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Determination of higenamine in human plasma and urine using liquid chromatography coupled to positive electrospray ionization tandem mass spectrometry

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ABSTRACT

Higenamine is an active ingredient of Aconite root in Chinese herbal medicine and might be used as a new agent for a pharmaceutical stress test and was approved to undergo clinical pharmacokinetic study. Therefore, there exists a need to establish a sensitive and rapid method for the determination of higenamine in human plasma and urine. This paper described a sensitive and rapid method based on liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) for the determination of higenamine in human plasma and urine. Solid-phase extraction (SPE) was used to isolate the compounds from biological matrices followed by injection of the extracts onto an Atlantis dC18 column with isocratic elution. The mobile phase was 0.05% formic acid in water–methanol (40:60, v/v). The mass spectrometry was carried out using positive electrospray ionization (ESI) and data acquisition was carried out in the multiple reaction monitoring (MRM) mode. The method was fully validated over the concentration range of 0.100–50.0 ng/mL and 1.00–500 ng/mL in plasma and urine, respectively. The lower limits of quantification (LLOQs) were 0.100 and 1.00 ng/mL in plasma and urine, respectively. Inter- and intra-batch precision was less than 15% and the accuracy was within 85–115% for both plasma and urine. Extraction recovery was 82.1% and 56.6% in plasma and urine, respectively. Selectivity, matrix effects and stability were also validated in human plasma and urine. The method was applied to the pharmacokinetic study of higenamine hydrochloride in Chinese healthy subjects.

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1. Introduction

Stress echocardiography is a widely used technique for noninvasive evaluation of coronary artery disease (CAD), especially for patients who cannot exercise. Higenamine, 1-[(4-hydroxyphenyl)methyl]-1,2,3,4-tetrahydroisoquinoline-6,7-diol, is an active ingredient of Aconite root in Chinese herbal medicine. It was reported to possess cardiac β-adrenoceptor stimulating, vasodilating and platelet anti-aggregating activities through α-adrenoceptor interaction and might be used as a new agent for a pharmaceutical stress test [1–5]. Moreover, higenamine hydrochloride exhibited good tolerance among healthy Chinese subjects in a tolerance clinical trial, which demonstrated the highest safe dose of 24 μg/kg [6]. Based on the efficacy and safety profiles exhibited in these pre-clinical and clinical studies, higenamine hydrochloride was approved to undergo clinical pharmacokinetic study.

To date, there has been only one reported determination method for higenamine in rabbit plasma and urine. This method used high performance liquid chromatography with electrochemical detection (HPLC-ECD) [7]. The LLOQs were 2.6 and 10.6 ng/mL in plasma and urine, respectively. Based on our preliminary pharmacokinetic study results (unpublished results), detection of higenamine in human plasma and urine required a LLOQ of 0.100 ng/mL and 1.00 ng/mL, respectively. Therefore, it was essential to develop a more sensitive and rapid determination method of higenamine in human plasma and urine samples.

In the present study, a specific, sensitive and rapid method based on LC–MS/MS was developed and fully validated to quantify higenamine in human plasma and urine. This method was successfully applied to determine the pharmacokinetic profile of higenamine after intravenous administration of higenamine hydrochloride to Chinese healthy subjects.

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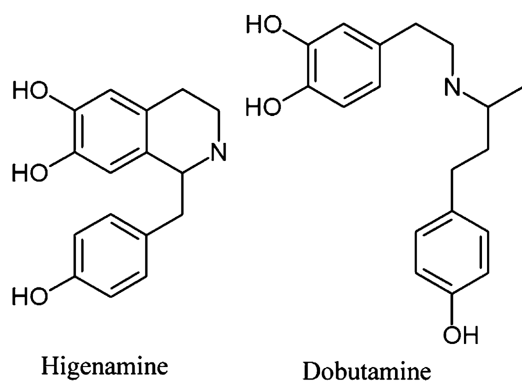

Fig. 1. Chemical structures of higenamine and dobutamine.

2. Experimental

2.1. Chemicals and solvents

Higenamine (purity 99.4%) and its internal standard dobutamine (Fig. 1) were provided by Zhuhai Rundumingtong Pharma Inc. (Zhuhai, China) and the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), respectively. Methanol was of chromatographic grade and obtained from Burdick & Jackson Lab (NJ, USA). Formic acid was of analytical grade and purchased from Beijing Chemical Reagents Company (Beijing, China). Drug-free human plasma (anticoagulant: sodium heparin) and urine were obtained from six different healthy subjects who were drug-free for at least two weeks. Distilled water was prepared with a Milli-Q water purifying system (Millipore, Bedford, MA, USA).

