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Short communication

Determination of p-aminohippuric acid in rat plasma by liquid chromatography-tandem mass spectrometry

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ABSTRACT

A rapid, simple and sensitive method was developed for the determination of para-aminohippuric acid (PAH) in rat plasma using liquid chromatography tandem mass spectrometry (LC-MS-MS). Acetaminophen was used as the internal standard. Chromatographic separation was performed using a Symmetry C_{18} column and the mobile phase was composed of A: 2 mM ammonium formate and 0.1% formic acid in water and B: 2 mM ammonium formate and 0.1% formic acid in acetonitrile (ACN) (A:B, 30:70, v/v). Detection was performed on a triple–quadrupole tandem mass spectrometer using positive ion mode electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode. The MS/MS ion transitions monitored were m/z 195.2 → 120.2 and 152.1 → 110.1 for PAH and acetaminophen, respectively. Good linearity is observed over the concentration range of 0.1–500 μg/ml. The method was proved to be accurate and reliable and was applied to a pharmacokinetic study in rat.

1. Introduction

For many decades, effective renal plasma flow (ERPF) has been essential for evaluating renal functions in clinical and laboratory study [1]. *para*-Aminohippuric acid (PAH) is the commonly well known marker for determining the ERPF for its characteristics of freely filtered through glomeruli and actively secreted in proximal tubules [2]. Several analytical methods have been developed for determining PAH in plasma and urine, including colorimetric [3–5] and high performance liquid chromatography (HPLC) [1,2,6–10]. However, limitations and drawbacks occurred within these methods. Recently, Han et al. described a liquid chromatography tandem mass spectrometry (LC-MS-MS) method for the determination of PAH in human plasma and urine [11]. Han et al. suggested that ion suppression effects may influence MS signal and using a HILIC column may overcome such a problem. In this study, a matrix effect study was performed along with a full validation. Although ion suppression was observed for PAH in plasma, the relative matrix effect of PAH was limited and this implied that there is no individual matrix variation concern. In other words, the impact from the extracted plasma was negligible. Furthermore, the present study described a more sensitive method with a lower limit of quantification (LLOQ) of 0.1 μg/ml and provided a much shorter analytical run time.

In this study, a fully validated, rapid, simple and sensitive method was established for the determination of PAH in rat plasma. The validated method was also applied to a pilot pharmacokinetic study in rats.

2. Materials and methods

2.1. Chemicals and reagents

Sodium 4-aminohippurate hydrate (p-aminohippuric acid sodium salt) and para-aminohippuric acid were obtained from Alfa Aesar (Ward Hill, MA, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively. Acetaminophen was also obtained from Sigma–Aldrich (St. Louis, MO, USA). Ammonium formate and formic acid, both analytical grades, were obtained from Riedel-deHaën (Seelze, Hanover, Germany) and Sigma–Aldrich (St. Louis, MO, USA), respectively. All other chemicals were analytical grades and commercially available. Water was prepared using a Milli-Q water purification system (Millipore, Beford, MA, USA).

2.2. LC-MS-MS conditions

A Shidmazu LC system (SHIMADZU LC-10AD*VP*, Japan) coupled to an Applied Biosystems-Sciex API 3000 (Foster City, CA, USA) triple–quadruple mass spectrometer was used for the analysis. The ionization was conducted using an electrospray ionization

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Table 1
Absolute and relative matrix effect of PAH.

Concentration (μg/ml)	PAH	Acetaminophen
0.3	41.31 (9.17)	81.31 (3.20)[a]
80	49.09 (10.37)	83.83 (7.73)[a]
400	52.95 (2.28)	88.34 (5.26)[a]

[a] 180 μl of 10 μg/ml acetaminophen was spiked in 0.3 (low), 80 (medium) and 400 (high) μg/ml of interests. Results are expressed as absolute matrix effect % (relative matrix effect %). Six different sources of blank plasma were used.

