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Determination of aconitine, hypaconitine and mesaconitine in urine using hollow fiber liquid-phase microextraction combined with high-performance liquid chromatography

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ABSTRACT

Hollow fiber liquid-phase microextraction (HF-LPME) coupled with high-performance liquid chromatography was used to simultaneously determine three *Aconitum* alkaloids, including aconitine (AC), hypaconitine (HA) and mesaconitine (MA) in human urine sample. Analytes were extracted from 5 mL urine sample containing 1.0 mmol/L NaOH into 1-octanol membrane phase impregnated in the pores of hollow fiber wall, and then back extracted into acidified aqueous solution in the lumen of the hollow fiber. After extraction, 10 μL of the acceptor phase was analyzed directly by HPLC. In this method, some important extraction parameters, such as organic solvent, extraction time, stirring rate, pH of donor phase and acceptor phase, temperature, and the volume of acceptor phase were optimized. This method provided 98- to 288-fold enrichment factors within 60 min of extraction and good repeatability with RSDs of 0.99–7.22%. The calibration curves were linear over the ranges of 16.0–128.0 μg/L for AC, 11.0–88.0 μg/L for HA and 8.1–64.8 μg/L for MA in human urine sample, with correlation coefficients of 0.9949, 0.9969 and 0.9904, respectively. Limits of detection were from 0.7 to 1.5 μg/L, and recoveries from spiked urine sample varied from 84.4% to 106.2% for AC, 77.3% to 85.6% for HA and 90.1% to 100.8% for MA.

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1. Introduction

The *Aconitum* species (Ranumculaceae) are widespread throughout Europe, Asia, and North America. Aconite plants contain highly toxic diester diterpence-type *Aconitum* alkaloids such as aconitine (AC), hypaconitine (HA) and mesaconitine (MA), which are neurotoxic and cardiotoxic [1]. *Aconitum* tubers after detox processing have been used to treat cold, polyarthralgia, diarrhea, skin wood, hear failure, depression, beriberi and edema in East Asia for over 2000 years [2,3]. But unexpected poisoning incidents caused by these toxic alkaloids remained in the herbal medicines have occurred from time to time because of improper processing [4]. Moreover, there are also some reports concerning suicidal [5] and homicidal [6] cases that have involved with aconite plants. As the aconitines are relatively unstable, and only trace amounts could be detected in biofluid after ingestion of *Aconitum* plant extracts [7,8], it is necessary to develop a sensitive, efficient, and rapid analytical method for the identification and determination of these three alkaloids in biofluid for toxicological research, clinical study, forensic analysis and medicine safety areas.

Several methods have been reported for the determination of AC, HA and MA in herbal plants, medicines and body fluid, including CE [9,10], HPLC [11–13], LC–MS [2,14,15], LC–MS–MS [3,16,17], GC–MS [1,18], and MS [19,20]. Most of these methods need complex sample pretreatments, such as liquid-phase extraction, solid phase extraction and pre-column derivation, which are time-consuming, tedious, not sensitive enough for trace analysis of aconitines in biofluid, and need large amounts of toxic organic solvents. Thus, an effective enrichment method is necessary, especially for the analysis of biological sample. In recent years, miniaturized extraction techniques have arisen continuously, such as solid phase microextraction (SPME) and liquid-phase microextraction (LPME). Despite of its simplicity, free organic solvent consumption and efficient extraction performance, SPME still suffers from some problems such as sample carry-over, relatively high cost, fiber breakage, stripping of coatings, and fiber instability and swelling in organic solvents (greatly restricting its use with HPLC) [21]. As a solvent-minimized procedure of liquid–liquid extraction, LPME

Abbreviations: AC, aconitine; HA, hypaconitine; MA, mesaconitine; HF-LPME, hollow fiber liquid-phase microextraction; HPLC, high-performance chromatography; LODs, limits of detection; LOQs, limits of qualification.