2.2. Calibration standard (CS) and quality control (QC) samples in human plasma and urine

Stock solutions of higenamine for CS and QC were prepared separately in methanol–water–formic acid (50:50:1, v/v/v) after correction for purity. The concentrations of stock solutions of higenamine for CS and QC were both 400 μg/mL. A stock solution of dobutamine was also prepared in methanol–water–formic acid (50:50:1, v/v/v). These stock solutions were further diluted to yield working solutions at several concentration levels.

Calibration standards and QC samples in plasma and urine were prepared by diluting corresponding working solutions with drug-free human plasma and urine, respectively. The final concentrations of calibration standards in plasma and urine were 0.100, 0.200, 0.500, 1.00, 2.00, 5.00, 10.0, 50.0 ng/mL and 1.00, 2.00, 5.00, 10.0, 20.0, 50.0, 100, 500 ng/mL, respectively. The final concentrations of QC in plasma and urine were 0.200, 4.00, 40.0 and 2.00, 40.0, 400 ng/mL, respectively. Internal standard working solutions (2.00 ng/mL and 20.0 ng/mL for plasma and urine, respectively) were prepared with 1% formic acid in water. Plasma and urine samples were both stored at −70 °C.

2.3. Sample preparation

Biological samples were extracted by a solid phase extraction (SPE) method using a Waters Oasis HLB plate (10 mg). Plasma, 100 μL, was mixed with 200 μL of internal standard (2.00 ng/mL). The SPE plate was conditioned with 600 μL methanol and 600 μL 1% formic acid in water. The mixture, 300 μL, was then loaded onto the plate. SPE columns were washed with 500 μL of 1% formic acid in water. The elution of these compounds was carried out with 150 μL methanol–1% formic acid in water (7:3, v/v) twice. The eluates were evaporated to dryness under nitrogen gas at room temperature. Methanol–0.2% formic acid in water (15:85, v/v), 200 μL, was finally added to the residues and well mixed before injection. Analysis of urine samples was prepared with a similar method except for the internal standard (20.0 ng/mL) and the volume of reconstituting solution (100 μL). The operation of mixing the biological matrices and internal standard was done in an ice bath.

2.4. Liquid chromatography–tandem mass spectrometry

Plasma samples and urine samples were analyzed using different LC–MS/MS systems due to the different requirement of sensitivity. The former was 20A HPLC system (Shimadzu Co., Kyoto, Japan) coupled with API 4000 tandem MS (Applied Biosystems, CA, USA), and the latter was 2695 Alliance HPLC system (Waters Co., MA, USA) coupled with API 3000 tandem MS (Applied Biosystems, CA, USA). Both systems used the same chromatographic condition and were equipped with electrospray interfaces.

Chromatography separation for plasma was carried out on a Waters Atlantis dC18 column (50 mm × 2.1 mm, 5 μm) at room temperature. The autosampler temperature was 10 °C. The mobile phase was 0.05% formic acid in water–methanol (40:60, v/v). The flow rate was 0.2 mL/min and the run time was 3.5 min with an injection volume of 10 μL. The run time for urine was longer at 4.5 min under the same conditions as the plasma sample analysis, owing to urine samples being dirtier.

For plasma samples, analysis was performed with an ionizing voltage of 5000 V. The ion source temperature was set at 350 °C with ultrahigh-purity nitrogen as curtain gas (10 psi), nebulizer gas (45 psi) and auxiliary gas (45 psi). Multiple reaction monitoring (MRM) was carried out using nitrogen as collision gas (6 psi), and with a dwell time of 200 ms for each transition. The analytes were detected by monitoring the transitions m/z 272.1 → 107.1 and 302.2 → 137.1 with the collision energy 35 eV and 31 eV for higenamine and dobutamine, respectively. Analysis of urine samples proceeded with similar MS condition except for the curtain gas (8 L/h). The product ion mass spectra of the analyte and internal standard are depicted in Fig. 2.

