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Ultrasound-assisted ionic liquid dispersive liquid–liquid microextraction coupled with high performance liquid chromatography for sensitive determination of trace celastrol in urine

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ABSTRACT

Ultrasound-assisted ionic liquid dispersive liquid–liquid microextraction (UA IL-DLLME) coupled with high-performance liquid chromatography (HPLC) has been developed for the determination of celastrol in human urine samples. In the microextraction procedure, ionic liquid (IL) was used as extraction solvent and dispersed into the aqueous sample solution as fine droplets by means of dispersive solvent and ultrasonication which promoted the analyte to migrate into IL phase more easily. Several important parameters affecting the extraction efficiency were studied and optimized, including the type and volume of extraction solvent and dispersive solvent, sample pH, ultrasonication time, cooling time, centrifugation time and salting-out effect. Under the optimized conditions, 110-fold enrichment factor was obtained and the limit of detection (LOD) was 1.6 μg/L at a signal-to-noise ratio of 3. The calibration curve was linear over the range of 10–1000 μg/L for celastrol in human urine sample, with a correlation coefficient of 0.9980. Intra- and inter-assay precision were 0.43% and 2.78%, respectively. The proposed method was successfully applied to the real human urine samples and good spiked recoveries in the range of 93.2–109.3% were obtained.

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1. Introduction

Celastrol, a quinine methide triterpene also named as tripterine, is a natural compound from a traditional Chinese medicine, *Tripterygium wilfordii* Hook. F. [1]. This herbal medicine, also known as "*Thunder of God Vine*", belongs to the family Celastraceae and has been used in China for hundreds of years owing to its excellent therapeutic effects [2]. Celastrol was first reported in 1936 [3], and recent researches have shown that it had great therapeutic potentials in several inflammatory diseases, e.g. rheumatoid arthritis [4], amyotrophic lateral sclerosis [5] and allergic asthma [6]. Especially, after the new anticancer mechanism of celastrol was found in 2006 [7], more and more researches have focused on its anticancer activity. However, not to be overlooked is that it will exhibit some degree of toxicity and cause serious adverse effects at high dose. Accordingly, it is significant to establish a simple, sensitive and effective analytical method to monitor the concentration of celastrol in biofluid for its toxicological research, clinical use and medicine safety evaluation.Nowadays, several methods such as HPLC [8] and HPLC-MS [9] have been used to analyze celastrol. Due to matrix interference and insufficient instrumental sensitivity for drugs in real biological samples, direct chromatographic separation and determination is becoming difficult [10]. In order to obtain sensitive, accurate and reliable results, an appropriate preconcentration method should be developed. By now, only a SPE method has been developed for preconcentrating celastrol [11]. The SPE method required a specific device loaded with certain adsorption material as well as a high-pressure delivery system that would be relatively expensive [12].Recently, dispersive liquid–liquid microextraction (DLLME) has attracted increasing attention for its superior advantages of high enrichment factor, perfect recovery, low cost, rapid and easy operation [13]. In this method, a cloudy solution is prepared by the means of injecting the mixture of extraction solvent and dispersive solvent into aqueous sample rapidly. As a result, the analyte in the sample is extracted into the fine droplets of extraction solvent. Subsequently, phase separation is performed by centrifugation and the enriched analyte in the sedimented phase is determined by chromatographic or spectrometric methods [14]. At the stage, chlorinated solvents, e.g. chlorobenzene [15], chloroform [16] and carbon tetrachloride, are commonly used as the extraction solvents [17,18]. Recently, ionic liquid (IL) has been used as the extraction solvent to replace the conventional organic solvents for

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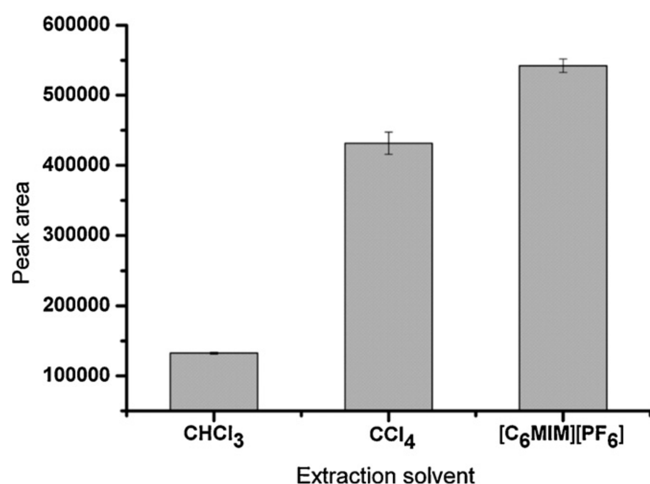

Fig. 1. Effect of extraction solvent on the peak area of celastrol ($n = 3$). Extraction conditions: extraction solvent volume, 50 μL; dispersive solvent, 0.5 mL methanol; sample pH, 2.0; sonication time, 6 min, cooling time, 15 min; centrifugation time, 10 min at 3000 rpm.

