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Article

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Determination of the Metabolite of Ephedrine, 4-Hydroxyephedrine, by LC–MS-MS in Rat Urine and Its Application in Excretion Profiles After Oral Administration of *Ephedra sinica Stapf* and Processing *Ephedra sinica Stapf*

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Abstract

This study aims to study the changes of metabolism of *Ephedra sinica Stapf* caused by processing. A sensitive and rapid high-performance liquid chromatographic tandem mass spectrometry (LC–MS-MS) method was developed for the quantitation of the major metabolite of ephedrine, 4-hydroxyephedrine, utilizing lidocaine as the internal standard in rat urine. Urine samples were precipitated with acetonitrile. Chromatographic separation was achieved on an Ultimate C18 analytical column. Detection was performed by a multiple reaction monitoring mode via an electrospray ionization source operating in the positive ionization mode. The method was linear over the concentration range of 0.05–1.0c for all components. The intra- and inter-day precision values were <13.2% and the deviations ranged from −8.4% to 7.5%. The recoveries at three levels were more than 66.2%. The fully validated method was used to study the pharmacokinetic profile of 4-hydroxyephedrine in rat urine to investigate the effects caused by processing.

Introduction

Traditional Chinese medicine (TCM) has been widely used over thousands of years. However, mechanisms of action of TCM are still unclear. TCM has received more and more concern nowadays. In order to elucidate their possible mechanisms of action, there were extensive literatures on phytochemical (1, 2) and compatibility (3, 4) while few research work on processing. In fact, processing is a key procedure for the potency of herbs. Processing drugs is a traditional pharmaceutical technology transforming crude herbs to decoction pieces, to meet different requirements of medical treatment, concoction, preparation, storage according to theories of TCM. The function of processing includes: reduce the toxicity and irritation; change the properties (flavor, trend, channel tropism, curative effect) of herbs; storage ease; augment the ingredients dissolving out of herbs. From modern science angle, the principle of processing is to change the chemical composition of herbs through processing thus adjust their medical function.

The violent nature of some herbs, which may cause adverse effects in clinic practice, could be adjusted or moderated by processing. *Ephedra sinica Stapf* is a widely used Chinese medicinal plant (Chinese name: Ma Huang) to treat asthma, inflammation, immunosuppression in a long history. Due to the acute potency, *E. sinica Stapf* is not suitable for the elderly and weak directly. In comparison, *E. sinica Stapf* after processing provides relief to some degree.

Ephedrine alkaloids (norpseudoephedrine, ephedrine, pseudoephedrine, methylephedrine, methylpseudoephedrine, etc.) are the most important pharmacologically active constituents. These alkaloids could transform mutually *in vivo* (5–8), or transform into new metabolites. Ephedrine, one of the main active ingredients of *E. sinica Stapf*, is a very active adrenergic receptor agonist, both α and β

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receptors. Ephedrine could agitate the adrenergic receptor directly, or indirectly through promoting the adrenergic nerve endings to release norepinephrine. Since ephedrine alkaloids always decompose fast in blood, metabolite studies are helpful to understand the medication safety, toxification and detoxification mechanism. Earlier publications have described methods for ephedrine alkaloids determination in biological samples, and the identification of metabolites (9–12). As far as we know, no methods for the determination of metabolites in biological fluids after oral administration of *E. sinica Stapf* have been published. 4-Hydroxyephedrine (Figure 1) is one of the major metabolites of ephedrine (9). This paper employed the "relative quantification" (13) to develop a LC–MS-MS method for the determination of 4-hydroxyephedrine in rat urine for the first time. The method was then applied to a comparative pharmacokinetic study on *E. sinica Stapf* and processed *E. sinica Stapf* (fried with honey) in rat urine to investigate how the processing affects the nature of *E. sinica Stapf*.

In addition, ephedrine hydrochloride could enhance largely the performance of athletes in sports, being recognized as the provocative. These kinds of drugs are strictly forbidden by the International Olympic Committee because of serious side effects. Meanwhile, ephedrine hydrochloride is the main basic material for synthesizing methamphetamine (ice) and has been listed in the precursor chemicals under the state's control. The analytical method of 4-hydroxyephedrine in urine of this paper could act as a supplementary method for monitoring the presence of ephedrine.