2.5. Method validation

The method was validated for selectivity, sensitivity, linearity, recovery, matrix effects, precision, accuracy and stability according to the US Food and Drug Administration (FDA) [8] and Chinese State Food and Drug Administration (SFDA) guidelines [9] for the validation of bioanalytical methods.

The specificity of this method was evaluated by comparison of LC–MS/MS chromatograms of higenamine at the LLOQ to those of six individual human blank plasma and urine samples.

Calibration standards in human plasma and urine were prepared and analyzed in three independent runs. The calibration curves were constructed by weighted ($1/x^2$) least-square linear regression analysis of the peak area ratio of analyte to its internal standard against nominal analyte concentration. The LLOQ was determined as the lowest concentration with values for precision and bias within ±20%.

The extraction recovery of higenamine was calculated by comparing the responses of QC samples spiked before and after extraction procedure. The matrix effects were evaluated by comparing the areas of higenamine in spiked QC sample with or without biological matrices with plasma and urine samples from five drug-free volunteers at three concentration levels. During the preparation of QCs at the same concentration level, each individual's biological matrix was used only once. The corresponding peak areas of compounds in spiked QCs in biological matrix (A)

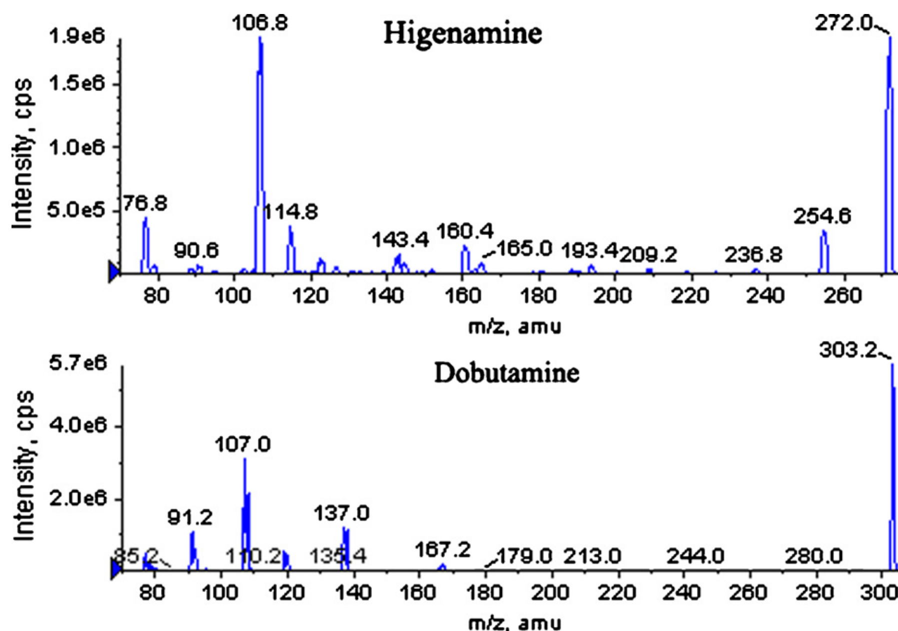

Fig. 2. Product ion spectrum of higenamine (upper) and dobutamine (IS, lower).

were then compared to those of the aqueous standards in the mobile phase (B) at equivalent concentrations. The ratio ($A/B \times 100$) is defined as the matrix effects. The inter-subject variability of matrix effects at every concentration level should be less than 15% [10–12].

Intra- and inter-batch precision and accuracy were determined by measuring the concentrations of analyte in plasma and urine in five replicates of QC samples at three different concentrations for three separate batches.

The stability of higenamine in biological matrices and in working solution at different storage condition was evaluated as follows: the short-term stability of analyte in biological matrices and in working solution was assessed after 6 h of storage in an ice bath and at room temperature, respectively. The long-term stability of higenamine in human plasma and urine was assessed after 40 days of storage in a freezer at −70 °C. The stability of analyte was assessed after two freeze–thaw cycles (−70 °C to room temperature). The stability of analyte in extracts was also tested after 6 h at 10 °C.

3. Result and discussion

3.1. Extraction procedure optimization

Because the polarity of higenamine is high, recovery by the liquid–liquid extraction (LLE) method using diethyl ether, ethyl acetate and dichloromethane is low. The matrix effects are significant for the precipitating protein method. We therefore chose the SPE method for which recovery is higher than with LLE and inter-subject variability is lower.

