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Determination of higenamine in plasma and urine by high-performance liquid chromatography with electrochemical detection

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Abstract

A method for the determination of higenamine in plasma and urine based on high-performance liquid chromatography (HPLC) with electrochemical detection was developed. The plasma or urine sample was treated by adsorption with acidic alumina and then higenamine was released by acid treatment. HPLC was performed on an ODS column with a mobile phase of acetonitrile–0.1% phosphoric acid (9:91) and an electrochemical detector at an oxidation potential of 0.75 V. The lower limits of the assay for higenamine in plasma and urine were 2.645 and 10.58 ng/ml, respectively. The recoveries of higenamine after alumina treatment in rabbit plasma and urine were *ca.* 77.5 and 84.4%, respectively. Intra- and inter-day precision and accuracy reported as coefficients of variation in plasma and urine were less than 7%. The application of the assay was demonstrated successfully by the determination of the concentration of higenamine in rabbit plasma and urine samples after doses of higenamine hydrochloride at 20 mg/kg intravenously and 50 mg/kg orally.

1. Introduction

Higenamine is a potent cardiotonic principle isolated from the root of *Aconitum japonicum* [1,2], and also from other natural sources such as *Anona squamosa* [3,4], *Asiasarum heterotropodes* [5], *Gnetum parvifolium* [6] and *Nelumbo nucifera* [7]. It acts directly on the adrenergic β_1- and β_2-receptors [8,9] and also inhibits strongly in human liver dihydropteridine reductase [10]. *Aconitum* root has been used traditionally in herbal medicines in oriental countries.

Several methods for the determination of higenamine in crude drugs and its preparations have been described. These include high-performance liquid chromatography (HPLC) with electrochemical detection (ED) and UV detection [11–13]. An HPLC-UV procedure has been developed to monitor blood concentrations of higenamine in rabbits [14]. However, this method could only detect higenamine in blood at a concentration of 6 μg/ml. Purification of higenamine in blood required a large amount of sample (3 ml) and was performed by protein precipitation followed by solid-phase extraction.

To our knowledge, no detailed pharmacokinetic study on higenamine obtained from ex-

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perimental animals or humans has been reported. The published method for the determination of higenamine in blood [14] is not sensitive enough for pharmacokinetic studies. We report here a simple, sensitive, accurate and reproducible assay of higenamine in rabbit plasma and urine using HPLC-ED.

2. Experimental

2.1. Instrumentation and chromatographic system

Chromatographic analysis was performed with a Model LC-9A high-pressure pump (Shimadzu) with a pulse damper (Alltech) and a Model LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA). The detector was operated at setting of +0.75 V *versus* Ag/AgCl. The system was automated with an SIL-9A autosampler (Shimadzu) equipped with a 250-μl loop injector and a CHEM-LAB (Shiunn-Hwa, Taiwan) data system for sampling control, recording and integration. The analytical column was a Cosmosil (C_{18}, 5 μm) packed column (250 mm × 4.6 mm I.D.) (Nacalai Tesque) run at ambient temperature. The mobile phase was acetonitrile–0.1% phosphoric acid (9:91) containing 1.5 μM EDTA disodium salt, the pH being adjusted to 2.5 with 40% (w/v) NaOH. The mobile phase was filtered through an FP Vericel membrane filter, (0.22 μm) (Gelman Sciences, Ann Arbor, MI, USA) and degassed with helium (99.995% pure) for 30 min. The mobile phase was continuously purged with helium prior to equilibration of the column and during each sample run. The flow-rate of the mobile phase was 1 ml/min.

2.2. Chemicals and reagents

Higenamine hydrochloride was synthesized by the previously described method [15]. An internal standard, N-(3,4-dihydroxyphenylethyl)-*p*-hydroxyphenylethylamine hydrobromide (Fig. 1), was prepared in our laboratory and confirmed by NMR, IR and GC–MS. Tris(hydroxymethyl)aminomethane (Trizma base) and EDTA disodium salt were purchased from Sigma (St. Louis, MO, USA) and alumina was obtained from Bioanalytical Systems. All other chemicals were of analytical-reagent grade and used without further purification. Methanol and acetonitrile were of HPLC grade (Lab-Scan, Dublin, Ireland). Tris buffer solution is consisted of 24.2 g of Trizma base and 4.0 g of EDTA disodium salt in 200 ml of distilled, deionized water (obtained with a Millipore water purification system) and adjusted to pH 8.6 with concentrated HCl.