Experimental

Chemicals and reagents

Ephedra sinica Stapf and processed *E. sinica Stapf* were purchased from the Northeast Pharmacy (Shenyang, China). The reagents used for LC–MS-MS measurement were of HPLC grade and were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade (Shenyang Chromatogram Scientific Instruments Limited Company, Shenyang, China). Double-distilled water was used throughout the study.

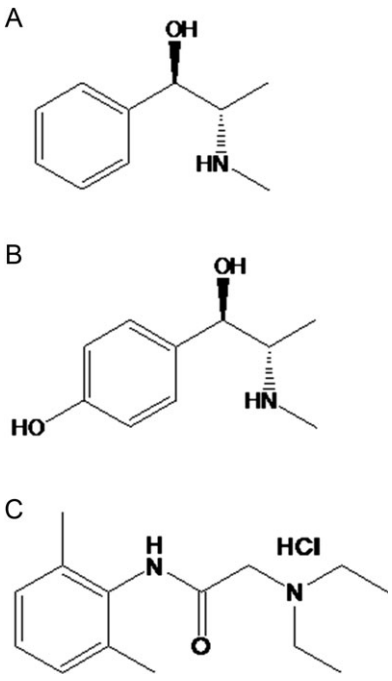

Figure 1. The chemical structure of (A) ephedrine, (B) 4-hydroxyephedrine and (C) lidocaine (IS).

LC–MS-MS conditions

The liquid chromatography was performed on Shimadzu UFLC system (Chiyoda-Ku, Kyoto, Japan) consisting of two LC-20AD pumps, DGU-20A3 on-line degasser, SIL-20ACHT autosampler, CTO-20AC column thermostat. The analytes were injected into an Ultimate C18 column (5 μm, 2.1 × 50 mm, USA) protected by a Phenomenex ODS guard column (5 μm, 4.0 mm × 3.0 mm i.d., Torrance, CA, USA) at 25°C. Separation and elution were achieved using 10 mM aqueous ammonium formate (adjust pH3.5 with formic acid)/methonal (30:70, v/v) as the mobile phase, at a flow rate of 0.2 mL/min. The total run time of each sample was 3.5 min.

Mass spectrometric analysis was performed on an API 3200 LC–MS-MS System (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ion source. The mass spectrometer was operated in the positive ion and multiple reaction monitoring mode with precursor to product transition *m/z* 182→164 for 4-hydroxyephedrine and *m/z* 235→86 for lidocaine (IS). The spray voltage and source temperature were 5,500 V and 550°C, respectively. The other gas source parameters were set as followings: curtain gas 35 psi, nebulizer gas 40 psi, auxiliary gas 60 psi, collision activated dissociation 4 psi. Data acquisition and analysis were achieved using the Analyst 1.5 Software (Applied Biosystems).

Preparation of calibration standards and quality control samples

An aliquot of 1 mL mixed urine 24 h after the administration of *E. sinica Stapf* was added with 4 mL acetonitrile. The mixture was vortex-mixed for 2 min and centrifuged at 10,000 × *g* for 5 min. The supernatant was separated out and blown to dryness under a gentle stream of nitrogen at 40°C. The residue was reconstituted in 250 μL of the mobile phase as the standard stock solution of metabolites, presuming a concentration of 2c. Calibration standards were prepared by serially diluting the stock solution with mobile phase to yield urine concentrations of 0.04c, 0.10c, 0.20c, 0.40c, 0.80c, 1.6c and 2.0c. A 100 ng/mL IS working solution was prepared by diluting a stock standard solution of lidocaine with mobile phase.

Then 100 μL of the working solution was added to 200 μL of blank rat urine, vortex-mixed for 30 s. The concentration levels of metabolites in urine ranged from 0.02c to 1.0c. Quality control (QC) samples were prepared in a similar manner at low, medium and high levels (0.05c, 0.20c, 0.80c). All of the spiked urine samples were then treated according to the sample preparation procedure. The standards and QC were stored at –20°C until analysis.

Sample preparation

To 200 μL rat urine in a 1.0 mL Eppendorf tube, 50 μL of the internal standard solution (100 ng/mL) and 800 μL of acetonitrile were added. This mixture was vortex-mixed 2 min and centrifuged at 4,000 × *g* for 5 min. The supernatant was separated out and blown to dryness with nitrogen at 40°C. Then the residue was reconstituted in 100 μL mobile phase and mixed to make final testing samples. A 10 μL aliquot of the final testing samples was injected onto the LC–MS-MS system for analysis.

