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고성능 액체 크로마토그래피의 중공섬유 액상 미세추출에 의한 인간뇨의 Matrine Alkaloids의 분석

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Analysis of Matrine Alkaloids in Human Urine by Hollow Fiber Liquid-phase Microextraction with High-performance Liquid Chromatography

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요약. 인간뇨의 마트린 알칼로이드의 결정을 위한 민감한 정량적 방법은 HPLC에 결합된 중공섬유 액상 미세추출을 기반으로 개발되었다. pH를 포함하여 HF-LPME 효율의 다양한 인자의 영향과 공급 용액의 이온세기, 수용체용액의 pH, 교반율 그리고 추출시간이 조사되었다. 최상의 HF-LPME 조건은 다음과 같다: 1-octanol이 중공섬유의 기공안에 주입된다, 중공섬유의 루멘에 pH 1.50의 100 mmol/L of H_3PO_4 수용체 용액을 주입한다, 1 mol/L NaOH는 공급 용액의 pH, 600 rpm의 교반속도와 60분의 추출시간을 조정하여 사용된다. LPME의 방법은 실제의 소변 샘플에서 마트린과 소포카르핌의 분석에 성공적으로 적용되었다.

주제어: 중공섬유 액상 미세추출, 인간 뇨, Matrine Alkaloids

ABSTRACT. A sensitive quantitative method for the determination of matrine alkaloids in human urine was developed based on hollow fiber liquid-phase microextraction (HF-LPME) combined with HPLC. The influence of the different factors on the HF-LPME efficiency including the pH and ion strength of the donar solution, the pH of the acceptor solution, stirring rate and extraction time were examined. The best HF-LPME conditions were as follows: 1-octanol impregnated in the pores of the hollow fiber, 100 mmol/L of H_3PO_4 at pH 1.50 as the acceptor solution injected into the lumen of the hollow fiber, 1 mol/L NaOH used to adjust the pH of the donor solution, stirring rate of 600 rpm and extraction time of 60 min. The LPME method was applied successfully to the analysis of matrine and sophocarpine in real urine samples.

Keywords: Hollow fiber liquid phase microextraction, Human urine, Matrine alkaloids

INTRODUCTION

Matrine and sophocarpine are two common alkaloids exist in the roots of *Sophora flavescens Ait* (SFA), which is commonly used as an important ingredient in traditional Chinese herbal medicines to treat viral hepatitis, cancer, cardiac diseases (such as viral myocarditis) and skin diseases (such as psoriasis and eczema).[1] Some herbal medicines must be used with caution because they may be toxic to human beings. There are some reports about those excessive doses of matrine alkaloids can have adverse effects.[2-4] Therefore, it is necessary to determine and control the contents in biological samples, such as plasma and urine.

Several methods have been developed for the determination of matrine or sophocarpine including high-performance capillary electrophoresis (HPCE),[5] high-performance liquid chromatography (HPLC),[6] gas chromatography-mass spectrometry (GC-MS)[7] and liquid chromatography-mass spectrometry (LC-MS).[8] But most of them utilize plasma protein precipitation to delete the matrix disturbance, which have low accuracy and are difficult to operate. Sample extraction that cleans-up the sample matrix and concentrates trace amounts of the analyte are essential aspects of the analysis of biological sample. Traditional methods, such as liquid-liquid extraction (LLE)[9] and solid phase extraction (SPE),[10] are tedious, time-consuming and consume large amounts of solvent. Recently, new microextraction techniques such as solid phase microextraction (SPME),[11,12] and liquid phase microextraction (LPME)[13] have been developed

because they are simple, fast and solvent-free or with less consumption of organic solvent. Among these techniques, liquid phase microextraction using a hollow fiber (HF-LPME)[14] provides a simple, effective, economic and environmentally benign extraction method. In addition, HF-LPME can also show some selectivity. Large molecules, such protein molecules cannot be extracted into the hollow fiber because the limitation of the pore of the fiber. HF-LPME includes two modes. In two-phase LPME, the analytes are extracted from an aqueous sample matrix into an organic solvent acceptor phase. In three-phase LPME, the unionized analytes are first extracted from the aqueous sample, entering into a thin layer of an organic phase inside the wall pores of the hollow fiber, and are then further extracted into the lumen of the hollow fiber, which holds a small volume of an aqueous-based acceptor phase. HF-LPME methods have been applied widely in many fields, such as pesticides,[15,16] tetracycline antibiotics,[17] diuretics[18] and other analytes.[19-21]