After the SPE method was chosen, the composition of the elution solvent was optimized. It was found that if the samples were washed with 1% formic acid in water and eluted using methanol–1% formic acid in water (7:3, v/v) during the SPE procedure, the highest and most robust extraction efficiency was obtained.

Because higenamine is unstable in human plasma and urine at room temperature, mixing the biological matrices and internal standard was done in an ice bath. Higenamine is stable during this sample preparation procedure for 6 h.

3.2. LC–MS/MS optimization

Because of the polarity, higenamine has no retention on simple reverse-phase columns. Though it has retention on amide columns, the sensitivity is low. We therefore chose the Atlantis column which is suitable for the analysis of polar analytes. The peak shape and sensitivity for higenamine and the internal standard are both good.

Since higenamine is unstable in basic solution, a series of aqueous formic acid with different pH values were investigated. When 0.05% formic acid in water is used as the aqueous phase, a good chromatographic profile and sensitivity was achieved.

The analyte was introduced into the mass spectrometer using an electrospray interface, and the parameters such as ionizing voltage (IS), declustering potential (DP) and entrance potential (EP) were optimized to obtain protonated molecular ions $[M+H]^+$ based on the structures. In order to achieve high specificity and sensitivity, the MRM scan mode was selected to assay the analyte at the most suitable collision energy (CE). Negative ionization was also applied, but compared to the positive mode, the sensitivity was much lower.

3.3. Validation procedure

3.3.1. Selectivity, linearity and LLOQ

Chromatograms of a blank plasma and urine, a calibration standard and a healthy subject's plasma and urine sample are shown in Figs. 3 and 4, respectively. No peaks eluting at the retention times of the higenamine or internal standard were detected in samples from 6 lots of human plasma and urine.

A calibration curve was established ranging from 0.1 to 50.0 ng/mL for plasma and from 1.00 to 500 ng/mL for urine. Both calibration curves were regressed using linear equation with a weighting factor of $1/x^2$. Coefficients of correlation of all calibration curves were more than 0.99. The LLOQs were 0.100 and 1.00 ng/mL in plasma and urine, respectively.

3.3.2. Recovery and matrix effects

The observed recovery for the extraction method from plasma and urine (mean value and RSD%, $n = 5$) is shown in Table 1. Recovery at different concentrations of higenamine was 82.1% and 56.6% in plasma and urine respectively with little inter-subject variability.