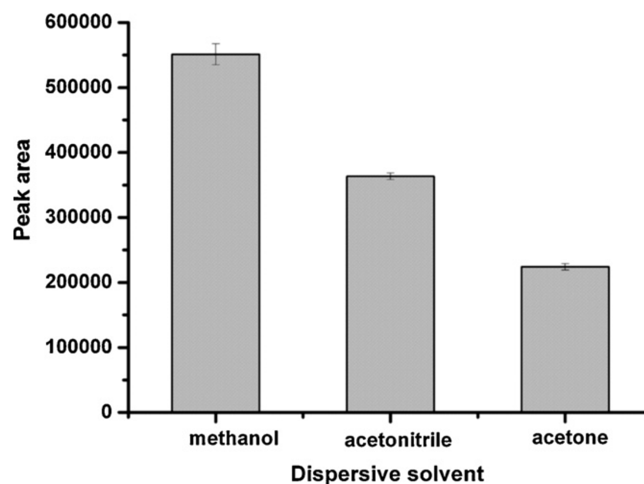

Fig. 2. Effect of dispersive solvent on the peak area of celastrol ($n = 3$). Extraction conditions: extraction solvent, 50 μL $[C_6MIM][PF_6]$. Other extraction conditions are the same as Fig. 1.

its poor solubility in water, low volatility and low toxicity. Owing to its high viscosity, it is difficult for IL to disperse in the sample solution, and the degree of dispersion of IL plays a crucial role in determining the enrichment efficiency of DLLME. The smaller fine droplet of IL forms, the higher enrichment performance achieves [19]. To perfect the dispersive process, a temperature-assisted ionic liquid DLLME using high temperature to help IL completely disperse in the sample solution and increase the chance of mass transfer into IL phase was developed [20,21]. Whereas, Zhou has proposed his method to disperse IL completely by ultrasonication in 2009 [19] which promotes the analytes more easily to migrate into the fine droplets of IL, and so high enrichment performance was obtained. And this method has been used in trace aromatic amines [19] and metal ions [22] in aqueous samples, respectively.

In this research, ultrasound-assisted ionic liquid dispersive liquid–liquid microextraction (UA IL-DLLME) method was utilized as a sample preparation technique for the determination of celastrol in urine samples. Several factors affecting the enrichment were evaluated, such as the type and volume of extraction solvent and dispersive solvent, sample pH, ultrasonication time, cooling time, centrifugation time and salting-out effect. It was a new enrichment method for celastrol, and was applied to biological samples.

2. Experimental

2.1. Chemicals and reagents

Celastrol (structure shown in Fig. 5(b)) was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China) and the purity was above 99.0%. Chromatographic grade acetonitrile and methanol were obtained from Shandong Yuwang Industrial Co., Ltd. (Yucheng, China). Analytical grade acetone, dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) were obtained from Tianjin Chemical Reagent Factory (Tianjin, China). Sodium chloride (NaCl) and hydrochloric acid (HCl) were of analytical grade and obtained from Beijing Shuanghuan chemical reagents Co., Ltd. (Beijing, China). Ionic liquids of 1-butyl-3-methylmidazoliumhexafluorophosphate ($[C_4MIM][PF_6]$), 1-hexyl-3-methylmidazoliumhexafluorophosphate ($[C_6MIM][PF_6]$), and 1-octyl-3-methylimidazoliumhexafluorophosphate ($[C_8MIM][PF_6]$) were obtained from Lanzhou Institute of Chemical Physics of the CAS (Lanzhou, China). Ultrapure water was used for the preparation of mobile phase and sample solution.

2.2. Apparatus

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. Chromatographic separation of the analyte was performed 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 was consisted of acetonitrile–0.1% phosphoric acid (90:10, V/V) at a flow rate of 1.0 mL/min. The column temperature was maintained at 25 °C. The detection wavelength was 425 nm.