In this study, a HF-LPME method was used to extract matrine alkaloids from urine samples for the first time. The optimized parameters affecting the HF-LPME extraction efficiency including the pH and ionic strength of the donor solution, the pH of acceptor solution, stirring rate and extraction time were evaluated. The detection limits, linear ranges and precision of the technique as well as the feasibility of applying this method to human urine were studied.

EXPERIMENTAL

Reagents, Materials and Solutions

Matrine (purity, 99.8%) and sophocarpine (purity, 99.8%) were obtained from the National Institute for the Control of Pharmaceuticals and Biological Products of China, Beijing, China, and used without further purification. Acetonitrile (HPLC grade), and diethylamine (99.5%) were obtained from Duksan Pure Chemical Co., LTD (Ansan, Korea). All the other reagents used in the experiment were HPLC or analytical grade. Double distilled water was filtered with a vacuum pump (Division of Millipore, Waters, U.S.A.) and filter (HA-0.45, Division of Millipore, Waters, U.S.A.) before use. All the samples were filtered by using a filter (MFS-25, 0.2 μm TF, WHATMAN, U.S.A.) before injection into the HPLC system.

Stock solutions of matrine and sophocarpine were prepared by dissolving 10 mg of standards in 10 mL methanol. Blank human urine (pH 6.0) from healthy volunteers was stored at -20 ^{o}C and kept at 4 ^{o}C before use.

Chromatography

Chromatography was performed with a Waters 600 s multisolvent delivery system, a Waters 616 liquid chromatograph, and a Waters 2487 variable wavelength, dual-channel, UV detector (Waters Associates, Milford, MA, USA). A six-port Rheodyne injector (20-μL sample loop) was also used. Data processing was performed with Millennium 3.2 software resident in an HP Vectra 500PC. Compounds were separated on a 250 mm × 4.6 mm, 5-μm particle, OptimaPak C_{18} column (RS Tech, Daejeon, Korea). HPLC separation of matrine alkaloids was conducted by using methanol/water/diethylamine (40/60/0.10, v/v/v) as mobile phase at a flow rate of 0.5 mL/min and the detection was carried out at a wavelength of 220 nm.

Distilled water was filtered with a vacuum pump and filter (HA-0.45 μm; Millipore, Waters, USA) before use. All samples were filtered (Whatman, USA; MFS-25, 0.2 μm TF filter) before injection for HPLC analysis.

Extraction Procedure

All the extractions were carried out using a polypropylene hollow fiber (Wuppertal, Germany) with a 0.2 μm pore size, 600 μm internal diameter and 200 μm wall thickness. The hollow fibers were cut into 7.0 cm long pieces. 2 mL of a 1 mol/L NaOH solution was palced into a 4 mL vial. Subsequently, 200 μL of a 0.05 mg/mL matrine and sophocarpine mixture was added. Finally, 1800 μL human urine was added to make a total volume of 4.0 mL. The hollow fiber was dipped in 1-octanol for 5 s to ensure that the pores of the hollow fiber had been impregnated with the extracting solvent. After solvent impregnation, the fiber was ultrasonificated for 10 s in a water bath to remove the excess of the solvent. Subsequently, 20 μL of a 100 mmol/L H_3PO_4 solution (acceptor solution) was injected into the lumen of the hollow fiber with a micro syringe. The impregnated and filled fiber was then placed in the sample vial as shown in *Fig.* 1 for immediate extraction. Both open ends of the medical syringe needles were connected to the syringe bodies in order to prevent solvent volatilization during extraction. During the extraction, the sample solution was stirred continuously at room temperature with a magnetic stirrer