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Method validation

The method was validated according to the currently accepted USA Food and Drug Administration (FDA) bioanalytical method validation guidance.

The linearity was evaluated by analyzing calibration standards in duplicate at each concentration level over three consecutive days. The accuracy and precision were determined by analyzing QC samples in six replicates at three concentration levels on three validation days. The extraction recovery was determined at three concentration levels and for the IS at one concentration level by comparing the peak areas of the analytes obtained from six plasma samples with the analytes spiked before and after extraction. Matrix effect was assessed by comparing the peak areas of the analytes obtained from six urine samples with the analytes spiked after extraction to those for the neat standard solutions at the same concentrations. The stability of analytes in urine was evaluated under a variety of storage and process conditions using low and high QC samples.

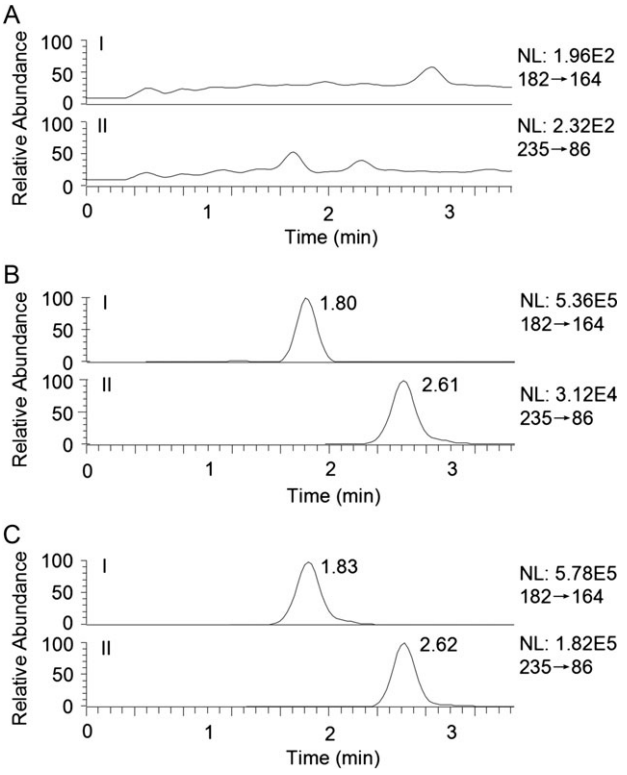

Figure 2. Representative selective reaction monitor chromatograms of metabolite (I) and IS (II): (A) a blank urine sample; (B) a blank urine spiked with IS 50 ng/mL, metabolite 0.02c; (C) a urine sample 2 h after administration.

Application to pharmacokinetic study

Six Sprague-Dawley rats weighing 220–250 g were fasted for 12 h. The rats were divided into two groups to complete the crossover design for pharmacokinetic experiment over a washout period of 7 days. Each rat was administered an oral dose of 2.5 g/kg *E. sinica Stapf* or processed *E. sinica Stapf* powder suspended in an aqueous solution containing 0.5% carboxymethyl cellulose sodium. Urine samples were collected before drug administration (0 h), and subsequently between 0 and 2, 2 and 4, 4 and 6, 6 and 8, 8 and 12, and 12 and 24 h following administration. After the accurate volume was measured, the samples were stored at −70°C until analysis.

Results

Method development

Different mobile phases consisting of methanol–water or acetonitrile–water were attempted. The selected mobile phase was composed of 10 mM aqueous ammonium formate (adjust pH3.5 with formic acid)/methonal (30:70, v/v), and provided a clear separation of 4-hydroxyephedrine and IS.

The LC–MS-MS conditions were optimized to provide a high mass spectral signal, low background noise and proper retention time. Comparing with the blank rat urine, we found the ion at *m/z* 182 at 1.83 min in the urine samples after the administration of medicines. Its MS^2 spectrum displayed the fragment ion at *m/z* 164, and was identified as 4-hydroxyephedrine by referring to the literature (9).

Sample preparation

In this study, a simple protein precipitation procedure was used. Different kinds of extraction procedures, including liquid–liquid extraction, were evaluated during our method development. The results showed that the protein precipitation with acetonitrile 4–5 times to rat urine (v/v) obtained good recoveries and low interfering peaks. Also the extraction procedure was very simple and little time-consuming and therefore utilized in the study.