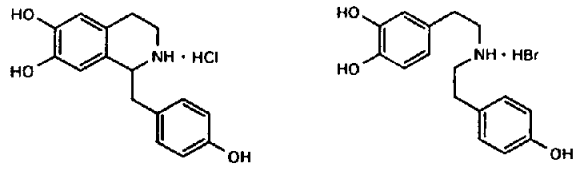
2.3. Preparation of calibration graphs for higenamine in plasma and urine

Blank plasma (0.2 ml) was spiked with higenamine hydrochloride corresponding to higenamine concentrations of 2.645, 5.289, 10.58, 26.45, 52.89, 105.8, 528.9, 1058, 2645 and 5289 ng/ml. The spiked plasma were treated as described for sample preparation.

Blank urine from rabbit was acidified to pH 2–3 with concentrated phosphoric acid. The blank urine (0.2 ml) was spiked with higenamine hydrochloride equivalent to higenamine concentrations of 10.58, 26.45, 52.89, 105.8, 528.9, 1058 and 2645 ng/ml. These samples were treated following the procedure described for sample preparation. A calibration graph for plasma or urine was freshly constructed for each assay run.

2.4. Plasma and urine sample preparation

The sample preparation consisted of clean-up and extraction procedures. Before urine sample



Higenamine Hydrochloride Internal Standard

Fig. 1. Structures of higenamine hydrochloride and the internal standard, N-(3,4-dihydroxyphenylethyl)-*p*-hydroxyphenylethylamine hydrobromide.

collection, 3 ml of concentrated phosphoric acid and 50 mg of EDTA disodium salt were placed in a 500-ml erlenmeyer flask for *ca.* 100–150 ml of urine (pH ≈ 2–3) for 24 h. The plasma or dilute urine sample (0.2 ml) in a borosilicate glass tube was mixed with 280 ng of internal standard, 50 mg of alumina and 1 ml of Tris buffer solution (pH 8.6). The mixture was vortex-mixed for 3 min and the supernatant was aspirated out as much as possible. The alumina was washed with 1 ml of water three times and then twice with 1 ml of methanol. The residual alumina was vortex-mixed with 400 μl of 0.1 *M* perchloric acid for 5 min to release higenamine. A 5–20-μl volume of 0.1 *M* perchloric acid solution was injected into the HPLC system.

2.5. Administration of higenamine to rabbits by intravenous and oral routes

Higenamine hydrochloride was dissolved in water for injection and injected into the ear vein of male albino healthy rabbits weighting 1.5–3 kg at a dose of 20 mg/kg. Blood samples were withdrawn at 1, 3, 5, 7, 9, 12, 15, 20, 30, 45, 60, 90 and 120 min. Plasma was separated by centrifugation at 6000 *g* for 5 min at 4°C and samples were acidified and stored immediately at −10°C until analysis. Urine samples were also collected for 24 h after dosing, then acidified and stored at 0°C before analysis.

The rabbits were fasting at least 24 h before administration and higenamine hydrochloride was given at a dose of 50 mg/kg by introduction through a gastric tube into the stomach. Blood samples were withdrawn at specific time intervals as above. Plasma was collected by centrifugation at 6000 *g* for 5 min at 4°C. The plasma samples were acidified and stored immediately at −10°C until analysis. Urine samples were also collected for 24 h after dosing, then acidified and stored at 0°C before analysis.

3. Results and discussion

Higenamine, possessing a catechol moiety, is labile at high pH and it can be stabilized by changing the pH to 3–5 or adding a chelating agent [12]. Plasma and urine samples or stock solutions of higenamine must be kept at low pH and addition of EDTA disodium salt is highly recommended.

Park *et al.* [14] reported an HPLC–UV method for the determination of higenamine in rabbit blood, but the detection limit was only 6 μg/ml. Owing to insufficient sensitivity with UV detection, the data for higenamine concentration in blood could not completely correlated with the pharmacological responses on changes of heart rate and mean arterial pressure in rabbits.