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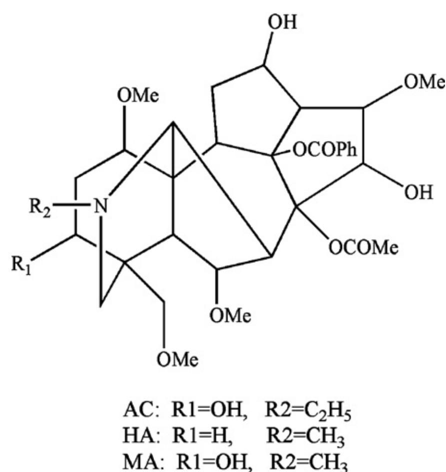


Fig. 1. The chemical structures of AC, HA and MA.

combines extraction, preconcentration and sample introduction into a single step and possesses many advantages such as simplicity, lower cost, negligible consumption of organic solvent, and high enrichment efficiency [22–25]. LPME can be divided into three main categories: single drop microextraction (SDME), hollow fiber liquid-phase microextraction (HF-LPME), dispersive liquid–liquid microextraction (DLLME). SDME shows some technical difficulties in maintaining a stable microdroplet in a stirred solution, whereas HF-LPME protects microdroplet of organic solvent in hollow fiber. In addition, HF-LPME has excellent sample clean-up ability which SDME and DLLME does not have at all and can be used for complex matrix sample. Therefore, HF-LPME has been given considerable attention in biomedical analysis since it was introduced by Pedersen-Bjergaard and Rasmussen [22].

The objective of this study was to investigate the suitability of HF-LPME for extraction of AC, HA and MA in urine sample. According to the alkaline nature of the analytes, three-phase extraction mode was utilized. Parameters such as organic solvent, extraction time, stirring rate, pH of donor phase and acceptor phase, temperature, and the volume of acceptor phase were controlled and optimized. The extract was injected directly into HPLC system for analysis. The optimized parameters were applied to the analysis of real human urine sample.

2. Experimental

2.1. Chemicals and materials

Aconitine, hypaconitine and mesaconitine (molecular structures shown in Fig. 1) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Chromatographic-grade acetonitrile was obtained from Merck Co. (Darmstadt, Germany). Other chemicals are of analytical grade and were purchased from Tianjin Chemical Reagent Co. (Tianjin, China). Distilled and deionized water was from Hangzhou Wahaha Co. (Hangzhou, China). The Accurel Q3/2 polypropylene hollow fiber (200 μm wall thickness, 600 μm internal diameter, 0.2 μm pore size) was purchased from Membrana GmbH (Wuppertal, Germany).

2.2. Apparatus and chromatography

The HPLC system (Waters Corp., Milford, MA, USA) consisted of a Waters quaternary pump (Model Delta 600E), a photodiode array detector (Model 2996), a manual injector, and Waters Millennium^{32} software (Version 3.2) for peak identification and integration. All separations were achieved on a Kromasil C_{18} column (5 μm, 4.6 mm × 250 mm i.d.) (Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China). The mobile phase consisted of acetonitrile–0.03% triethylamine (85:15, v/v) at a flow rate of 1.0 mL/min, and the wavelength used to measure the analyte was 230 nm. Helium (He) was used for degassing the mobile phase. The temperature of the column during analysis was maintained at 25 °C.

2.3. Preparation of standard solutions and biological samples

Aconitine, hypaconitine and mesaconitine were dissolved in methanol to obtain stock solutions with concentration of 0.20 mg/mL, 0.11 mg/mL and 0.09 mg/mL, respectively. Calibration standard working solutions of the three alkaloids at five concentration levels were freshly prepared by appropriate dilution of the stock solutions with distilled water. Human drug free urine samples were supplied by a healthy volunteer, stored below 0 °C and brought to room temperature before use.

2.4. HF-LPME procedure

The experimental setup of HF-LPME, as illustrated in Fig. 2, was adopted from Lee [26]. A 50 μL microsyringe (needle tip 0.46 mm o.d.) purchased from Hamilton Co. (Reno, Nevada, USA) was used to introduce the acceptor phase and support the hollow fiber. 5.0 mL of aliquot sample solution was placed in a 10-mL sample vial with a screwcap and a silicon septum. The sample vial was clamped to fix its position above the magnetic stirrer. A hollow fiber was cut manually into segments of 5.3 cm, and then ultrasonically cleaned in acetone for 1 min to remove any contaminations and dried in air. After rinsing the microsyringe with methanol, 15 μL of 10.0 mmol/L HCl was withdrawn into it, and then it was inserted into a hollow fiber segment which had been impregnated in 1-octanol for 10 s. After that, HCl was introduced into the hollow fiber, and one end of the hollow fiber was flame-sealed. The prepared hollow fiber together with the syringe was pierced through the silicon septum in the screw cap and then immersed into the sample. At the end of

