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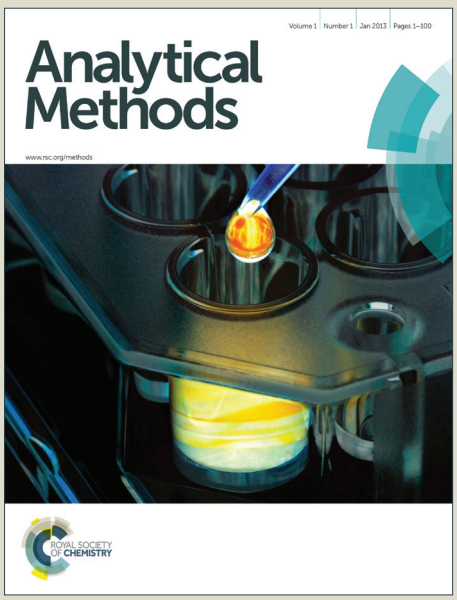

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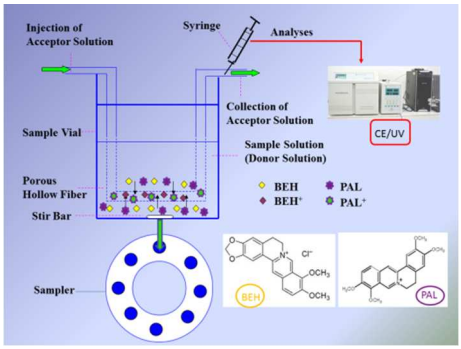

A new assay for determination of alkaloids using capillary electrophoresis combined with polypropylene hollow fiber liquid-liquid-liquid microextraction.

Analytical Methods

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ARTICLE

Simultaneous determination of berberine and palmatine in human plasma and in urine by capillary electrophoresis combined with polypropylene hollow fiber liquid-liquid-liquid microextraction

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A sensitive and precise method for determination of berberine hydrochloride (BEH) and palmatine (PAL) in human plasma and in urine simultaneously was developed using capillary electrophoresis (CE) combined with polypropylene hollow fiber liquid-liquid-liquid microextraction (PHF-LLLME). Because of the reports of human poisoning as a consequence of unregulated use of some drugs containing alkaloids, development of a rapid and precise assay for alkaloid measurement in the clinical setting is desirable. We report herein the development of a sensitive and precise assay for the measurement of alkaloids in the settng of a clinic. Ten participants (6 men and 4 women with a mean age of 26.3) who resided in a closed research unit participated in the detection of alkaloids. All of the volunteers were healthy. BEH and PAL were selected as model compounds to develop and evaluate the performance of the proposed method. Under the optimum conditions, the linear range was 20-1600 ng/mL for BEH, 50-1600 ng/mL for PAL. The limits of quantification for BEH, PAL were found to be 8.7 and 11.3 ng/mL. The average extraction recoveries in urine ranged from 87.3 to 102.4% and in plasma were 78.2 to 109.7%. The RSDs were less than 4.2 and 10.5, respectively. The proposed method greatly improves the extraction efficiency and simplifies the experimental procedure considerably, which can be used as an automatic experiment for quantitative determination of alkaloids in the clinical setting.

Introduction

Alkaloids represent a group of natural medicinal products, comprising berberine hydrochloride (BEH), palmatine (PAL), tetrahydropalmatine (THP) coptisine (COP) and others, which have been separated from Rhizoma corydalis.[1] The alkaloids display broad-spectrum antibacterial activity against a variety of organisms including bacteria, fungi, viruses, helminthes and Chlamydia, which is widely used in the clinical application.[1,2] Their antibacterial activity is based on a selective inhibition of bacterial DNA replication.[3-5] Additionally, studies also indicated their anticancer activities.[6,7] However, excessive consumption of alkaloids may lead to adverse effects, such as astriction, tetter, as well as antibiotic resistance.[2,8] Therefore, it is crucial to find an applicable method to determine the alkaloids in biological fluids. Nowadays, high performance liquid chromatography (HPLC),[2,9] liquid chromatography-mass spectrometry (LC-MS),[10-12] ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)[13] and CE[14,15] have been used to determine the amount of alkaloids. However, these methods suffer from drawbacks, such as consumption of a great deal of time up to several hours, cost, and underproduction and labor intensiveness. Hence, a more rapid and effective method needs to be developed.