2.3. Preparation of stock solution and biological samples

The stock solution (0.1 mg/mL) of celastrol was prepared in methanol and stored at 4 °C before use. Then the aqueous samples solutions were prepared daily by diluting the stock solution with ultrapure water and used to optimize the influencing factors of DLLME procedure.

Human urine samples were collected from a healthy volunteer and stored below 0 °C. Before use, the samples were thawed to room temperature. The urine samples were used for validation of method and application in real sample. Calibration standard working solutions at seven concentration levels, i.e. 10, 60, 100, 300, 500, 700, 1000 μg/L, were freshly prepared by appropriate dilution of the stock solution with urine.

2.4. Ultrasound-assisted IL-DLLME procedure

5 mL aqueous sample was placed in a 10 mL screwcap glass conical tube, and then HCl was added to adjust the sample solution to pH 2.0. After 0.1 mL methanol containing 45 μL $[C_6MIM][PF_6]$ was rapidly injected into the tube using a 1.0 mL syringe, the cloudy solution was formed in the tube. Then the tube was immersed in an ultrasonic water bath (KQ500DB, Kunshan, China), and sonicated at 200 W power for 1 min at room temperature, following by transferring into an ice–water bath for 10 min. After centrifuging the solution for 10 min at 3000 rpm, the upper aqueous phase was removed with a syringe, and the sedimented phase was diluted with 50 μL methanol and then filtrated through 0.45 μm filter membrane. Finally, 10 μL filtrate was injected into the HPLC system for analysis.

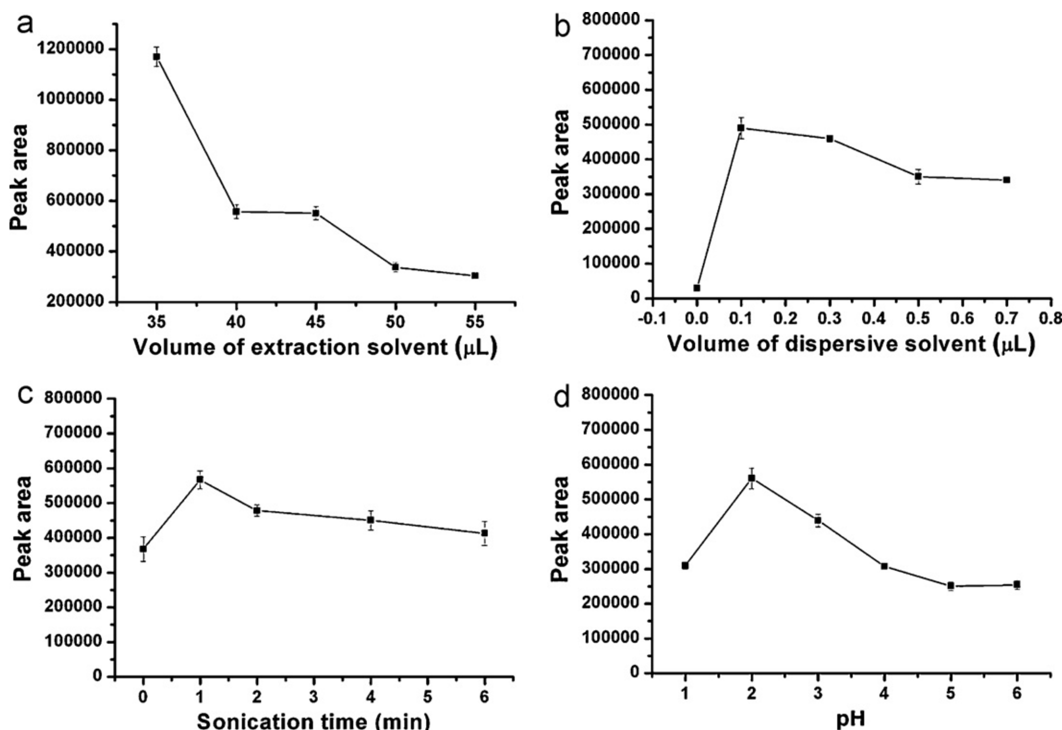

Fig. 3. Effect of volume of extraction solvent $[C_6MIM][PF_6]$ (a), volume of dispersive solvent (b), sonication time (c) and sample pH (d) on the peak area of celastrol ($n = 3$). Extraction conditions, (b): extraction solvent, 45 μL $[C_6MIM][PF_6]$; (c): extraction solvent, 45 μL $[C_6MIM][PF_6]$; dispersive solvent, 0.1 mL methanol; (d): extraction solvent, 45 μL $[C_6MIM][PF_6]$; dispersive solvent, 0.1 mL methanol; sonication time, 1 min; the others are the same as Fig. 1.