(ESI) interface in positive mode. Ion source temperature was maintained at 400 °C and ionspray voltage was 5.5 kV. Multiple reaction monitoring (MRM) mode was used for the quantification of the analytes. The selected transitions of m/z were 195.2 → 120.2 and 152.1 → 110.1 for PAH and acetaminophen, respectively. High purity nitrogen gas was used as collision-induced dissociation (CAD) gas (setting 7), curtain gas (setting 10) and nebulizer gas (setting 9). All the other mass spectrometry parameters were shown in Table 1. The chromatography separation was performed on a Symmetry C_{18} column (4.6 mm × 100 mm, i.d., 3.5 μm particle size, Waters, Ireland). Mobile phase A consisting of 2 mM ammonium formate and 0.1% formic acid in water and B composed of 2 mM ammonium formate and 0.1% formic acid in ACN (A:B, 30:70, v/v) were used. The flow rate was set at 0.4 ml/min and the run time was 3 min. The injection volume was 5 μl.

2.3. Analytical method

2.3.1. Preparation of standards and quality control samples

Stock solution of PAH was prepared with MeOH:H_2O (1:1, v/v) to a concentration of 5 mg/ml. Acetaminophen as the internal standard was prepared with deionized water to a concentration of 1 mg/ml. The calibration curve was prepared with 180 μl of 10 μg/ml IS added into 18 μl of blank plasma with 2 μl of standard working solution. The linearity was studied in the range from 0.1 μg/ml to 500 μg/ml. Quality control (QC) samples were prepared with blank plasma at 0.3 μg/ml (low), 80 μg/ml (medium) and 400 μg/ml (high).

2.3.2. Sample preparation

All samples were thawed at room temperature before analysis. 180 μl of ACN containing 10 μg/ml IS was added into 20 μl of plasma sample. Samples were vortexed for 1 min and centrifuged at 13,000 rpm for 5 min. The supernatant was removed and 5 μl aliquot was injected onto HPLC.

2.3.3. Method validation

The analytical method was validated following the criteria suggested by the US Food and Drug Administration (FDA) bioanalytical method validation guidance [12]. The following assays were determined for the validation of the analytical method developed for PAH: selectivity, matrix effect, linearity, lower limit of quantification (LLOQ), precision and accuracy, recovery and stability.

2.4. Selectivity

Selectivity was evaluated by comparing the chromatograms of blank plasma from six different sources for the presence of endogenous interfering peaks.

2.4.1. Matrix effect

Six different sources of blank plasma were used to assess the matrix effect. The absolute and relative matrix effects were previously defined by Matuszewski et al. [13]. Concentration at 0.3, 80 and 400 μg/ml was used as low, medium and high QC levels to evaluate matrix effect of PAH where IS was measured with the same concentration spiking in different PAH QC levels. The absolute matrix effect was evaluated by comparing the peak areas of analytes added to extracted blank plasma with those of extracted water. The relative matrix effect was expressed as the R.S.D. of the mean peak areas of the analytes in the extracted blank plasma.

2.4.2. Linearity, lower limit of quantification, precision and accuracy

A calibration curve was conducted using the peak area ratio of PAH against IS with a weighted linear regression ($1/X^2$). Lower limit of quantification, LLOQ, was defined as the lowest plasma concentration in the calibration curve that can be quantitatively measured with acceptable precision and accuracy (within 20%). The precision and accuracy were determined in six replicates of QC samples at three concentration levels of PAH. The precision and accuracy were expressed as the relative standard deviation (R.S.D.) and relative error (R.E.), respectively. The acceptance values used for validation of R.S.D. and R.E. were within 15%, except LLOQ (within 20%).

2.4.3. Recovery

The extraction recovery was determined by comparing the response ratio of extracted plasma QC samples with those of extracted blank plasma spiked with corresponding concentrations. The response was defined as the peak area of the analyte divided by the peak area of the IS.

2.4.4. Stability

2.4.4.1. Stock solution stability. Stock solution was prepared as aforementioned. The stock solutions for both PAH and IS were stored at ambient temperature for 6 h and diluted to 25 μg/ml and 5 μg/ml, respectively for analysis. The peak areas of the diluted samples were compared to those of the freshly prepared samples.