Capillary electrophoresis (CE) has become an important separation technique for both ionic and neutral compounds due to high separation efficiency, low consumption of analytes and short separation time (usually requiring 30 minutes).[16] CE has been employed in the analysis of medicine, proteins, DNA, chemicals and many other applications.[16-21] Generally, most of CE is combined with a UV detector on the narrow fused-silica capillaries, which is used for sample separation. Because only a tiny sample volume is injected in traditional CE, high detection limits and poor concentration sensitivity are the shortcomings of CE due to the limited volume.[22] It is difficult to use CE for trace analysis applications, especially in biological and environmental samples.[23] To overcome these disadvantages, many on-line preconcentration techniques are developed before CE analysis, containing analyte stacking and sweeping, field amplification or other more sensitive

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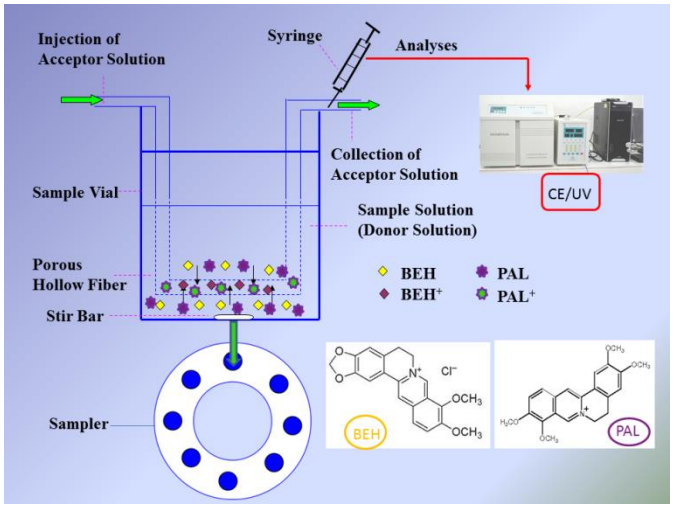

Fig.1 Schematic illustration of the extraction mechanism of proposed method using berberine hydrochloride (BEH) and Palmatine (PAL).

detectors such as mass spectrometry (MS)[24] and laser-induced fluorescence.[25,26]

Sample preparation and pretreatment are also very important steps in analytical assay, especially in chromatographic analysis. Thus far, a large number of traditional methods have been established to isolate, extract, and concentrate analytes from various samples, including liquid-liquid extraction (LLE)[24,27] and solid-phase extraction (SPE).[28] However, being time-consuming and labor-intensive are their common demerits. Additionally, a large amount of a toxic and environmental unfriendly organic solvent is required for LLE.[27] In view of the increasing awareness to protect the environment, many microextraction techniques which are environmental friendly have been proposed and developed. A widely used method, solid phase microextraction (SPME) which was solvent-free, was proposed by Mitani and Kataoka.[29] However, this technique requires expensive and fragile fibers, leading to problems of reproducibility.[30]

In our study, polypropylene hollow fiber liquid-liquid-liquid microextraction (PHF-LLLME) was modified and tested as a novel preconcentration methodology for CE analysis, and a series of parameters influencing the extraction recovery were studied systematically. An illustration of the extraction mechanism of the proposed method using BEH is shown in Fig. 1. BEH and PAL were selected as the model substances to evaluate the proposed method. This assay was successfully applied to determine the BEH and PAL in human urine and plasma, suggesting that it could be a useful method for the determination of drugs in clinics.

Materials and Methods

Chemicals and reagents

Berberine hydrochloride (BEH) and palmatine (PAL) were purchased from Changchun Dirui Pharmaceutical Co. Ltd. (Changchun, China). Quinoline (internal standard, IS) was obtained from the Control of Pharmaceutical and Biological Products (Beijing, China). The stock solutions of BEH, PAL and IS standard solutions were prepared by dissolving the commercial products in methanol. Working solution was prepared by spiking BEH, PAL and IS standard solution to NaOH solution to adjust to the optimum pH. Hydrochloric acid, sodium hydroxide, sodium tetraborate, n-octanol, iso-octanol, isooctane, butyl acetate, hexyl ether and toluene of analytical grade were all purchased from Sigma-Aldarich (China). Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, phosphoric acid, boric acid, acetic acid, and methanol were all purchased from Guangzhou Chemical Regent Plant (Guangzhou, China).

All chemicals and solvents used throughout the experiments without other illustration were analytical grade or the best commercially available grade. Ultrapure water obtained by a Milli-Q water purification system (18.2 MΩ cm, Millipore, Bedford, MA, USA) was used throughout the whole experiment.

Instrumentation

A CL1030 capillary electrophoresis system (Cailu, Beijing, China) coupled with a UV detector was employed to determine the samples. Throughout the experiment, fused silica separation capillary of 48 cm (40 cm to the detector) × 50 μm i.d. × 360 μm O.D. (Yongnian Optical Fiber, Hebei, China) with 0.3 cm of detection window was used at 25 ℃.

All pH measurements were made with a DELTA 320-S acidity meter (Mettler-Toledo Instruments Co. Ltd., Shanghai, China) equipped with a combined glass-calomel electrode. Analysis of the experimental design data and calculation of predicted responses were carried out, using HW-2000 Chromatography Workstation (Qianpu, Shanhai, China).

