mg/L to 100 mg/L in plasma or diluted urine by weighted ($1/x^2$) least squares linear regression using the criterion of a correlation coefficient (R^2) of 0.99 or better. The limit of detection (LOD) and limit of quantitation (LOQ) were determined at a signal-to-noise ratio of 3 and 5 respectively. The intra-day assay variability was evaluated by determining the PAH concentration in five replicates of QC samples at three concentrations (low, medium and high) on the same day, and the inter-day assay variability was performed over three separate days. Accuracy (at 0.2 mg/L, 0.5 mg/L, 1 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L and 100 mg/L) was expressed as a percentage of the theoretical concentration (accuracy (%) = mean observed concentrations/theoretical concentration × 100). Precision was determined as the coefficient of variation (%CV). The acceptance criteria used for validation of both accuracy and precision were a 15% deviation from the theoretical value except at the lower limit of quantitation (LLOQ), which was set at 20%.

2.5.3. Recovery and stability

For evaluation of extraction recovery, triplicate plasma samples at each of the three QC concentrations (low, medium, and high) were prepared as described above. In addition, another set of unextracted standards were prepared at the same QC concentrations by spiking deproteinised plasma with PAH, and subjecting them to the same preparative procedure. The recovery was determined by comparing the peak area of PAH for the extracted samples with the unextracted standards. The use of deproteinised plasma spiked with PAH as a reference sample for the recovery experiment allowed for the extraction recovery to be determined without the influence of matrix effects (e.g. ion suppression). Recovery was not determined for the urine samples as they were processed using a dilution step and were therefore not extracted. The stability of PAH in plasma and urine samples was assessed using three sets of QC samples at each of the low and high concentrations under the following conditions: (1) short-term stability at room temperature for 24 h, (2) long-term stability at −80 °C for 20 days, (3) autosampler stability at 20 °C for 6 h, and (4) freeze–thaw stability after three freeze–thaw cycles at −80 °C.

2.6. Pharmacokinetic study

The proposed analytical method was applied to a pharmacokinetic study. This study protocol was approved by the Princess Alexandra Hospital Human Research Ethics Committee and the University of Queensland Medical Research Ethics Committee. A single intravenous dose of 440 mg PAH (Merck & Co., Inc., USA)

Table 2
Intra- and inter-day accuracy and precision for the PAH QC samples.

Concentration (mg/L)		Accuracy (%)	Precision (%CV)
Theoretical	Measured (mean ± SD)		
Plasma (intra-day)			
0.4	0.42 ± 0.05	105.5	10.8
20	20.63 ± 0.43	103.1	2.1
75	72.93 ± 0.83	97.2	1.1
Plasma (inter-day)			
0.4	0.42 ± 0.04	105.5	8.8
20	20.78 ± 0.40	103.9	1.9
75	72.11 ± 1.24	96.1	1.7
Urine (intra-day)			
0.4	0.39 ± 0.02	96.7	3.9
20	20.49 ± 0.38	102.4	1.8
75	73.15 ± 1.68	97.5	2.3
Urine (inter-day)			
0.4	0.38 ± 0.02	95.7	5.4
20	20.16 ± 0.63	100.7	3.1
75	74.19 ± 1.96	98.9	2.7

Table 3
Stability data for PAH.

	Plasma		Urine	
Theoretical concentration (mg/L)	0.4	75	0.4	75
24 h at room temperature				
Mean ± SD (mg/L)	0.43 ± 0.03	73.75 ± 1.31	0.40 ± 0.02	72.55 ± 1.97
Accuracy (%)	108.8	98.3	100.8	96.7
20 days at −80 °C				
Mean ± SD (mg/L)	0.42 ± 0.02	73.86 ± 1.61	0.38 ± 0.01	73.28 ± 0.52
Accuracy (%)	104.5	98.5	95.1	97.7
6 h in autosampler (20 °C)				
Mean ± SD (mg/L)	0.39 ± 0.01	73.38 ± 0.42	0.38 ± 0.01	74.64 ± 2.42
Accuracy (%)	98.2	97.8	96.0	99.5
Three freeze–thaw cycles				
Mean ± SD (mg/L)	0.41 ± 0.02	75.7 ± 2.45	0.40 ± 0.02	73.03 ± 1.96
Accuracy (%)	102.6	100.9	100.8	97.4

was administered to 32 healthy volunteers. Blood samples were collected into heparinised tubes (Sarstedt AG & Co., Germany) at predetermined time points within a 24-h period. After centrifugation of the blood samples, the plasma was harvested and stored frozen at −80 °C until analysis. Urine was collected, the volume measured, and pooled for each individual over the time intervals 0–3 h, 3–6 h, and 6–24 h post-dose. A portion of the urine was stored frozen at −80 °C until analysis.