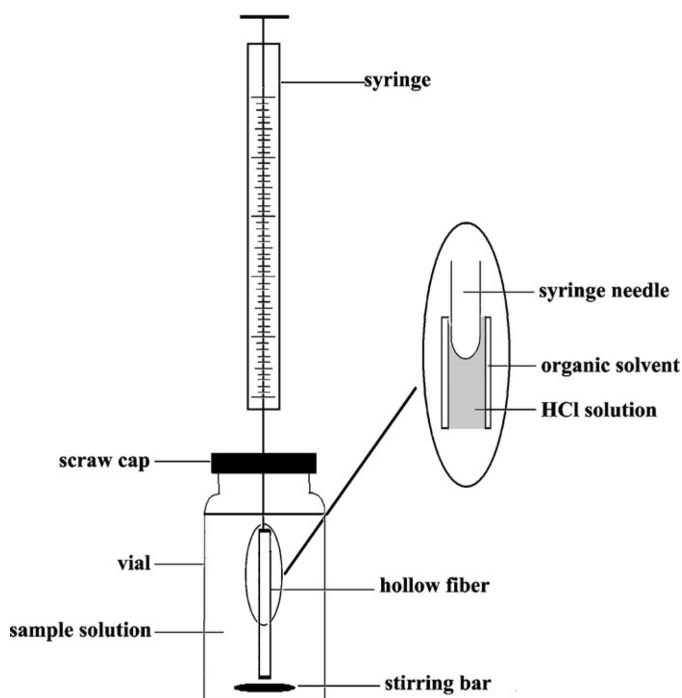


Fig. 2. Schematic illustration of HF-LPME device.

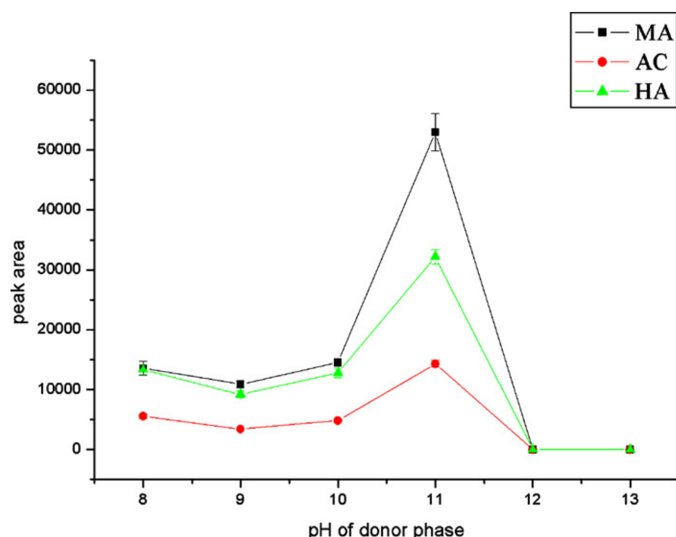


Fig. 3. Effect of donor phase pH on the peak area of AC, HA and MA extracted with LPME. Extraction condition: organic solvent: 1-octanol; acceptor phase: 10.0 mmol/L HCl; extraction time: 60 min; stirring rate: 800 rpm.

the extraction, which was performed by the magnetic stirrer, 12 μL of the acceptor phase was retracted into the syringe and 10 μL was injected for HPLC analysis.

3. Results and discussion

3.1. Optimization of HF-LPME procedure

Because AC, HA, and ME are ionizable basic compounds, and they are transported between the acceptor phase and the donor phase by the gradient of pH, a three-phase extraction is suitable for the analytes. Relevant experimental parameters, including organic solvent, extraction time, stirring rate, pH of donor phase and acceptor phase, temperature, and the volume of acceptor phase were optimized. Five milliliters of aqueous solution containing 80 μg/L of AC, 77 μg/L of HA and 63 μg/L of MA were used for all the optimization experiments. In optimization, three parallel experiments were performed for each experimental condition.