Sample collection and pretreatment

Blank urine sample and blood samples were provided by 6 healthy male and 4 female volunteers (with a mean age of 26.3) who had not taken any kind of medication three days before the day of the test which was completed in Shantou University, China. Volunteers were recruited by Shantou University during the period February 2011 - August 2011. All of participants who were recruited for the study provided informed consent. The collection of clinical samples was approved by the Ethics Committee of Shantou University.

About 5 ml fasting blood sample per volunteer was collected into purple top EDTA tubes between 7:00 and 9:00 am. The blood was centrifuged (2000 rpm) at 4 ℃ for 20 min. After centrifugation 1.0 ml of plasma was placed into 1.5 ml ependorf tube by clean pipette technique, then plasma was labeled with tracking number and stored plasma at -80 ℃ freezer for further study.

The plasma were deproteinized by adding 1 mL acetonitrile followed by centrifugation (10 min at 10000 rpm) at room temperature. Then acetonitrile was removed under reduced pressure on a rotary evaporator in a 55 ℃ water bath. The human serum samples without proteins were stored at 4 ℃ for further use. The urine samples were collected and stored under the same conditions of plasma.

Before testing, human plasma and urine samples were put into the centrifuge tubes and centrifuged for 4 min at 10000 rpm, then filtered using 0.45 μm polyether sulfone filters (PES4547100, STERLITECH). Finally, the pH of the samples was adjusted to provide the optimum pH as donor solutions for PHF-LLLME.

Capillary electrophoresis conditions

Before sample measurement, the capillary was rinsed consecutively with 0.2 M NaOH for 20 min, deionized water for 15 min and running buffer (12 mM $Na_2B_4O_7$-35 mM NaH_2PO_4) with a pH value of 7.5 for 15 min. In addition, the running buffer was used to flush the capillary for 3 min between runs. All solutions were filtered with 0.25 μm filters before analysis. The instrument was operated at 15 kV. Samples were introduced by hydrodynamic injection at 50 mbar for 5s.

Results and Discussion

Extraction procedure

The experimental setup for the PHF-LLLME was a U-shaped configuration, as shown in Figure 1. A 3.0 mL sample solution was introduced into a 5 mL amber vial which was placed on a heating-magnetic stirrer with a magnet placed in the solution to ensure efficient stirring during the extraction. An 8 cm piece of Q3/2 Accurel KM polypropylene hollow fiber (Wupertal, Germany) was used to separate the donor phase and acceptor phase. The inner diameter of the hollow fiber was 600 μm, the thickness of the wall was 200 μm, and the pore was 0.2 μm. The hollow fiber, fixed on the end needles of two microliter syringes: one is used for injection of acceptor solution, and the other is used to collect the acceptor solution. The extraction procedure is as follows: A suitable amount of typically n-octanol was firstly injected into polypropylene hollow fiber to fill the pores. After that, n-octanol (Organic layer) was injected into the polypropylene hollow fiber for impregnation and filling the pores of the hollow fiber due to the high viscosity and good stability. Because of the existing of organic layer, there were three phase including donor, organic and acceptor phase in the PHF-LLLME extraction system. Donor solution was in the sample vial. Organic phase was in the inwall and pores of polypropylene hollow fiber. And acceptor phase was in the hollow fiber. The extraction system was three phases liquid-liquid-liquid microextraction. n-octanol separated the donor phase and acceptor phase and allowed the two target alkaloids infiltrated into the acceptor phase. Secondly, 20 μL of acceptor (0.1 M HCl) as the acceptor phase was injected into the hollow fiber using a microliter syringe. Then, the fiber was placed into the donor phase which was the sample solution in the sample vial. The sample solution was stirred at 600 rpm for 10 min using a constant temperature magnetic stirrer. After extraction, the acceptor solution was collected by the other microliter syringe, and transferred into a microvial for the CE analysis.

Principle

The selection of organic solvent and acceptor phase should be immiscible solvents, one immobilized in the pores of the hollow fiber and the other filled in the lumen of fiber. It is critical in three-phase PHF-LLLME. The organic membrane solvent serves to separate sample solution (donor phase) and acceptor phase. It should be compatible with the fiber so as to fill the pores of the fiber wall and to create a suitable medium for extraction. It also must be immiscible with water and acceptor phase and stable enough over the extraction time.

In this system, the target analytes were extracted from the aqueous sample into the organic membrane based on diffusion, in which extraction was promoted by high partition coefficients, and then, easily back extracted into acceptor phase by concentration gradient between both of immiscible solvents. On the other hand, the acceptor phase (0.1 M HCl) had low solubility in n-octanol and effectively remained in lumen of the fiber during the extraction period without leakage to organic membrane and solvent loss due to evaporation. It is known that the existence form of certain analytes will change with the change of solution pH and thereby affect their water solubility and extractability. For basic drugs, the aqueous sample phases were commonly strongly alkalized to keep the analyte in its neutral form and consequently reduce their solubility within the samples. Therefore, the non-ionized BEH and PAL were existed in sample solution. When BEH and PAL exchanged into the acceptor solution, they expressed in charged ions in fiber. The extraction procedure is illustrated in Fig.1.