2.4.4.2. Autosampler stability. QC samples were analyzed and stored in autosampler for 12 h followed by reinjection. Autosampler stability was determined by comparing the concentration of the reinjecting QC samples to the initial analyzed QC samples.

2.4.4.3. Freeze and thaw stability. Freeze and thaw stability was evaluated by comparing the observed concentration of QC samples processing three freeze–thaw cycles to that of freshly prepared QC samples.

2.4.4.4. Short-term stability. QC samples were prepared and analyzed immediately and after storing at ambient temperature for 6 h. Short-term stability was evaluated by comparing calculated concentration of 6 h stored QC samples to the concentration of initial QC samples.

2.4.4.5. Long-term stability. QC samples were prepared and stored at −80 °C for two weeks before processing and analysis. Long-term stability was evaluated by comparing the obtained concentrations with initial values.

2.5. Animals

Male Wistar rats, weighing 380–430 g, were used throughout the study. The animals were housed in a 12-h light/dark temperature-controlled environment with free access to water and food and acclimatized to the environment for at least 1 week before the study. Anestesia was induced by intraperitoneal application using pentobarbital sodium (50 mg/kg b.w.). Animals were placed on thermoregulated heating blanket throughout the study to maintain body temperature at 37 °C. The study was approved by the

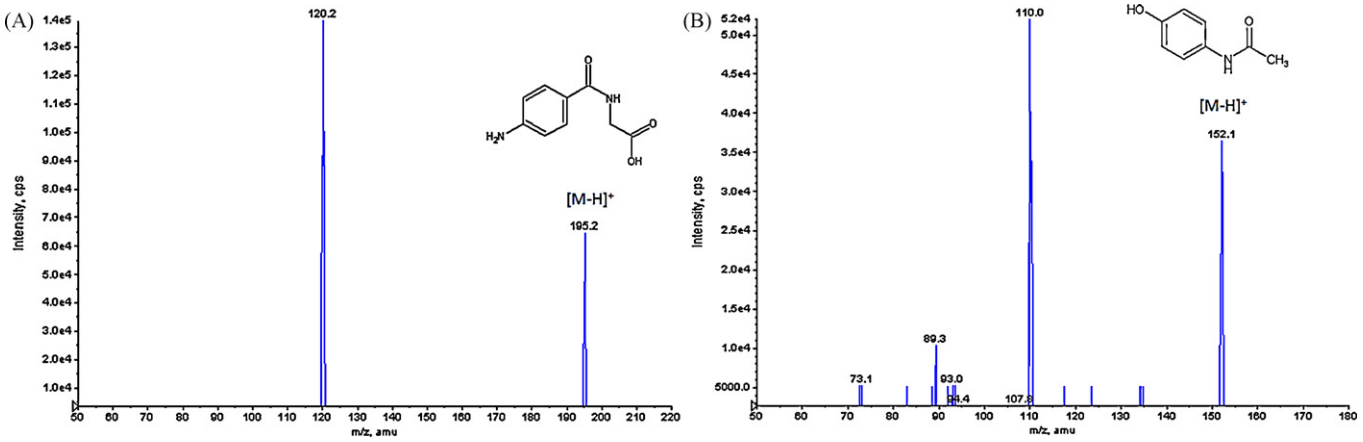

Fig. 1. Full scan product ion spectra of (A) PAH and (B) acetaminophen.

Institutional Animal Care and Use Committee of National Defense Medical Center.

2.6. Pharmacokinetic study

The pharmacokinetic study was similar to the previous studies [14,15] with some modifications. The femoral artery and vein of the left leg were cannulated with a PE-50 catheter. A single bolus/dose of PAH (30 mg/kg b.w.) was administered. Blood samples were obtained at 0 min, 0.25 min, 0.5 min, 1 min, 2 min, 5 min, 10 min, 15 min, 30 min, 45 min and 60 min. 100 μl of blood was drawn from each time point and an equivalent volume of isotonic saline was infused that restored the amount of blood removed. Plasma was obtained after centrifugation of blood sample at 13,000 rpm for 5 min. All samples were stored at −80 °C until analysis.