3.1.1. Selection of organic extraction solvent

Ideally, organic solvent in HF-LPME should be non-volatile, immiscible with water, compatible with the HPLC mobile phase and strongly immobilized within the pores of the hollow fiber, and should be able to provide high solubility for the target analytes. On the basis of these considerations, six organic solvents, chloroform, dichloromethane, *n*-heptane, toluene, xylene and 1-octanol were separately investigated. With 1 mmol/L NaOH as the donor phase, 10 mmol/L HCl as the acceptor phase, and 30 min extraction at a stirring rate of 800 rpm, the results showed that 1-octanol was the most suitable extraction solvent, as it extracted all the three analytes, provided much higher enrichment factors and selectivity, and exhibited low solvent loss.

3.1.2. Selection of pH of donor phase and acceptor phase

The compositions of both donor and acceptor phase are very important parameters, which can influence the transfer of analytes from donor phase to acceptor phase, thus they were optimized to obtain the highest enrichment factors.

For the three basic analytes, donor phase should be alkalized to insure their deprotonation and consequently reduce their solubility in sample solution. In a series of experiments, the pH of donor phase was varied from 8.0 to 13.0. The results in Fig. 3 showed that the peak area fluctuated with increasing pH from 8.0 to 10.0, and at pH 11.0 (the concentration of NaOH in the donor phase was 1.0 mmol/L), the largest peak area was obtained. None of the three analytes, however, could be extracted when pH exceeded 11.0. The above results may be explained as follows: with increasing pH value from 8.0 to 11.0, the weakly basic aconitines would be deprotonated more easily and their solubility decreased in sample solution, and more amounts of analytes diffused into 1-octanol which retained in the pores of hollow fiber. But when pH value exceeded 11.0, at which the basic nature of sample solution was greater than that of the analytes, and the analytes was precipitated from sample solution, then no analytes could diffused into 1-octanol. Therefore, the optimum pH of donor phase was selected as 11.0.

Acceptor phase should be acidized to insure the ionization of the analytes and promote their dissolution. Several HCl aqueous solutions with pH values ranging from 2.0 to 5.0 were measured as the acceptor phase. Higher concentrations of HCl were not used to avoid possible problems with the hollow fiber, column and injector. The results showed that the extraction efficiency of these analytes increased as the pH became lower, and none of the three analytes could be extracted when pH was higher than 3.0. This is in accordance with what the literature pointed out that the pH of acceptor phase should be at least 2–3 units different from the pK_a values of the analytes [24], and the pK_a of these aconitines is nearly 6 [27]. Thus, 10.0 mmol/L HCl (pH 3.0) was adopted as the acceptor phase in the following studies.

3.1.3. Selection of extraction time

A series of exposure times was investigated by extracting spiked solutions at a stirring rate of 800 rpm, with 1-octanol as organic solvent, 1.0 mmol/L NaOH as donor phase and 10.0 mmol/L HCl as acceptor phase. As shown from Fig. 4, the peak areas of the aconitines extracted by HF-LPME increased with increasing extraction time from 20 to 60 min. After 60 min, the peak area barely increased for AC and HA, and increased slightly for MA. Considering there is a potential solvent loss with continuing prolongation of exposure time and it is not necessary to attain equilibrium if extracting conditions remain constant [28], 60 min was selected as extraction time for the subsequent experiments.

3.1.4. Selection of stirring rate

The effect of stirring rate in the range of 0–1000 rpm was evaluated. As seen from Fig. 5, extraction efficiency of the three analytes improved as the agitation rate increasing from 0 to 800 rpm.

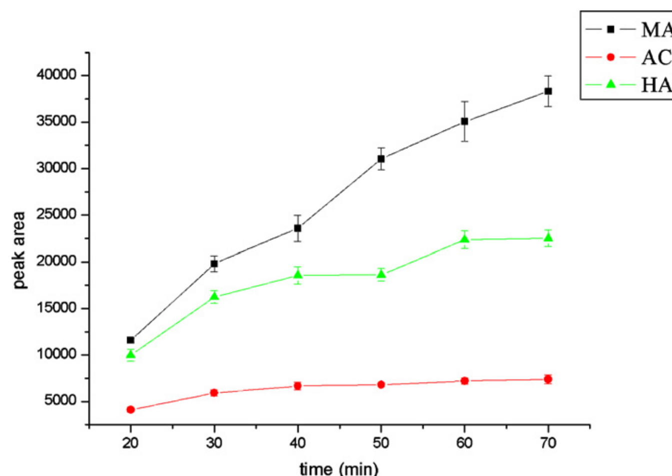


Fig. 4. Effect of extraction time on the peak area of AC, HA and MA extracted with LPME. Extraction condition: organic solvent: 1-octanol; donor phase pH: 11; acceptor phase: 10.0 mmol/L HCl; stirring rate: 800 rpm.