Extraction parameters

Preconcentration factor (PF) is the most critical parameter in evaluating the extraction, which can be calculated by the following equation:[31]

$$PF=C_1/C_0 \quad (1)$$

where, PF, $C_1$and C_0 are the preconcentration factor, concentration of the analytes in the aqueous back-extractive, and initial concentration of the analyte in the aqueous sample phase, respectively. C_1 was calculated from a calibration graph which was gained by direct injections of standard solution with concentration in the range of 2-50 μg/ml under the optimum electrophoresis conditions.

Optimization of PHF-LLLME

Before the analysis, preliminary studies were performed to investigate the interaction that variables effected on the analyte, such as sample pH, acceptor phase concentration, stirring rate, extraction time, temperature, as well as ionic strength. . In order to obtain the optimal extraction efficiency, orthogonal design has been used for studying the main effect of pH, stirring rate, back-extraction extraction time and temperature. Results were given on the optimized conditions.

Effect of solvent

The organic solvent plays a vital role in the sample separation in order to obtain a satisfactory selectivity and good extraction

Table 1. The effect of solvent on the preconcentration factor of analytes. Extraction conditions: water sample volume, 3.0 mL; organic solvent (n-octanol, p-octanone, acetone, hexylether); 10 µL extraction solvent (0.1 M HCl); concentration of analytes, 4 µg/mL; pH 11.8.

Solvent	Solubility in water (g/L)	Density(g/mL)	Viscosity (C.P.)	Preconcentration factor[a]	
				BEH	PAL
n-octanol	0.003	0.83	10.64	134	142
*Iso*octanol	0.077	0.82	8.2	117	120
Isooctane	0.001	0.86	7.7	102	113
Butyl acetate	0.83	0.88	0.63	90	75
Hexyl ether	0.79	1.24	1.68	-[b]	-[b]
Toluene	0.53	0.87	0.59	95	62

[a] Results varied within 20% RSD (n=5).

[b] No signal was observed for BEH and PAL.

efficiency. Generally, the organic solvent should have a lower density than that of water, with a low solubility in water and a high extraction capability for analytes. Because organic solvent with high viscosity can form a well-settled phase and low volatility organic solvent prevents loss during extraction process. Due to these requirements, several organic solvents (n-octanol, iso-octanol, isooctane, butyl acetate, hexyl ether and toluene) with different characteristics were studied in this assay (Table 1). A 20 µL aliquot of each of the organic solvents mentioned above was tested. Iso-octanol and isooctane had lower viscosity than n-octanol, they were not suitable solvent here due to the instability of the extraction system in long time. Butyl acetate and toluene, due to the low viscosity, the extraction time cannot be long because of dissolution and loss of solvent. Besides, butyl acetate and toluene were not stable of the aqueous. Hexyl ether which has high volatility and low viscosity is not suitable for back-extraction phase maintained for a long time. Therefore, n-octanol with high viscosity and satisfactory extraction efficiency was chosen for further studies.

Effect of pH of donor and acceptor phase

pH gradient is the driving force in the transport of BEH and PAL from the donor phase to the organic phase stable in the pores of hollow fiber. The pH of aqueous phase was important parameter which may affect the extraction efficiency in aqueous samples. It is known that the existence form of certain analytes will change with the change of solution pH and thereby affect their water solubility and extractability. For basic drugs, the aqueous sample phases were commonly strongly alkalized to keep the analyte in its neutral form and consequently reduce their solubility within the samples. Thus, the extractions of the alkaloids were performed in the aqueous sample with pH range of 8.8-12.8. The results showed that extraction efficiency was the highest at pH 11.8 (Fig. 2a). Thus, pH 11.8 was chosen for donor solution in the extraction experiment. In this study, NaOH solution was selected as the donor phase for its better dissolving of the alkaloids and compatibility of the running buffer. The effect of the concentration of NaOH was studied. 0.1 M NaOH