3. Results and discussion

3.1. Method development

The proposed LC-MS-MS method provides a simple procedure for the determination of PAH. The product mass spectra of PAH and acetaminophen are illustrated in Fig. 1. The most intensive precursor ion observed for PAH and acetaminophen was at m/z 195.2 and 152.1, respectively. The parameters of mass spectrometry were optimized with collision energy of 13 eV and 21 eV for PAH and acetaminophen, respectively.

3.2. Method validation

3.2.1. Selectivity

Representative chromatograms of drug free plasma, plasma spiked with PAH at LLOQ and acetaminophen at 10 μg/ml and real samples 60 min after the injection of PAH are shown in Fig. 2(A)–(C). The retention times for PAH and acetaminophen were 2.09 min and 2.18 min, respectively. The retention time for PAH was much shorter compared to that in the previous study done by Han et al., where a longer run time was observed for PAH (6.38 min) [11]. No significant interferences were observed in the chromatograms of six blank plasma samples at the retention times of the analytes.

3.2.2. Matrix effect

The absolute and relative matrix effect is shown in Table 1. Ion suppression was observed for both PAH and IS at low, medium and high concentrations. However, the variabilities of the response of the analytes (relative matrix effects) were within 10.4%. This suggested that the impact from the extracted plasma was consistent and was limited.

3.2.3. Linearity, lower limit of quantification, precision and accuracy

The calibration curve of PAH with a $1/X^2$ weighted regression was linear over the concentration range of 0.1–500 μg/ml. LLOQ was 0.1 μg/ml in this study, which is lower than the previous study by Han et al. [11]. Precision and accuracy for the standard used in the

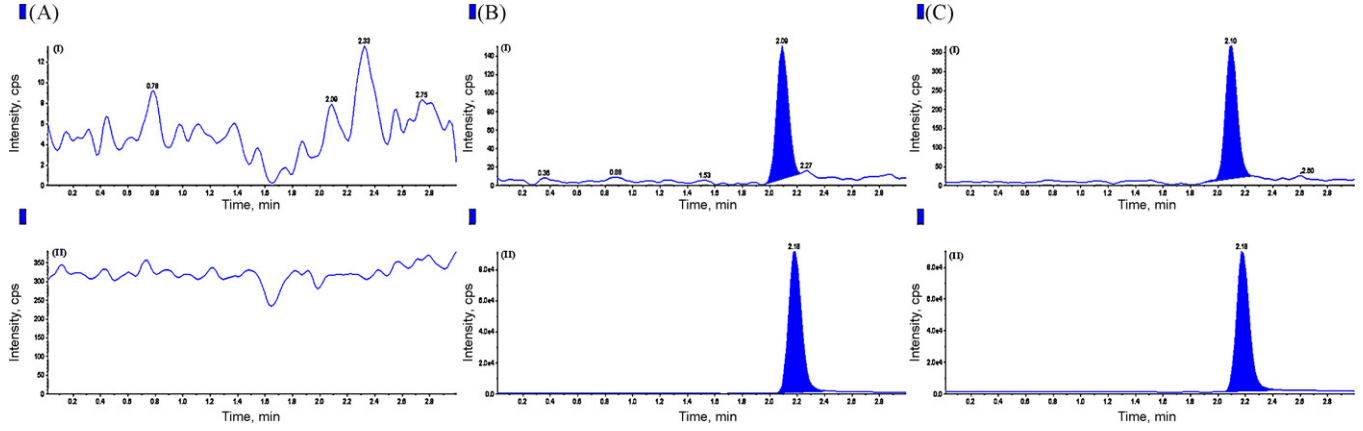

Fig. 2. MRM chromatograms of (A) blank plasma, (B) blank plasma spiked with PAH at LLOQ and acetaminophen at 10 μg/ml and (C) real sample 60 min after injection of PAH.