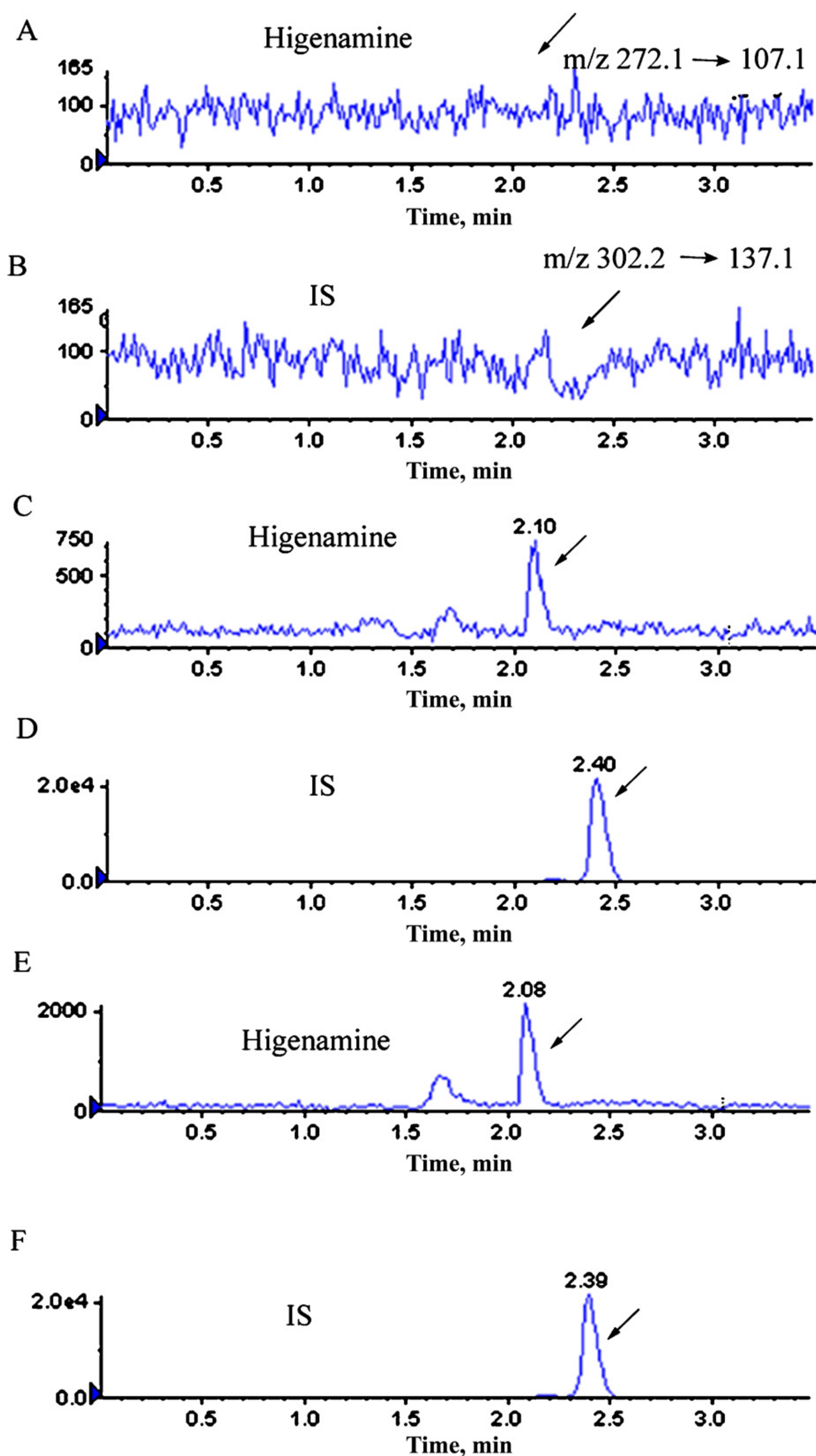

Fig. 3. MRM chromatograms of higenamine and dobutamine (IS) in blank plasma (A and B), LLOQ (C and D) and plasma from a subject 3 min post-dose of higenamine hydrochloride (E and F).

Matrix effects and inter-subject variability data from plasma and urine of individual subjects who had not received higenamine are summarized in Table 1. The inter-subject variabilities were up to 8.5% and 10.7% in plasma and urine, respectively. This indicated little or no difference in ionization efficiency of higenamine from different plasma and urine lots.

3.3.3. Accuracy and precision

Precision and accuracy values were determined on three different batches by measuring five replicates of QC samples at three concentration levels. The results are listed in Tables 2 and 3. In both plasma and urine, intra-batch bias ranged from −6.5% to 7.5%, while precisions were less than 6.2%. Inter-batch