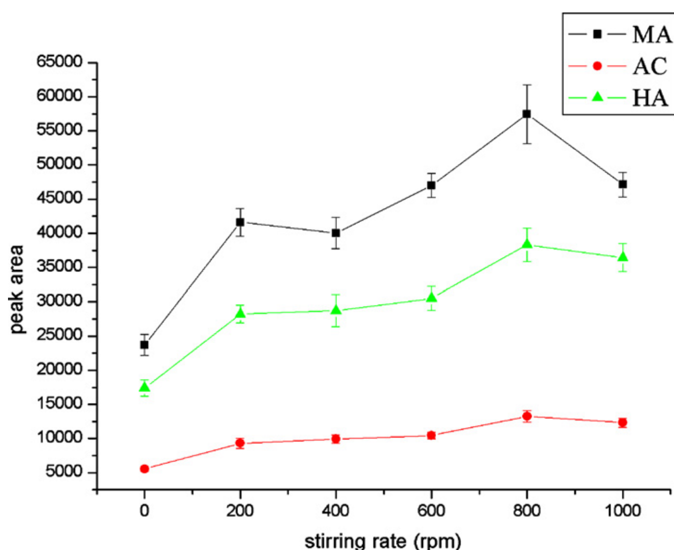


Fig. 5. Effect of stirring rate on the peak area of AC, HA and MA extracted with LPME. Extraction condition: organic solvent: 1-octanol; donor phase pH: 11; acceptor phase: 10.0 mmol/L HCl; extraction time: 60 min.

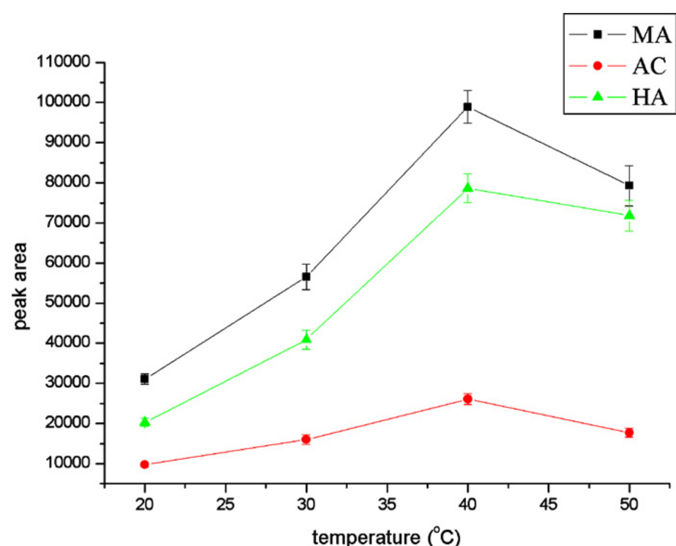


Fig. 6. Effect of temperature on the peak area of AC, HA and MA extracted with LPME. Extraction condition: organic solvent: 1-octanol; donor phase pH: 11; acceptor phase: 10.0 mmol/L HCl; extraction time: 60 min; stirring rate: 800 rpm.

However, when it exceeded 800 rpm, instead of increasing, peak area decreased. This phenomenon can be accounted for the fact that although agitation could accelerate distribution equilibrium by facilitating mass transfer, overhigh stirring speed would generate air bubbles adhering on the surface of hollow fiber and promote solvent loss [26]. Therefore, 800 rpm of stirring speed was selected.

3.1.5. *Selection of extraction temperature*

Increasing extraction temperature can be expected to increase enrichment factor owing to an increase in the analytes diffusion coefficient across the hollow fiber membrane. But overhigh temperature would result in the evaporation loss of organic solvent and the formation of air bubbles in the hollow fiber. Fig. 6 illustrates the effect of extraction temperature ranging from 20 °C to 50 °C on extraction efficiency. The peak areas of the analytes increased with increasing temperature, and reached maximum at 40 °C, and then decreased when temperature exceeded 40 °C. Therefore, 40 °C was used for the microextraction.