Modification of the previous method [12] used for the determination of higenamine in medicinal plants enhanced the detection sensitivity to a level suitable for the simultaneous determination of higenamine in plasma and urine for pharmacokinetic studies. The biological sample was mixed with alumina at pH 8.6 to adsorb higenamine. After washing with water and methanol, higenamine was released with 0.1 *M* perchloric acid. The acidic solution then was injected into the HPLC system.

The chromatograms of blank, spiked rabbit plasma and urine samples in Figs. 2 and 3 show that higenamine and the internal standard were well resolved. The approximate retention times for higenamine and the internal standard were 12.27 and 14.06 min, respectively, in plasma and 11.44 and 13.19 min, respectively, in urine. Further, no endogenous compound appears to interfere with higenamine in plasma or urine samples.

The peak-height ratios between higenamine and the internal standard were linear over the concentration ranges 2.645–5289 ng/ml for plasma and 10.58–2645 ng/ml for urine. The calibration graphs were divided into low (2.645–105.8 ng/ml for plasma and 10.58–105.8 ng/ml for urine) and high (2.645–5289 ng/ml for plasma and 10.58–2645 ng/ml for urine) concentration ranges in order to obtain a more precise fit of the linear regression lines. The linear regression of higenamine in plasma is $y = 1.957 \cdot 10^{-3} + 1.170 \cdot 10^{-3}x$ ($r^2 = 1.000$) for the low concentration range and $y = -1.325 \cdot 10^{-2} + 1.237 \cdot 10^{-3}x$ ($r^2 = 1.000$) for the high concentration range (y = peak-height ratio; x = concentration in ng/ml). The linear regression of higenamine

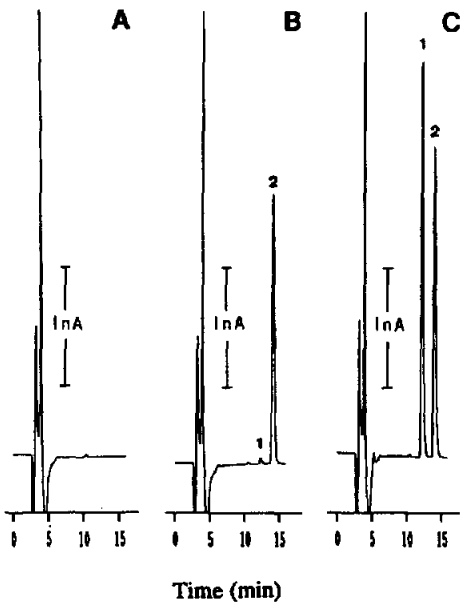


Fig. 2. Chromatograms of (A) drug-free plasma, (B) plasma spiked with 26.45 ng/ml of higenamine and (C) higenamine plasma sample (1478 ng/ml) obtained 20 min. after intravenous injection of 20 mg/kg of higenamine hydrochloride into a rabbit. Peaks: 1 = higenamine; 2 = internal standard.

in urine is $y = 1.576 \cdot 10^{-3} + 9.546 \cdot 10^{-4}x$ ($r^2 = 0.999$) for the low concentration range and $y = -8.450 \cdot 10^{-4} + 9.782 \cdot 10^{-4}x$ ($r^2 = 1.000$) for the high concentration range. The detection limits for higenamine in plasma and urine were 2.645 and 10.58 ng/ml, respectively, employing 0.2 ml of plasma or urine sample.

The absolute recoveries for higenamine by alumina treatment in plasma and urine are given in Table 1. The average recoveries of higenamine in plasma and urine calculated by the slope of the mean peak-height ratios were found to be 77.5 and 84.3%, respectively, at four different concentrations. Higenamine in plasma and urine on storage was stable for 4 weeks without degradation.