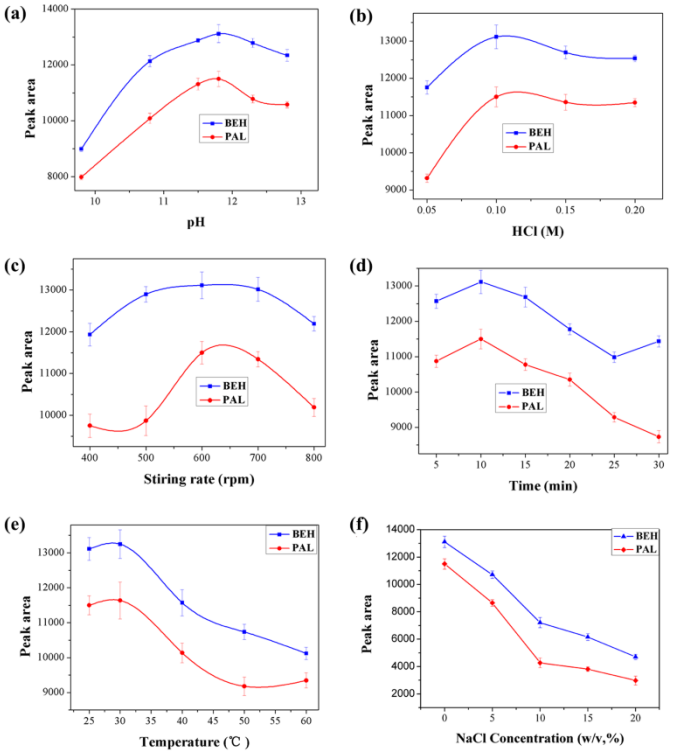

Fig. 2 Optimization of the experimental conditions. (a) Effect of the pH of aqueous sample phase on the extraction efficiency. Condition: 10 min at 600 rpm for back-extraction; 10 µL of 0.1 M HCl as acceptor solution: 25 ℃; (b) Effect of extraction solvent (HCl) concentration on the preconcentration factor of analytes. Conditions: 3.0 mL sample; 10 min at 600 rpm for back-extraction; 25 ℃; pH 11.8; (c) Effect of stirring rate in back-extraction on the extraction efficiency. Conditions: sample volume, 3.0 mL; 10 min for back-extraction; 25 µL of 0.1 M HCl as acceptor phase: 25 ℃; pH 11.8; (d) Effect of back-extraction time on the extraction efficiency. Conditions: 3.0 mL sample; 10 min at 600 rpm for back-extraction; 25 µL of 0.1 M HCl as acceptor phase: 25 ℃; pH 11.8; (e) Effect of temperature on the extraction efficiency. Conditions: 3.0 mL sample; 10 min at 600 rpm for back-extraction; 25 µL of 0.1 M HCl as acceptor phase; pH 11.8; (f) Effect of NaCl concentration on the extraction efficiency. Conditions: 3.0 mL sample; 10 min at 600 rpm for back-extraction; 25 µL of 0.1 M HCl as acceptor phase: 25 ℃; pH 11.8. Analytes concentrations: 4 µg/mLof berberine hydrochloride (BEH), 4 µg/ mLof Palmatine (PAL). Data are shown as mean ± SD (n=3).

was selected as the donor phase due to its excellent extraction efficiency for the analytes.

The effect of acceptor solution (HCl) concentration on the preconcentration factor of analytes is shown in Fig. 2b. It indicated that the samples with 0.1 M HCl had the highest extraction efficiency. Therefore, 0.1 M HCl was chosen for further analysis.

Effect of stirring rate

The effect of stirring rate on extraction was investigated in the range of 400-800 rpm (Fig. 2c). Generally, the higher the stirring rate, the better the extraction efficiency was. However, the acceptor phase

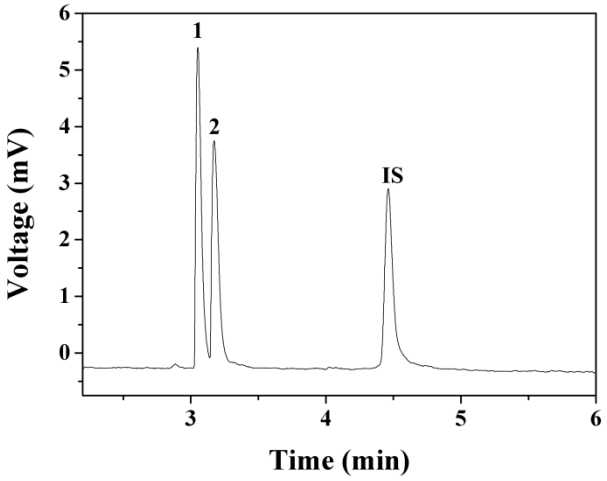

Fig. 3 Electropherogram of two alkaloids solution. CE conditions: running buffer (12 mM Na2B4O7-35mM NaH2PO4) with a pH value of 7.5; applied voltage, 15 kV; injection time 5s. Analytes concentrations: 1, 8 μg/mL of BEH; 2, 8 μg/mL of PAL; and 20 μg/mL of IS.

would drop when the stirring rates was over 600 rpm. Thus a stirring rate of 600 rpm was selected in the study.