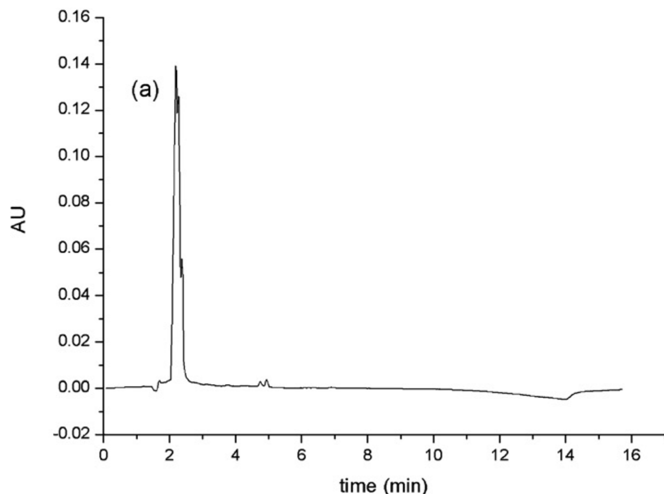
3.1.6. *Selection of the volume of acceptor phase*

The influence of acceptor volume was studied from 6 μL to 18 μL, with a constant donor volume of 5 mL. Enrichment factors of the three analytes increased with increasing acceptor volume from 6 μL to 9 μL, reached maximum at 9 μL, but dropped a little when acceptor volume was 12 μL, and then continually dropped with the increasing of acceptor volume. On the other hand, recoveries of the three analytes increased from 6 μL to 12 μL, reached maximum at 12 μL, and subsequently dropped largely. On the basis of the above observations, volume 12 μL was selected since it provided acceptable results for both recovery and enrichment factor.

3.2. *Method evaluation*

3.2.1. *Enrichment factor, linearity and limit of detection*

Under the optimized conditions, the three analytes were extracted with satisfactory enrichment factors of 98-, 172- and 288-fold, respectively. The enrichment factor (EF) is calculated according to the following equation $EF = C_{org}/C_o$, where C_{org} is the concentration of analyte extracted in the organic solvent, and C_o is



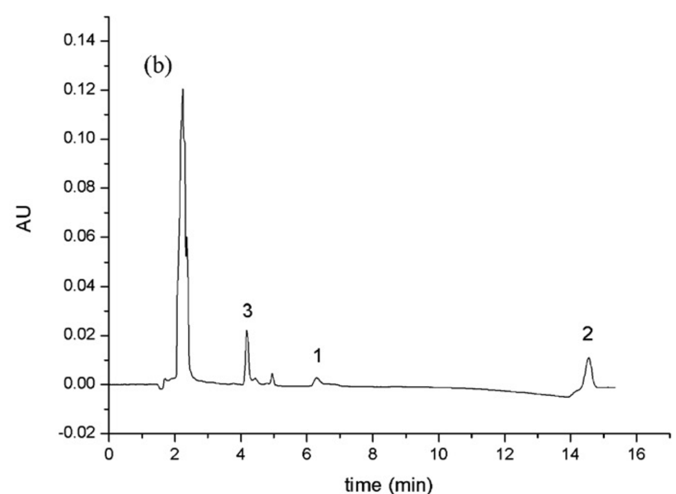


Fig. 7. Chromatograms of (a) human blank urine and (b) urine sample spiked with 64.0 μg/L of AC (1), 44.0 μg/L of HA (2) and 32.4 μg/L of MA (3) extracted by HF-LPME. Mobile phase: acetonitrile–0.03% triethylamine (85:15, v/v); flow rate: 1.0 mL/min; detection wavelength: 230 nm.

Table 1
Calibration graph data, LODs and LOQs for the analytes.

Analyte	Correlation equation	Correlation coefficient	Linear range (μg/L)	LOD (μg/L)	LOQ (μg/L)
AC	$y = 751x - 2010$	0.9949	16.0–128.0	0.8	2.6
HA	$y = 3545x + 2517$	0.9969	11.0–88.0	1.5	5.0
MA	$y = 4983x - 8737$	0.9904	8.1–64.8	0.7	2.3

the concentration of analyte originally presented in the sample.