The validation of the HPLC procedure was designed to test the precision and accuracy of the method. Validation was accomplished by assaying five different concentrations of higenamine in rabbit plasma and urine each day for five consecutive days. The coefficient of variation (C.V.) served as a measure of precision. The accuracy was assessed by calculating the relative error (R.E.) of the mean of five determinations of

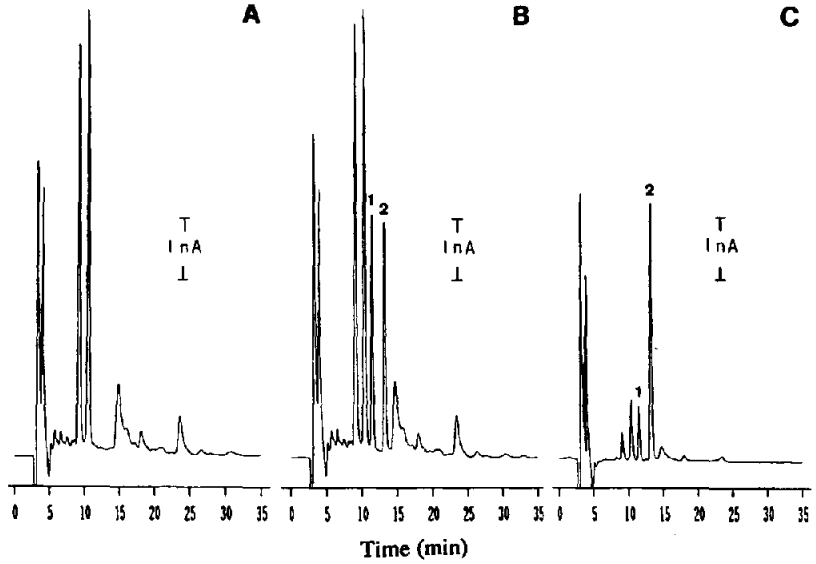


Fig. 3. Chromatograms of (A) drug-free urine, (B) urine spiked with 1058 ng/ml of higenamine and (C) higenamine urine sample collected after 24 h following administration of 50 mg/kg of higenamine hydrochloride to a rabbit. Peaks: 1 = higenamine; 2 = internal standard.

Table 1
Recovery tests for higenamine in rabbit plasma and urine with and without alumina treatment ($n = 3$)

Spiked concentration (ng/ml)	Peak-height ratio (mean ± S.D.)		Recovery (%)
	Without alumina	With alumina	
Plasma			
21.16	0.0213 ± 0.0007	0.0165 ± 0.0001	77.5
211.6	0.1974 ± 0.0022	0.1507 ± 0.0044	76.3
423.1	0.3837 ± 0.0048	0.3084 ± 0.0127	79.6
846.2	0.7757 ± 0.0023	0.5992 ± 0.0103	77.2
Urine			
21.16	0.0213 ± 0.0007	0.0149 ± 0.0007	70.0
211.6	0.1974 ± 0.0022	0.1645 ± 0.0075	83.3
423.1	0.3837 ± 0.0048	0.3215 ± 0.0048	83.0
846.2	0.7757 ± 0.0023	0.6547 ± 0.0023	84.4

each concentration relative to the known concentration. The intra- and inter-day assay precision and accuracy in plasma and urine are summarized in Tables 2 and 3. The C.V. and R.E. were less than 6%.

To verify the acceptance of plasma sample runs, quality control (QC) samples in duplicate at four concentrations (9.256, 42.31, 264.5, and 2116 ng/ml) were incorporated in each run. The result for the QC samples provide the basis of accepting or rejecting a run. The QC samples of plasma fitted with low- and high-range calibration graphs for four concentrations were 82.9 and 83.1% at 9.256 ng/ml, 93.2 and 99.8% at 42.31 ng/ml, 106.1 and 104.1% at 264.5 ng/ml and 100.6 and 107.0% at 2116 ng/ml, which meet the requirement of the report of the Conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies [16]. Using QC samples to assess plasma samples subjected to HPLC–ED analysis, the results demonstrated that the plasma data were acceptable and that the HPLC system is reliable. On the other hand, the chosen concentrations of the QC samples could also validate the precision and accuracy assay.