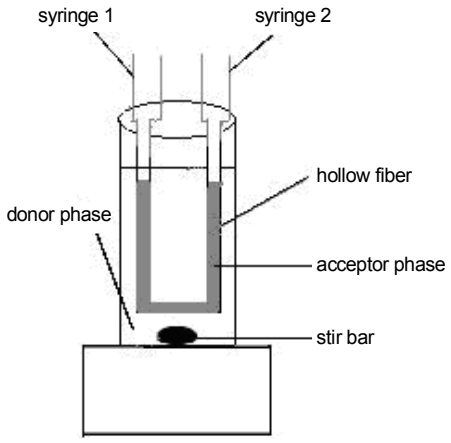

Fig. 1. Schematic illustration of HF-LPME.

at 600 rpm for 60 min. After extraction, one end of the fiber was detached from the medical syringe needle and 10 μL of the extraction solvent was analyzed directly by HPLC. Each segment of the hollow fiber was used only for a single extraction in order to delete the memory effect.

RESULTS AND DISCUSSION

The extraction recovery (R) and the concentration enrichment factor (E) were calculated using equations (1) and (2):[22]

$$R = \frac{n_{a,final}}{n_{s,initial}} \times 100\% = \frac{V_a}{V_s} \times \frac{C_{a,final}}{C_{s,initial}} \times 100\% \quad (1)$$

$$E = \frac{C_{a,final}}{C_{s,initial}} \quad (2)$$

In the equations, $n_{s,initial}$ and $n_{a,final}$ denote the number of moles of analyte present originally in the donor phase and finally collected in the acceptor phase, respectively. $C_{s,initial}$ and $C_{a,final}$ are the initial concentration of the analyte in the donor phase and the final concentration of the analyte in the acceptor phase, respectively. V_a is the volume of the acceptor phase, and V_s is the volume of undiluted sample.

The factors influencing HF-LPME extraction efficiency including different organic extraction solvent, the pH and ion strength of the donor solution, the pH of the acceptor solution, stirring rate and extraction time, were optimized to obtain the highest enrichment efficiency and high analytical sensitivity.

Selection of Organic Solvent

During the HF-LPME process, the type of organic extraction solvent immobilized in the porous hollow fiber is an essential consideration for efficient extraction. The organic solvent greatly affects the partition coefficients between the donor sample and the organic solvent, as well as between the organic solvent and acceptor phase. In three-phase HF-LPME, the ideal organic solvent should be easily immobilized on the hollow fiber, and be immiscible with the aqueous phase with low volatility. In this study, four different organic solvents, 1-octanol, toluene, cyclohexane and chloroform were examined. As shown in *Table* 1, the highest enrichment factor of matrine and sophocarpine was achieved by 1-octanol. Therefore, 1-octanol was selected as the extraction solvent for further optimization.

Table 1. Selection of organic phase

Compounds	Enrichment factor			
	Toluene	Cyclohexane	1-octanol	Chloroform
Matrine	14	27	93	51
Sophocarpine	10	40	123	34

Effect of pH of Donor Phase and Acceptor Phase

The acidity and basicity of the donor phase and acceptor phases can highly influence the extraction efficiency. The pKa of matrine and sophocarpine was 7.7 and 7.2, respectively. Hence, they belong to basic drugs. For basic drugs, the donor phase should be strongly alkalized in order to effectively deionize the analytes and reduce their solubility in the donor phase, while the acceptor phase should be acidified in order to promote dissolution of the basic analytes. Different concentrations of NaOH were added to the sample solution to adjust the pH of donor phase. As a result, the best extraction efficiency was observed at pH 13.7 (0.5 mol/L of NaOH).

100 mmol/L of H_3PO_4 as the acceptor solution at seven different pH (1.2, 2.0, 3.0, 4.0, 5.5, 6.0 and 7.0) was examined to optimize the acceptor pH. As shown in the *Fig*. 2, the enrichment factor of the matrine alkaloids decreased with increaseing pH of the acceptor solution. Therefore, a pH of 1.5 was selected as the acceptor solution.