3. Results and discussion

3.1. Optimization of ultrasound-assisted IL-DLLME

Several factors affecting the extraction efficiency of IL-DLLME were studied. 5 mL aqueous solution containing 80 μg/L of celastrol was used as the sample for the optimization experiments.

3.1.1. Selection of extraction solvent

An ideal extraction solvent in DLLME should possess some characteristics including low solubility in water, good extraction capability to interested compounds, higher density than water, low volatility and good chromatographic behavior. Most of the chlorinated solvents possess all of above properties, but these solvents are highly toxic so their application is not desirable. As a new green solvent, IL becomes an excellent and efficient alternative for conventional organic chlorinated solvents in DLLME. In this research, three chlorinated solvents, i.e. CH_2Cl_2, $CHCl_3$, CCl_4, and three ILs, i.e. $[C_4MIM][PF_6]$, $[C_6MIM][PF_6]$, $[C_8MIM][PF_6]$, were compared as extraction solvents. In the procedure of extraction, $[C_4MIM][PF_6]$ and CH_2Cl_2 hardly formed the emulsion, which is probably attributed to their relatively high solubility in water; and the $[C_8MIM][PF_6]$ is too sticky to disperse in the water. As Fig. 1 shows that $[C_6MIM][PF_6]$ provided higher enrichment than $CHCl_3$ and CCl_4. Hence, $[C_6MIM][PF_6]$ was selected as the extraction solvent for the subsequent research.

3.1.2. Selection of dispersive solvent

Dispersive solvent is another significant influencing factor of extraction. Solvent possessing good miscibility with both water and IL helps IL totally disperse into the water and form water-dispersive solvent-IL system, which enlarges the contacting area between IL and sample solution. In this research, methanol, acetonitrile and acetone were evaluated as dispersive solvents, respectively. As can be seen in Fig. 2, methanol performed the highest extraction efficiency. Then methanol was chosen as the dispersive solvent for the next optimization.

3.1.3. Effect of IL volume

In order to study the effect of IL volume on extraction efficiency, different volumes of IL ranging from 35 to 55 μL at the interval of 5 μL were tested under the above extraction conditions. As illustrated in Fig. 3(a), with the IL volume increasing, the settled phase volume increased from 6 to 20 μL, which caused the marked decreasing of peak area of celastrol. The volume of IL was chosen as 45 μL for the subsequent optimization of obtaining high enrichment factor and appropriate volume of settled phase.

3.1.4. Effect of the volume of dispersive solvent

Dispersive solvent could affect the emulsion formation and the dispersity of IL. Different volumes of methanol were tested in the range of 0–0.7 mL. Fig. 3(b) indicated that, before adding methanol, poor extraction efficiency was obtained, which might be due to the failure of emulsion formation. After adding 0.1 mL methanol, the peak area of celastrol increased remarkably. However, the peak area decreased with increasing methanol volume from 0.3 to 0.7 mL, which might be accounted for the fact that at a higher volume, the solubility of IL in the sample solution increased while the volume of sedimented phase decreased responsively. Accordingly, a 0.1-mL volume of methanol was chosen for the subsequent extractions.

3.1.5. Effect of ultrasonication time

Dispersion is the most important stage in determining whether ultrasound-assisted IL-DLLME can be successfully carried out or not [19]. So enough sonication time ensures IL to disperse entirely into the sample solution resulting in higher extraction efficiency. However, too long time of sonication may cause the generation of heat, which increases the solubility of IL in the sample solution. In this section, sonication time in the range of 0–6 min was optimized. The