Effect of extraction time

PHF-LLLME is a nonexhaustive extraction approach, and the extraction efficiency is governed by partitioning of the analyte between donor phase and the immobilized organic solvent and by partitioning between the acceptor solution and the immobilized organic solvent. Generally, when the extraction is at the parttitioning equilibrium, the maximum extraction efficiency would be attained. The extraction equilibrium of PHF-LLLME is time-dependent. The extraction time was also studied in the range of 5-30 min at 5 min intervals (Fig. 2d). It was found that the extraction efficiency increased with extraction time, until the equilibrium between acceptor phase and donor phase was achieved after extraction for 10 min. However, a longer extraction time would lead to poor extraction efficiency and reproducibility. The decrease for extraction efficiency might result from the acceptor phase dissolution and loss in liquid phase microextraction.[32] Therefore, 10 min was selected as the optimum extraction time.

Effect of sample temperature

Temperature of the aqueous sample phase was an important parameter that should also be considered, as the diffusion coefficient and partition coefficient of analytes between the acceptor solution and donor solution can be improved at higher temperatures. Fig. 2e shows the effect of temperature on the extraction efficiency in the range of 25-60 ℃. The peak areas were enhanced by increasing the temperature from 25 ℃ to 30 ℃, on the contrary, it decreased slowly when temperature exceeded 30 ℃. This might be due to higher temperatures resulting in increased solubility of analytes in the

Table 2 Quantitative results of PHF-LLLME combined with CE for berberine hydrochloride (BEH) and palmatine (PAL).

Analyte	Regression equation[a]	Linear range (ng/mL)	Correlation coefficient	R.S.D. (%)[b]	LOD[a] (ng/mL)[c]
BEH	Y=0.00159X+0.0161	20-1600	0.9972	5.7	8.7
PAL	Y=0.00396X+0.0139	50-1600	0.9998	8.1	11.3

[a]Calibration equation: the vertical coordinate of the standard curves showed the ratio of peak areas of analytes with IS, and the abscissa reflected the change of the concentration of analytes.
[b]RSD: relative standard deviation (n=3).
[c]LOD: limit of detection for S/N = 3.

aqueous sample, which reduced the extraction efficiency. Higher temperatures broke the stability of the aqueous back-extractive phase. The outside temperature usually stood at 25 ℃ and the peak areas did not show an obvious difference between 25 ℃ and 30 ℃. Therefore, 25 ℃ was selected as the optimum extraction temperature.

Influence of interfering substances

Due to the salting-out effect, salt was used to enhance the extraction of analytes in different liquid phase microextraction models. The effect of the ionic strength on the extraction efficiency of PHF-LLLME was investigated with NaCl at a concentration between 0 and 20% (w/v) in the sample solution (shown in Fig. 2f). The results demonstrated that the peak areas declined with the increase of NaCl concentration, which means that this method was affected by ionic strength and the relatively low ionic strength in the assay system should be maintained. The decrease of extraction efficiency may attributed to the addition of salt increased the viscosity of sample solution leading to the decline in the diffusion rate of analytes.[33] Therefore, low ionic strength is beneficial to this assay system. One more parameter that should be emphasized was adequately diluting the real samples, which could eliminate the effect of ionic strength in the assay system.

Optimization of CE conditions

The running buffer was an important factor for the separation of BEH and PAL. The effects of different buffers were tested included Britton-Robinson (BR) buffer solution and $Na_2B_4O_7$-NaH_2PO_4-H_3BO_3 buffer, and HCl and NaOH at different concentrations. The results showed that 12 mM $Na_2B_4O_7$ – 35 mM NaH_2PO_4 had better separation ability. The running buffer (12 mM $Na_2B_4O_7$-35 mM NaH_2PO_4) with a pH value of 7.5 was chosen as the optimal pH. Afterward, the effect of applied voltage on the CE in the range of 5-20 kV was investigated. Results indicated that 15 kV voltage had better separation ability. A electropherogram of 4 ng/mL of two alkaloids solution is shown in Fig. 3. Under the optimized CE conditions, the two target alkaloids could be well separated by CE.

Table 3 Comparison of PHF-LLLME with other methods for determination of Berberine and Palmatine.

Method	Berberine		Palmatine		Reference
	Linear range	LOD	Linear range	LOD	
NACE-UV	2.5-150 μg/mL	0.31μg/mL	2.5-150 μg/mL	0.34 μg/mL	[14]
HPCE-UV	4.986-498.6 μg/mL	-	5.058-505.8 μg/mL	-	[34]
HPLC-UV	0.907-18.13 ng/mL	13.12 ng/ mL	0.06-60 μg/mL	0.06μg/mL	[35],[36]
LC-MS/MS	0.2-100 ng/ mL	-	0.2-100 ng/ mL	-	[37]
PHF-LLLME	20.0-1600 ng/ mL	8.7 ng/mL	50.0-1600 ng/mL	11.3 ng/mL	This study

LOD: limit of detection

Analytical performance

Quantitative results of PHF-LLLME combined with CE for BEH and PAL was shown in Table 2. According to the procedure and using these conditions, we recorded ΔI peak area values for different concentrations. ΔI was proportional to concentration in the range of 20-1600 ng/mL for BEH, 50-1600 ng/mL for PAL. The regression equations were Y=0.00159X+0.0161 (BEH) and Y=0.00396X+0.0139 (PAL), respectively. A detection limit of BEH and PAL was calculated as the mean ± SD for measurement of a blank solution (n=20). The limits of detection (LOD) for BEH, PAL were found to be 8.7 and 11.3 ng/mL. Thus, we were able to use this method to determine a minute amount of sample.