Effect of Stirring Rate

Suitable stirring of the sample solution enhances extraction and reduces the time needed to reach the thermodynamic equilibrium. The effects of the stirring rate on the enrichment factor at stirring rates of 300 - 700 rpm for the two analytes was evaluated. *Fig*. 3 showed that the optimum stirring rate was 600 rpm. If the stirring rate is too fast, it can deteriorate the stability of the extraction droplets, increase the solubility of the organic solvent in the donor phase and reduce the extraction efficiency.

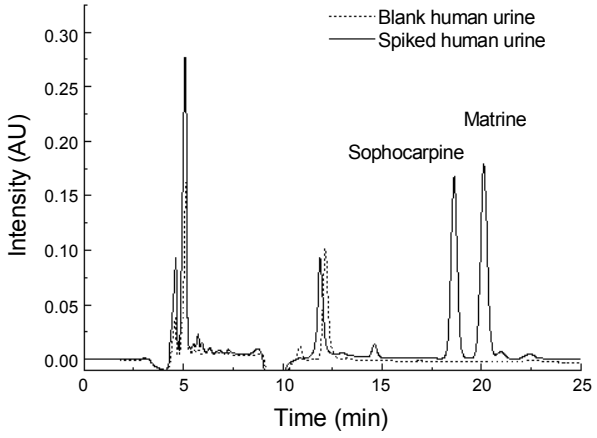

Fig. 2. Chromatograms of blank human urine and human urine spiked with 2.5 μg/mL matrine and sophocarpine extracted by HF-LPME. HF-LPME condition: donor solution: (2 mL human urine containing 5 μg/mL matrine and sophcarpine, then added 2 mL of 1 mol/L NaOH); Acceptor sample : 100 mmol/L H_3PO_4, stirring rate: 600 rpm and extraction time: 60 min.

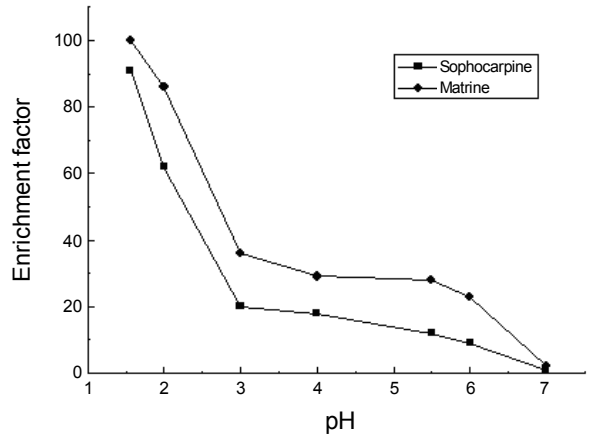

Fig. 3. Effect of the donor solution pH on extraction efficiency.

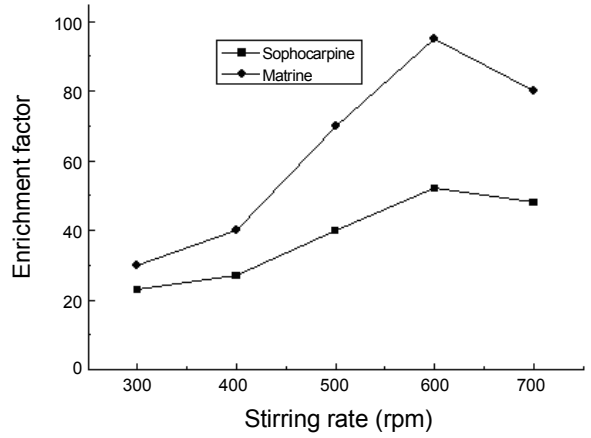

Fig. 4. Effect of stirring rate on extraction efficiency.