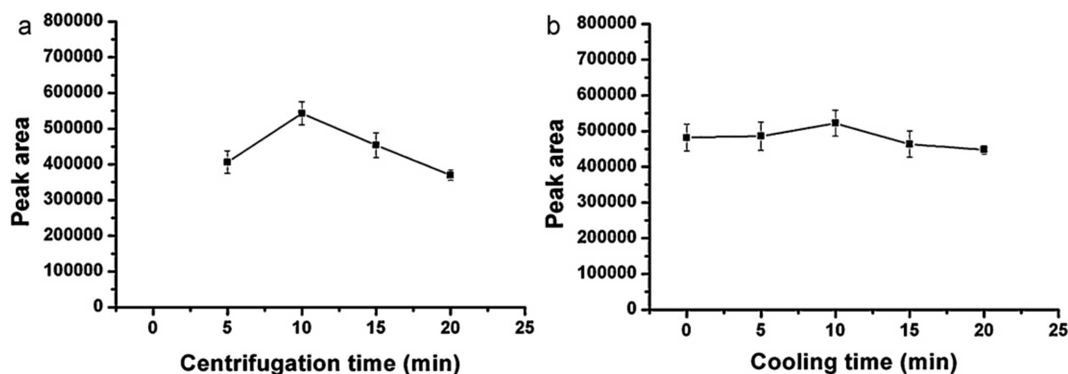

Fig. 4. Effect of cooling time (a) and centrifugation time (b) on the peak area of celastrol (n = 3). Extraction conditions were the same as Fig. 3(d).

results in Fig. 3(c) showed that the peak area of celastrol had a lot increase from 0 to 1 min and then decreased slowly, indicating that sonication really contributed to dispersion of IL into the sample solution. Hence, 1 min was chosen for the dispersion procedure.

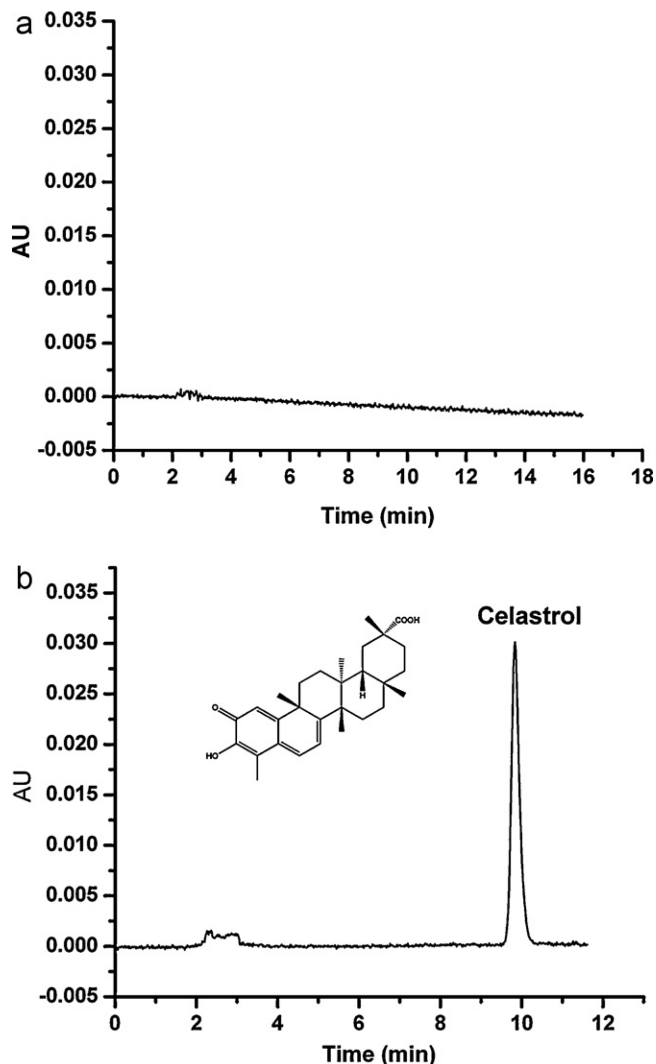

Fig. 5. Chromatograms of (a) human blank urine and (b) urine sample containing 160 μg/L celastrol extracted by DLLME. Mobile phase: acetonitrile–0.1% phosphoric acid (90:10, V/V); flow rate: 1.0 mL/min; detection wavelength: 425 nm.

3.1.6. Effect of sample pH

In general, pH value of sample solution determines the existential state of analytes, thus affecting extraction efficiency. Celastrol is a weakly acidic compound with a p*K*a of nearly 4 existing as neutral form in acidic solution. Considering this, the effect of sample pH was investigated in the range of 1.0–6.0 by adding appropriate amount of HCl to the sample solution. Fig. 3(d) showed that, the peak area of celastrol increased with pH increasing from 1.0 to 2.0, and reached the maximum at pH 2.0, and declined gradually with further increase in pH. Thus, the optimum pH value of the sample solution was selected as 2.0.