Quantitative analysis method validation

Six different lots of blank urine without analytes or internal standards were extracted and analyzed for evaluating the specificity of this method. No interference from endogenous substances was observed at the retention times of 4-hydroxyephedrine and IS (Figure 2). Calibration curves showed good linearity over the range of 0.02–1.0c with a correlation coefficient (R^2) of 0.9951. The lower limit of quantitation was 0.02c ± 0.003c.

Table I presents the precision and accuracy for 4-hydroxyephedrine by analyzing QC samples. The intra- and

Table I. Precision, Accuracy and Matrix Effect of 4-Hydroxyephedrine in Rat Urine

Nominal concentration (c)	Mean measured concentration (c)		Precision (RSD, %)		Accuracy (RE, %)		Matrix effect, %
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Mean ± SD
0.02	0.019	0.018	4.4	13.2	−3.0	−8.4	Not required
0.05	0.049	0.046	2.5	5.6	−1.3	−6.9	93.5 ± 3.1
0.20	0.19	0.22	2.0	2.9	−4.8	7.5	88.0 ± 2.3
0.80	0.84	0.83	3.1	7.8	5.0	4.6	90.2 ± 1.8

RSD, relative standard deviation.

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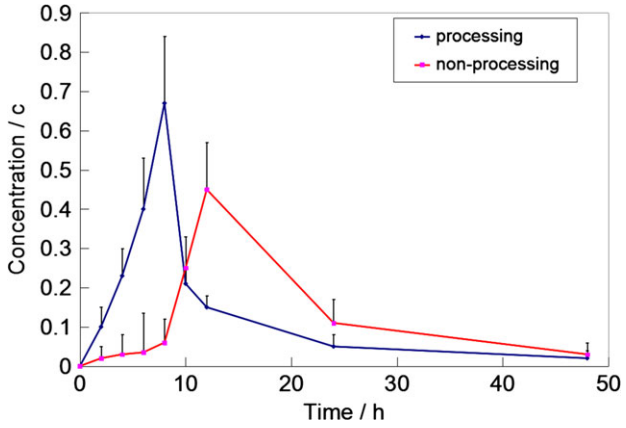

Figure 3. Mean urine concentration–time curves of the metabolite after oral administration of *E. sinica Stapf* and processed *E. sinica Stapf*.

inter-day precision was <5.3% and the accuracy was <2.7%. The mean extraction recoveries were 75.5 ± 3.8%, 66.2 ± 2.3% and 81.0 ± 2.5% at concentrations of 0.02c, 0.05c, 0.20c and 0.80c, respectively. The mean recovery of the IS was 73.3 ± 2.2%. With regard to matrix effect, all the calculated values were between 85% and 115%, which indicated that the co-eluting matrix components had little or no effect on the ionization of the analytes and the IS.

The stability study showed that 4-hydroxyephedrine was stable in urine at room temperature (25°C) for 2 h [relative error (RE) <7.9%], at −70°C for 15 days (RE <8.3%) and after three freeze–thaw cycles (RE <6.5%). The analyte was also shown to be stable after the reconstitution at 25°C for 24 h (RE <12.6%).

Pharmacokinetic investigation in rats

The validated method was applied to the pharmacokinetic study of 4-hydroxyephedrin after oral administration of *E. sinica Stapf* and processed *E. sinica Stapf* to six rats. The mean urine concentrations of 4-hydroxyephedrin versus time profile are presented in Figure 3.

Discussion

The results indicated that after processing, the main metabolite of ephedrine excreted into urine in less time, the time to peak reduced, the peak concentration increased, the area under the urine concentration–time curve increased, which means that the mean residence time of *E. sinica Stapf* reduced, that the metabolizing degree of ephedrine alkaloids increased. Hence, to the body, the acting time and strength of *E. sinica Stapf* reduced, which suggests that the medicinal potency weakened. The results matched the explanation in TCM theory that processing could weaken the medicinal potency of *E. sinica Stapf*. The metabolism research can contribute to understand the mechanism of processing.

Conclusion

The paper first presents a LC–MS-MS method for the determination of 4-hydroxyephedrin in rat urine. The method has been successfully applied to the pharmacokinetic studies of 4-hydroxyephedrin after oral administration of *E. sinica Stapf* and processed *E. sinica Stapf* to rats. The pharmacokinetic results obtained from this study can give some sorts of useful information to elucidate the mechanism of TCMs.

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