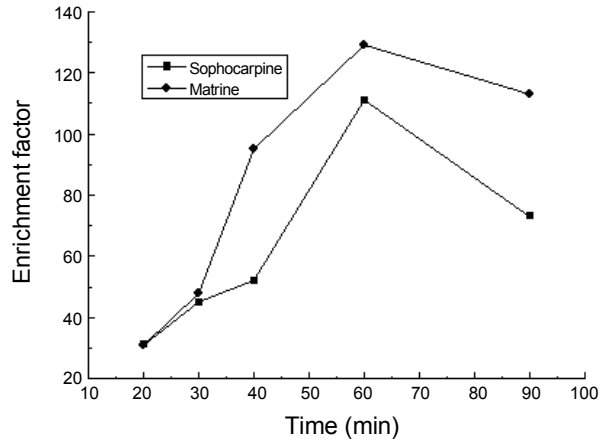

Fig. 5. Effect of extraction time on extraction efficiency.

Effect of Extraction Time

In the three-phase HF-LPME, obtaining a balance between the donor phase, organic phase and acceptor phase is a time dependent process. The effects of the extraction time on the extraction efficiency were studied from 20 to 90 min. The results in *Fig*. 4 suggest that the enrichment factor of two compounds increased with increasing the extraction time, but decreased when the extraction time exceeded 60 min. Therefore, 60 min was selected as the optimum extraction time.

Effect of Ionic Strength

The addition of salt improves the ionic strength of the donor phase and reduces the affinity of the organic compounds in the aqueous phase, which would improve the extraction efficiency of organic analytes in many conventional extraction techniques. NaCl is commonly added to analytical samples.

In this study, the ionic strength of the sample solution was optimized by spiking the donor phase with a series of NaCl concentrations ranging from 0 to 0.20 g/mL. The results showed that NaCl had almost no salting-out effect on the extraction efficiency. Therefore, this HPME system was conducted without the addition of salt.

Linearity, Reproducibility and Limits of Detection

A calibration curve of spiked urine samples was prepared by adding different volume stock solution to a 4 mL, followed by the addition of 2 mL 1mol/L NaOH and finally human urine to a total volume of 4.0 mL. The samples were then extracted using the HF-LPME procedure established above. Each extract was directly analyzed by HPLC. *Fig*. 5 shows the chromatograms of the blank human urine and spiked human urine extracted by HF-LPME. The linear ranges, regression equations and detection limits of matrine and sophocarpine are demonstrated in *Table* 2. Here *Y* and *X* represents the peak area of the analytes and the concentration of the analytes in the urine sample (μg/mL), respectively. The detection limits (S/N = 3:1) for matrine and sophocarpine were 0.025 and 0.042 μg/mL, respectively. Compared with SPE,[23] HF-LPME had lower detection limits and higher sensitivity. The proposed HF-HPME method provided very high enrichment factors (*Table* 3): 93-fold and 123-fold for matrine and sophocarpine, respectively, meaning that trace concentrations of matrine and sophocarpine which can not be detected by conventional extraction methods could be detected sufficiently after HF-LPME concentration.

Table 2. The regression equations, linear ranges, correlation coefficients and detection limits in human urine

Compounds	Regression equation	Linear range (μg/mL)	RSD (%) (n = 5)	r^2	Detection limit (μg/mL)
Matrine	$Y = 1.86 \times 10^6 X - 35054$	0.25 - 5.00	3.9	0.999	0.025
Sophocarpine	$Y = 1.64 \times 10^6 X + 479170$	0.25 - 5.00	5.1	0.997	0.042

Table 3. Extraction enrichment and recoveries of matrine and sophocarpine in human urine

Compounds	Enrichment (-fold)	Recovery (%)	RSD (%) (n = 5)
Matrine	93	77.8	5.7
Sophocarpine	123	61.5	6.4

CONCLUSIONS

The developed HF-LPME offers a simple and sensitive approach for the determination of matrine alkaloids in a biological matrix, and can lead to a high sample pre-concentration as well as an efficient sample cleanup effect. The LPME-sweeping method was applied successfully to the analysis of matrine and sophocarpine in real urine samples with high enrichment factors 93-fold and 123-fold, respectively. The results showed that HF-LPME is a promising combination for the analysis of basic drugs present at low levels in biological samples.

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