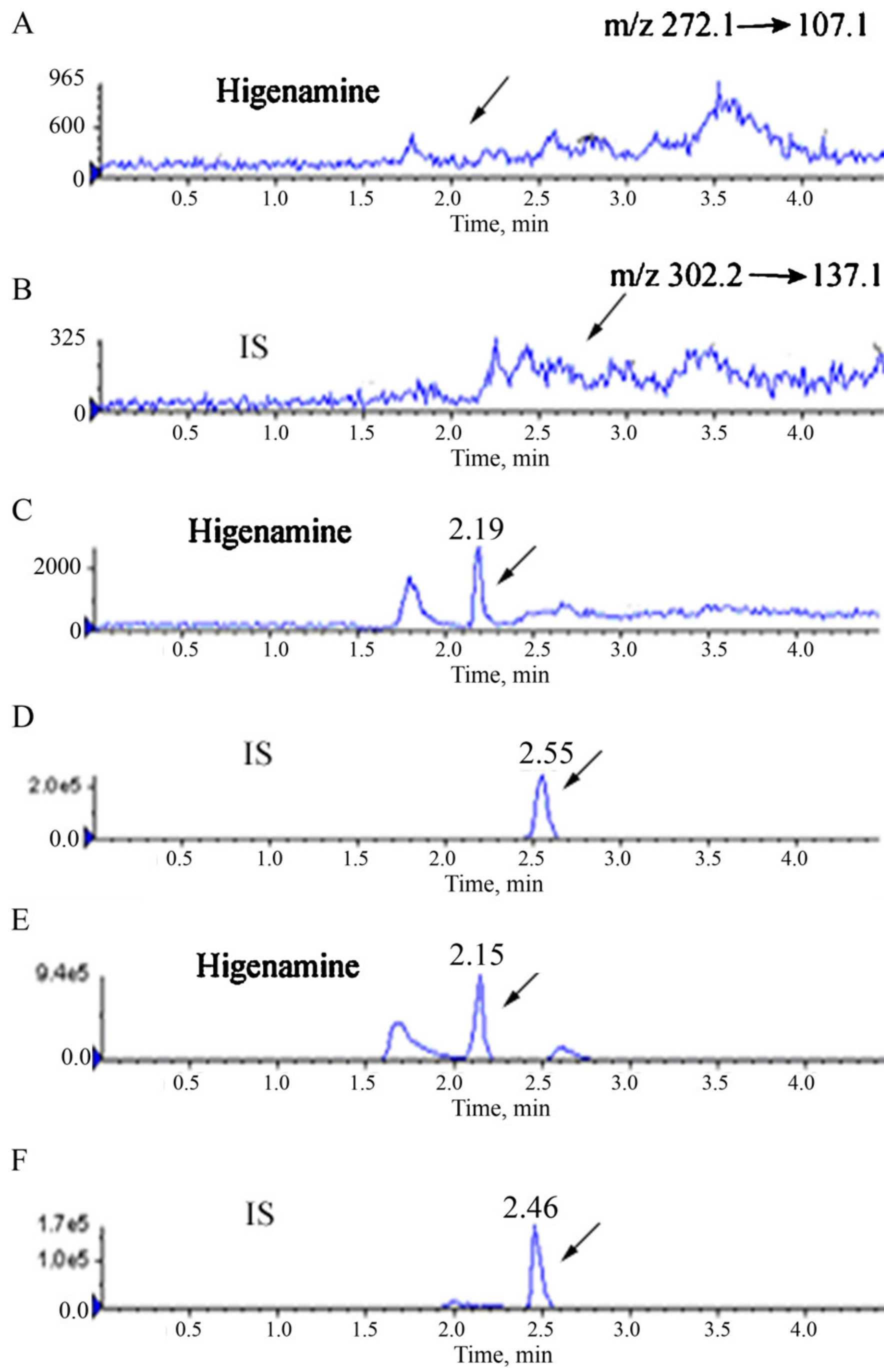

Fig. 4. MRM chromatograms of higenamine and dobutamine (IS) in blank urine (A and B), LLOQ (C and D) and urine from a subject 0–4 h post-dose of higenamine hydrochloride (E and F).

bias ranged from −5.0% to 8.0%, while precisions were less than 10.8%.

3.3.4. Stability

The stability tests of the analyte were designed to cover expected conditions of handling of clinical samples. The stability of the analyte in human plasma and urine was investigated under a variety of storage and processing conditions. Briefly, two freeze/thaw cycles and ice bath storage of the QC samples up to 6 h appeared to have no effect on results of quantification of higenamine in plasma and urine. QC samples stored at −70 °C remained stable for at least 40 days in plasma and urine. Processed samples were allowed to stand at 10 °C in extracts for 6 h prior to analysis, with no observed effect on results of quantification. When working solutions of higenamine were stored at room temperature for 6 h, the analyte was found to be stable.

3.4. Application of the method in pharmacokinetic studies

The LC–MS/MS method described in this paper was used to investigate the plasma and urine pharmacokinetic profiles of higenamine in Chinese healthy subjects after doses of 22.5 μg/kg of higenamine through intravenous administration ($n = 10$). The study was approved by the Ethics Committee of Peking Union Medical College Hospital and all subjects signed the Informed Consent Form before the study. The mean plasma

Table 1
Results of extraction recovery and matrix effects of higenamine in human plasma and urine ($n = 5$).

Nominal concentration (ng/mL)	Recovery% (RSD%)	Matrix effects% (inter subject variability%[a])
Human plasma		
0.200	77.3 (7.5)	121.3 (8.5)
4.00	78.8 (4.9)	105.4 (2.4)
40.0	90.2 (4.4)	107.2 (1.2)
Human urine		
2.00	59.7 (6.7)	61.5 (3.4)
40.0	61.7 (7.9)	46.7 (5.1)
400	48.4 (10.0)	64.4 (10.7)

[a] Expressed as RSD%.