Calibration curves were plotted using five spiked levels of AC in the concentration range of 16.0–128.0 μg/L, HA in the concentration range of 11.0–88.0 μg/L and MA in the concentration range of 8.1–64.8 μg/L. The samples were extracted with the established HF-LPME procedure, and analyzed by HPLC. Each extract was analyzed in triplicate. In the correlation equation, *y* represents the peak area of the analytes, and *x* represents the concentration of the analytes in the urine sample. These analytes exhibited good linearity squared regression coefficients from 0.9904 to 0.9969. Limits of detection (LODs) of these analytes studied in the urine sample, calculated on the ratio of signal to noise at 3 ($S/N = 3$), were in the range of 0.7–1.5 μg/L. Limits of quantification (LOQs, $S/N = 10$) were in the range of 2.3–5.0 μg/L. The performance of the LPME system in the terms of correlation equations, correlation coefficients, linear ranges, LODs and LOQs were calculated and summarized in Table 1.

3.2.2. Precision, repeatability and accuracy

The intra- and inter-day precision of the developed method was performed by replicate injection of a standard solution. Intra-assay precision was tested for five continuous injections during the same day, while inter-assay precision was tested on three consecutive days. The results are listed in Table 2.

The repeatability study was performed by extracting urine sample spiked at 64.0 μg/L of AC, 44.0 μg/L of HA and 32.4 μg/L of MA (five replicates) under optimal conditions, and the relative standard deviations were calculated to be from 0.99% to 7.12%.

The accuracy of the method was confirmed by spiked recovery test. 16.0 μg/L of AC, 11.0 μg/L of HA and 8.1 μg/L of MA was added in blank urine sample as original amounts, then three different quantities, i.e. 80% (low), 100% (medium) and 120% (high) of above concentrations of the analytes was spiked to the original sample, respectively. Then the three sets of spiked urine samples described above were extracted and analyzed. The relative recoveries and RSD of all three analytes in urine sample are listed in Table 3. As can be seen, the recoveries were in the range of 77.3–106.2%, and RSDs were from 5.6% to 11.7%.

3.3. Application in real sample

In order to evaluate the applicability of the newly developed method to real samples, the proposed procedure was applied to determine AC, HA and MA in real human urine sample. 1.0 mL of urine sample was diluted to a total volume of 10 mL and the pH was adjusted to 11 with 1.0 mmol/L NaOH. Then 5.0 mL of the above sample solution was added to 10-mL sample vial and extracted under the optimum condition. Fig. 7 shows the HPLC chromatogram of blank urine and urine sample containing 64.0 μg/L of AC, 44.0 μg/L of HA and 32.4 μg/L of MA after HF-LPME. It is obvious that no significant interferential peaks were detected, and so this method is applicable to analyze real human urine sample.

Table 2
The precision of the developed method.

Analyte	Intra-assay RSD (%, $n = 5$)		Inter-assay RSD (%, $n = 3$)	
	Peak area	Retention time	Peak area	Retention time
AC	1.49	0.62	2.49	1.93
HA	1.37	1.51	0.57	2.88
MA	1.00	0.24	2.92	0.59

Table 3
Spiked recoveries of the analyte in the urine sample ($n = 3$).

Analyte	Original amount (μg/L)	Added amount (μg/L)	Found amount (μg/L)	Recovery (%)	RSD (%)
AC	16.0	12.8	29.6	106.2	11.7
		16.0	30.8	92.5	
		19.2	32.2	84.4	
HA	11.0	8.8	17.9	78.4	5.6
		11.0	19.5	77.3	
		13.2	22.3	85.6	
MA	8.1	6.48	14.4	97.2	5.7
		8.1	15.4	90.1	
		9.72	17.8	100.8	

4. Conclusion

In the present work, a three-phase LPME in combination with HPLC was developed for the extraction of trace level amounts of AC, HA and MA in human urine sample. High enrichment factors and effective sample clean-up were obtained, so were good linearity and recovery. This method proved to be a simple, fast, inexpensive and sensitive analytical procedure for the determination of these three toxic *Aconitum* alkaloids. Overall, the advantages of the method allow its potential application for basic drugs analysis at low levels from biological matrix.

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