The assay was then applied to preliminary pharmacokinetic studies by administration of higenamine intravenously and orally to rabbits. The plasma concentration *versus* time profile for higenamine following intraveous and oral dosing are shown in Fig. 4. These data could be described by a two-compartment model with a biological half-life of 16 min. Higenamine was rapidly absorbed from the gastrointestinal tract after oral dosing; T_{max} was *ca.* 10 min. The cumulative urinary excretion of higenamine from a same rabbit after intravenous and oral dosing within 24 h was 4.73 and 0.82% of the administration dose, respectively.

Table 2
Precision and accuracy tests for higenamine in rabbit plasma ($n = 5$)

Spiked concentration (ng/ml)	Intra-day			Inter-day		
	Measured concentration[a] (mean ± S.D.) (ng/ml)	C.V. (%)	R.E. (%)[b]	Measured concentration[a] (mean ± S.D.) (ng/ml)	C.V. (%)	R.E. (%)[b]
21.16	22.18 ± 0.81	3.65	−4.82	21.86 ± 1.14	5.22	−3.31
211.6	213.5 ± 10.1	4.73	−0.90	211.0 ± 13.7	6.49	0.28
423.1	432.4 ± 12.0	2.78	−2.20	432.9 ± 10.0	2.31	−2.32
846.2	893.3 ± 6.4	0.72	−5.57	837.4 ± 31.2	3.73	1.04

Five sets of calibration measurements were made on consecutive days. The calibration graphs were divided into low-range (2.645–105.8 ng/ml) and high-range (2.645–5289 ng/ml).

[a] The measured concentration data for 0–105.8 ng/ml were obtained from the low-range graph and the data for 105.8–5289 ng/ml from the high-range graph.

[b] Relative error of mean (%) $= \dfrac{\text{true concentration} - \text{mean measured concentration}}{\text{true concentration}} \cdot 100.$

Table 3
Precision and accuracy tests for higenamine in rabbit urine ($n = 5$)

Spiked concentration (ng/ml)	Intra-day			Inter-day		
	Measured concentration[a] (mean ± S.D.) (ng/ml)	C.V. (%)	R.E.[b] (%)	Measured concentration[a] (mean ± S.D.) (ng/ml)	C.V. (%)	R.E.[b] (%)
21.16	21.91 ± 0.71	3.24	−3.54	21.48 ± 0.65	3.03	−1.51
211.6	214.3 ± 4.0	1.87	−1.28	210.1 ± 2.0	0.95	0.71
423.1	420.1 ± 2.9	0.69	0.71	417.1 ± 2.5	0.60	1.42
846.2	849.2 ± 7.0	0.82	−0.40	836.6 ± 7.2	0.86	1.13

Five sets of calibration measurements were made on consecutive days. The calibration graphs were divided into low-range (10.58–105.8 ng/ml) and high-range (10.58–2645 ng/ml).

[a] The measured concentration data for 0–105.8 ng/ml were obtained from the low-range graph and the data for 105.8–2645 ng/ml from the high-range graph.

[b] Relative error of mean (%) $= \frac{\text{true concentration} - \text{mean measured concentration}}{\text{true concentration}} \cdot 100.$

The HPLC–ED method reported here, with simplicity and high sensitivity, precision, accuracy and reproducibility, is suitable for the determination of higenamine in rabbit plasma and urine. Detailed pharmacokinetic studies on higenamine are in progress.

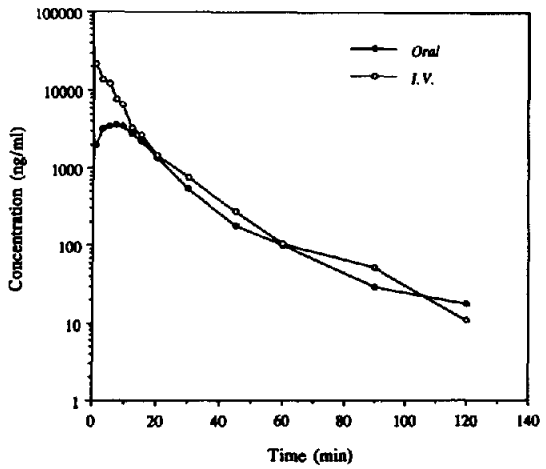


Fig. 4. Plasma concentration *versus* time profile of higenamine in rabbit after (○) an intravenous dose of 20 mg/kg and (●) an oral dose of 50 mg/kg.

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