Table 2
Accuracy and precision of intra- and inter batch for the detection of higenamine in plasma.

	Q1	Q2	Q3
Intra-batch ($n = 5$)			
Nominal concentration (ng/mL)	0.200	4.00	40.0
Mean	0.194	4.22	37.4
Accuracy (Bias%)	−3.0	5.5	−6.5
Precision (RSD%)	3.6	6.1	6.2
Inter-batch ($n = 3$)			
Nominal concentration (ng/mL)	0.200	4.00	40.0
Mean	0.190	4.23	39.6
Accuracy (Bias%)	−5.0	5.8	−1.0
Precision (RSD%)	7.0	4.7	10.8

Table 3
Accuracy and precision of intra- and inter batch for the detection of higenamine in urine.

	Q1	Q2	Q3
Intra-batch ($n = 5$)			
Nominal concentration (ng/mL)	2.00	40.0	400
Mean	2.15	41.3	407
Accuracy (Bias%)	7.5	3.3	1.8
Precision (RSD%)	4.3	1.8	4.1
Inter-batch ($n = 3$)			
Nominal concentration (ng/mL)	2.00	40.0	400
Mean	2.16	43.0	410
Accuracy (Bias%)	8.0	7.5	2.5
Precision (RSD%)	4.7	5.8	5.9

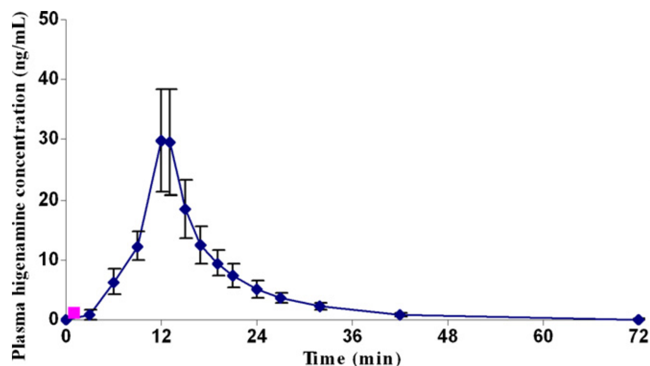

Fig. 5. The average concentration–time curve of higenamine in plasma from Chinese healthy subjects after intravenous administration of 22.5 μg/kg ($n = 10$) higenamine hydrochloride (mean ± SD).

concentration–time curve and urine cumulative amount–time curve of higenamine are shown in Figs. 5 and 6, respectively. Peak concentrations (C_{max}) for individuals were ranged from 15.1 to 44.0 ng/mL. The half-life was 0.133 h (range 0.107–0.166 h)

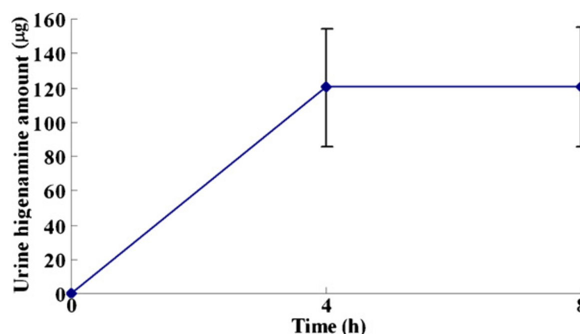

Fig. 6. The cumulative amount–time curve of higenamine in urine from Chinese healthy subjects after intravenous administration of 22.5 μg/kg ($n = 10$) higenamine hydrochloride (mean ± SD).

while the area under concentration–time curve (AUC) extrapolated to infinity was 5.39 ng h/mL (range 3.2–6.8 ng h/mL). The cumulative recovery of higenamine in urine within 8 h was 9.3% (range 4.6–12.4%). These values were obtained using non-compartmental analysis. In general, after the dose uniformizing, there were significant differences between the major pharmacokinetic parameters in Chinese subjects and those in rabbits [13].

4. Conclusion

A fast, sensitive and specific LC–MS/MS method based on SPE has been developed and validated for the determination of higenamine concentrations in plasma and urine of Chinese healthy subjects. The extraction procedure and LC–MS/MS conditions were optimized in order to improve the sensitivity and robustness of the method. The procedure was fully validated. This method was successfully applied to the determination of higenamine in human plasma and urine to study the pharmacokinetic profile of higenamine in Chinese healthy subjects.

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