Table 2
Precision and accuracy of p-aminohippuric acid in rat plasma.

Added concentration	Within-run			Between-run		
	Founded concentration	RSD	RE	Founded concentration	RSD	RE
0.1	0.11 ± 0.01	6.37	108.67	0.11 ± 0.01	12.47	111.47
03	0.30 ± 0.03	8.50	98.89	0.31 ± 0.02	7.72	103.33
80	78.18 ± 2.64	3.38	97.73	81.50 ± 2.65	3.25	101.88
400	375.00 ± 13.22	3.53	93.75	395.67 ± 13.75	3.48	98.92

Results are expressed as mean ± SD ($n = 6$). Concentrations are expressed as μg/ml. RSD and RE are expressed as %.

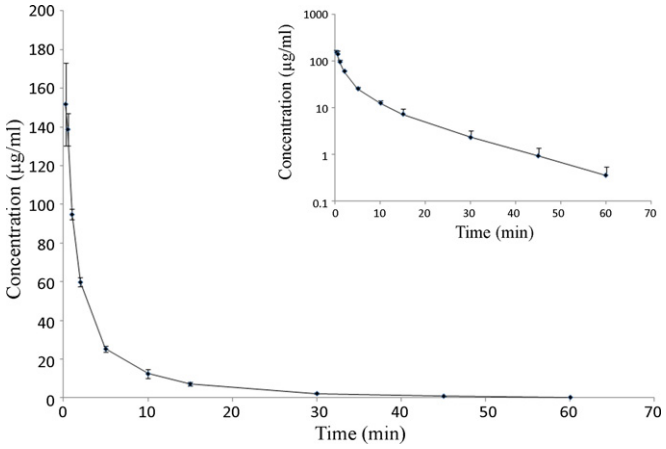

Fig. 3. Mean concentration–time profile of PAH in three Wistar rats. Inset panel was the semi-log of the mean concentration–time profile of PAH.

calibration curve are shown in Table 2. Precisions and accuracies of QC samples were all within 20% and 15% for LLOQ and QC samples, respectively.

3.2.4. Recovery

The extraction recovery of PAH and IS at each level of QC samples ranged from 81.97% to 87.91% and 88.85% to 93.54%, respectively.

3.2.5. Stability

PAH was shown to be stable in plasma after short-term storage for 6 h at room temperature and long-term storage for two weeks at −80 °C. Stock solution stability of PAH was 95.05 ± 3.28%. Autosampler and freeze and thaw stability of PAH were 102.17–113.03% and 87.7–99.16%, respectively. PAH was shown to be stable throughout all the stability tests, which suggested that this analytical method was applicable for routine analysis.

3.3. Pharmacokinetic study

The validated analytical method was applied to a pharmacokinetic study in rats following intravenous administration of 30 mg/kg PAH. The mean plasma concentration–time profile is shown in Fig. 3. The area under the curve (AUC_∞) and clearance (CL) are 588.76 ± 76.95 min* μg/ml and 20.97 ± 3.40 ml/min, respectively. The $t_{1/2}$, 10.96 ± 0.86 min, was longer compared to the previous study [16]. The longer $t_{1/2}$ resulted from the extended sample collecting times and lower quantitative detection limit. Moreover, as Fig. 3 showed, blood concentration of PAH at 30 min, 45 min and 60 min revealed a better approximation of the real elimination phase in rat. Thus, this analytical method with a higher sensitivity provided more optimal conditions for animal mechanism study.

4. Conclusion

The presented study describes and validates a simple and reliable LC-MS-MS method for the determination of PAH in rat plasma. This method was proved to be linear over the range of 0.1–500 μg/ml. Negligible matrix effect allowed the usage of a C_{18} column in this analytical study and greatly reduced the run time. This simple one-step protein precipitation method with a shorter running time was successfully applied to a pharmacokinetic study. The study also provides an alternative option for the quantification of PAH in rat plasma.

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