Comparison with other methods

Table 3 shows the comparison of analytical performance of the present method with that obtained by other approaches for alkaloids analysis. From table 3, it can be seen that the proposed method has low LOD and wide linear range compared to those reported in Refs.[14,34-37] And this method can be used to determine low content alkaloids in human plasma and urine. Besides, the proposed method using a general instrument can be used to accomplish the pretreatment of samples, with high extraction efficiency and lower cost. Moreover, the alkaloids can be selectively extracted and enriched, without further diluting the urine or plasma samples that would diminish the analytical sensitivity of analytes. The only shortcoming of this method is that lower stirring rates would affect its performance, which should be further studied to overcome the uncertainty of the results.

Table 4 Determination of berberine hydrochloride (BEH) and palmatine (PAL) in human urine and human plasma.

Sample	Concentration of analytes (ng/mL)		Relative recovery (%)	Relative standard deviations (RSD) (n=3)
Urine	BEH	100	87.3	4.2
		200	95.6	3.9
	PAL	100	102.4	4.1
		200	95.5	2.8
Plasma	BEH	100	78.2	6.1
		200	109.7	10.5
	PAL	100	92.2	4.9
		200	99.1	3.5

[a]Samples were provided by 10 health volunteers from the same institute, China.

Application of real samples

To evaluate the practical applicability of the proposed PHF-LLLME technique, BEH and PAL were preconcentrated from both human urine and plasma samples. Under the optimum conditions, the average extraction recoveries in urine ranged from 87.3 to 102.4% and in plasma were 78.2 to 109.7%. The RSD were less than 4.2 and 10.5, respectively, as shown in Table 4. The results showed that this method had high enrichment ability, which was a useful assay for the biological samples. Also, the presented approach could be seen as an effective method to determine the blood drug concentration in clinic.

Conclusion

In conclusion, this proposed analytical method is simple, rapid, precise and reproducible with a low detection limit and a wide linear range. In addition, the method requires only general equipment, is easy to perform and provides results comparable to those of existing laboratory assays. Moreover, the assay in the present study is suitable for determining alkaloid concentrations in urine and serum samples. The test time required ranges between 15 minutes to 20 minutes. The rapid and quantitative test results obtained directly from urine and plasma samples indicate that it could be a successful application of the PHF-LLLME combined with CE and it allows separation and preconcentration of BEH and PAL at a low concentration level in clinical tests with the potential of low-cost and better clinical care. The proposed method can be expected to develop easily into an automated method, in view of the advantages of simple processes and common instruments as well as a low cost, which can also become feasible in a laboratory which is short of sophisticated facilities.