3. Results and discussion

3.1. Detection and chromatography

The ESI-MS (negative ion mode) of PAH and the internal standard PAS revealed the deprotonated molecules as base peaks at m/z 192.9 and 152.1 respectively, which corresponded to their $[M-H]^-$ ions. The major fragment observed was at m/z 149.1 for PAH and at m/z 108.1 for PAS. The product mass spectrum of PAH and PAS are illustrated in Fig. 1. No visible interferences were observed in the chromatograms of six blank plasma and urine samples at the retention positions of PAH or the internal standard PAS, which occurred at 6.38 min and 6.18 min respectively, resulting in an overall chromatographic run time of 9 min. The co-elution of PAH and IS indicated that the IS was subjected to the same type and extent of matrix effect as the analyte, thus compensating for matrix effects inherent in the sample. Representative chromatograms of blank and spiked human plasma and urine samples, as well as a volunteer sample, are shown in Figs. 2 and 3. Sufficient chromatographic retention of PAH was achieved using a HILIC analytical column, thus avoiding the need for ion-pairing reagents which have a negative impact on the analyte MS signal. This approach allows for the separation of highly polar compounds using common aqueous-organic mobile phases suitable for HPLC–ESI-CID-MS/MS. The positively charged triazole stationary phase present in the Cosmosil HILIC column displays an anion-exchange mechanism, thus resulting in stronger retention of acidic compounds.

3.2. Calibration curves, accuracy and precision

The eight-point calibration curve for both plasma and diluted urine exhibited good linearity in the concentration range of 0.2–100 mg/L. A mean weighted ($1/x^2$) least squares linear regression correlation coefficient (R^2) of 0.99 or greater was obtained in all analytical runs. For each standard used in the calibration curve, the average accuracy in terms of the back-calculated relative to theoretical concentrations ranged from 94.7% to 107.4% in plasma and 95.8% to 108.5% in urine (Table 1). The LOD and LOQ were established at 0.1 mg/L and 0.2 mg/L respectively. The average accuracy at the three QC concentrations ranged from 96.1% to 105.5% in plasma, and 95.7% to 102.4% in urine, with the intra-day and inter-day precision values all less than ±11% (Table 2).

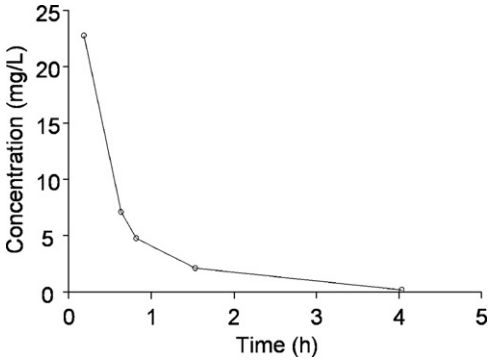

Fig. 4. Plasma concentration–time profile of PAH after administration of an intravenous bolus to a representative subject.

3.3. Recovery and stability

The extraction recovery of PAH from plasma at concentrations of 0.4 mg/L, 20 mg/L and 75 mg/L was consistent and reproducible, ranging from 79.7% to 84.4%. PAH was also stable in both plasma and urine after short-term storage for 24 h at room temperature, long-term storage for 20 days at −80 °C, storage in the autosampler for 6 h at 20 °C, and three freeze–thaw cycles at −80 °C, with precision values of less than ±7% at 0.4 mg/L and 75 mg/L (Table 3).

3.4. Plasma concentration–time profile

This HPLC–ESI-CID-MS/MS method was successfully applied to a pharmacokinetic study investigating renal plasma flow in healthy volunteers. The plasma concentration–time profile of PAH from a representative subject who received PAH as an intravenous 440 mg bolus over 1 min is shown in Fig. 4.

4. Conclusion

A sensitive and reliable HPLC–ESI-CID-MS/MS method was developed and validated for the quantitation of PAH in human plasma and urine. To the best of our knowledge, this is the first report for the determination of PAH concentrations in plasma and urine samples using HPLC–ESI-CID-MS/MS. Linear calibration curves were generated over a concentration range of 0.2–100 mg/L in plasma or diluted urine. This method provides significant advantages in terms of greater specificity and sensitivity when compared to other analytical techniques such as HPLC–UV. Furthermore, sample preparation requirements were minimal, thereby reducing

processing time and costs. This method was successfully applied to the determination of PAH in the plasma and urine samples of healthy volunteers participating in a pharmacokinetic study.

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