Acknowledgements

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References

1 L. L. Ren, X. Y. Xue, F. F. Zhang, Q. Xu, and X. M. Liang, *J. Sep. Sci.*, 2007, **30**, 833–842.
2. C. K. Lai and A. Y. W. Chan, *Clin. Chem*., 1999, **45**, 229–236.
3. C. Zongo, E. F. O. Akomo, A. Savadogo, L. C. Obame, J. Koudou, and A. S. Traore, *Asian J. Plant Sci*., 2009, **8**, 172–177.
4. L. Guittat, P. Alberti, F. Rosu, S. V. Miert, E. Thetiot, L. Pieters, V. Gabelica, E. D. Pauw, A. Ottaviani, J. F. Roiu, and J. L. Mergny, *Bioch*., 2003, **85**, 535–541.
5. J. N. Lisgarten, M. Coll, J. Portugal, C. Wright, and J. Aymami, *Nat. Struct. Mol. Biol*., 2002, **9**, 57–60.
6. K. V. Anis, G. Kuttan, and R. Kuttan, *Pharm. Pharmacol. Commun*., 1999, **5**, 697–700.
7. G. C. Jagetia and M. S. Baliga, *J. Med. Food*, 2004, **7**, 235–244.
8. Y. Shi, P. Y. Wang, and J. N. Li, *Chin. J. Microbiol*., 2009, **21**, 727–734.
9. P. H. Qiu, X. H. Chen, X. Chen, L. Lin, and C. C. Ai, *J. Chromatogr. B.*, 2008, **875**, 471–477.
10. P. Arbel áez, F. Borrull, R. M. Marc é, and E. Pocurull, *Talanta*, 2014, **125**, 65–71.
11. T. Lu, Y. Liang, J. Song, L. Xie, G. J. Wang, and X. D. Liu, *J. Pharm. Biomed. Anal*., 2006, **40**, 1218–1224.
12. H. D. Ma, Y. G. Wang, T. Guo, Z. G. He, X. Y. Chang, and X. H. Pu, *J. Pharm. Biomed. Anal*., 2009, **49**, 440–446.
13. J. Zhang, Y. Jin, J. Dong, Y. H. Xiao, J. T. Feng, X. Y. Xue, X. L. Zhang, and X. M. Liang, *Talanta*, 2009, **78**, 513–522.
14. W. H. Gao, S. Y. Lin, L. Jia, X. K. Guo, X. G. Chen, Z. D. Hu, *J. Sep. Sci.*, 2005, **28**, 92–97.
15. Q. Liu, Y. J. Liu, Y. Q. Li, and S. Z. Yao, *J. Sep. Sci*., 2006, **29**, 1268–1274.
16. W. H. Gao, G. P. Chen, Y. S. Chen, N. N. Li, T. Chen, and Z. Hu, *J. Chromatogr. A*, 2011, **1218**, 5712–5717.
17. C. Chartier, A. M. Boularan, A. M. Dupuy, S. Badiou, A. S. Bargnoux, C. Cognot, J. F. Eliaou, and J. P. Critol, *Clin. Biochem*., 2011, **44**, 1473–1479.
18. M. Kanoatov and S. N. Krylov, *Anal. Chem*., 2011, **83**, 8041–8045.
19. P. J. Li and B. Hu, *J. Chromatogr. A*., 2011, **1218**, 4779–4787.
20. C. C. Hung, C. P. Chen, S. P. Lin, S. C. Chien, C. N. Lee, W. F. Cheng, W. S. Hsieh, M. S. Liu, Y. N. Su, and W. L. Lin, *Clin. Chem*., 2006, **52**, 2203–2210.
21. C. M. Quich, A. Klipp, G. L. Woods, J. W. Theus, and J. A. Bornhorst, *Clin. Chem*., 2009, **55**, 594–596.
22. M. C. Breadmore, J. R. E. Thabano, M. Dawod, A. A. Kazarian, J. P. Quirino, and R. M. Guijt, *Electrophoresis*., 2009, **30**,230–248.
23. F. Pena-Pereira, I. Lavilla, and C. Bendicho, *Trends Anal. Chem*., 2010, **29**, 617–628.
24. H. Y. Huang C. L. Lin, and S. H. Jiang, *Anal. Chim. Acta*., 2012, **719**, 96–103.
25. S. Hustad, P. M. Ueland, and J. Schneede, *Clin. Chem*., 1999, **45**, 862–868.
26. J. G. Moller, H. Stass, R. Heinig, and G. Blaschke, *J. Chromatogr. B*., 1998, **716**, 325–334.
27. H. Prosen and L. Z. Kralj, *Trends Anal. Chem*., 1999, **18**, 272–282.
28. R. Shen, L. Guo, Z. Y. Zhang, Q. G. Meng, and J. W. Xie, *J. Chromatogr. A*., 2010, **1217**, 5635–5641.
29. K. Mitani and H. Kataoka, *Anal. Chim. Acta*, 2006, **562**, 16–22.
30. M. Palit, D. Pardasani, A. K. Gupta, and D. K. Dubey, *Anal. Chem*., 2005, **77**, 711–717.
31. A. S. Yazdi, Z. Es'haghi, *Talanta*, 2005, **66**, 664-669.
32. Y. C. Chen, H. F. Wu, *J. Sep. Sci*., 2009, **32**, 3013-3019.
33. Z. Lin, J. H. Zhang, H. M. Cui, L. Zhang, G. N. Chen, *J. Chromatogr. A*, 2010, **1217**, 4507-4510.
34. L. Xu, H. K. Lee, *J. Chromatogr. A*, 2009, **1216**, 5483-5488.
35. M. A. Farajzadeh, D. Djozan, and R. F. Bakhtiyari, *Talanta*., 2010, **81**, 1360–1367.
36. X. Ji, Y. Li, H. Liu, Y. Yan, and J. Li, *Pharm. Acta Helv*., 2000, **74**, 387–91.
37. Y Li and X Xu, *J. Chromatogr. B*., 2004, **810**, 165–168.
38. J. Zhao and W. Yan, *J. Pharm. Biomed. Anal*., 2005, **38**, 571–5.
39. G. Liu, W. He, H. Cai, X. Sun, W. Hou, M. Lin, Z. Xie and Q. Liao, *Anal. Methods*., 2014, **6**, 2998-3008.