3.1.7. Salting-out effect

Generally, the addition of salt decreases the solubility of analytes in aqueous sample and enhances their distribution in organic phase. However, it may have different effects when IL is used as extraction solvent. In this experiment, 0–20% (w/v) NaCl was added to evaluate the effect of ionic strength on the extraction efficiency. The experimental results showed that the peak area of celastrol decreased with NaCl concentration increasing, which may be attributed to the fact that addition of salt enhanced the solubility of IL in aqueous phase [23]. Hence salt addition was not used in all the subsequent optimization.

3.1.8. Effect of cooling time

The cooling time of 0, 5, 10, 15, 20 min in the ice–water bath was investigated. As seen in Fig. 4(a), the peak area of celastrol hardly changed with the variety of cooling time, and the highest peak area was obtained at 10 min. When the time was longer than 10 min, the peak area decreased slightly. The possible reason is that equilibrium state is achieved quickly owing to the large surface area between extraction solvent and aqueous phase under this dispersive mode, but the viscosity of IL increases with cooling time extending, and as a result, a few IL could not be separated from aqueous phase in the subsequent centrifugation.

3.1.9. Effect of centrifugation time

Centrifugation is a vital procedure for separating IL phase from aqueous phase. In order to achieve the best extraction efficiency, centrifugation time in the range of 5–20 min at 3000 rpm was evaluated. As Fig. 4(b) shown, the highest peak area was got at 10 min. Longer or shorter than which, the peak area decreased. The phenomenon might be attributed to the incomplete sediment of IL drops within shorter centrifugation time, and the generated heat leading to the dissolving partly of IL within longer time. Therefore, 10 min was adopted.

3.2. Evaluation of method performance

Enrichment factor (EF) is calculated according to the following equation: $EF = C_{fil}/C_o$, where C_{fil} is the concentration of analyte in the filtrate for HPLC analysis, and C_o is the concentration of analyte originally presented in the sample solution. Under the optimum condition, celastrol in urine sample was extracted with an enrichment factor of 110-fold.

The calibration standard working solutions were extracted under the optimized DLLME procedure, and then analyzed by HPLC. The calibration curve was constructed by plotting integrated chromatographic peak area of the analyte against the corresponding seven concentrations in three duplicate analyses. The linear regression equation was $Y = 3989\ X - 151{,}872$ with a correlation coefficient of 0.9980. The limit of detection of real sample was 1.6 μg/L at a signal-to-noise ratio of 3.

The precision of the proposed method was evaluated by performing intra-day ($n = 5$) and inter-day assays ($n = 3$) replicate injection of a standard solution. Intra-assay precision was measured for continuous injections during the same day whereas inter-assay precision was measured on 3 consecutive days. The RSD value of peak area was 0.43% for intra-day precision, and 2.78% for inter-day precision.

Five replicate experiments of the urine samples containing 300 μg/L celastrol were performed under optimal conditions to give a relative standard deviation of 3.78%.

The accuracy of the method was confirmed by spiked recovery test. Three sets of the urine samples containing 300 μg/L of celastrol were extracted and analyzed after spiking 240, 300, and 360 μg/L of celastrol, respectively. The recoveries of the analyte at low, medium and high spiked levels were 93.2, 104.1, 109.3% with RSDs of 4.62, 4.52, and 1.87%, respectively ($n = 3$).

3.3. Application in real sample

The applicability of the proposed method was tested by determining celastrol in real human urine samples. 1.0 mL of urine sample was diluted to a total volume of 10 mL and the pH was adjusted to 2.0 with HCl. Then 5.0 mL of the above sample solution was placed in a 10 mL screwcap glass conical tube and extracted according to the optimized procedure. The typical chromatograms of human blank urine and urine sample containing 160 μg/L celastrol extracted with ultrasound-assisted IL-DLLME were illustrated in Fig. 5.

4. Conclusion

A new method of UA IL-DLLME combined with HPLC was developed for the determination of celastrol in human urine sample. The ultrasonication process promoted IL to disperse into the sample solution and formed the cloudy solution. High linearity, repeatability, intra-day and inter-day precision and accuracy were confirmed. The proposed procedure provides several merits such as good enrichment performance, simplified and fast operation, and low consumption of organic solvent, and so forth. The method also offers reference value for analyzing natural compounds at trace levels in biological matrix to some extent.

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References

[1] H. Mou, Y. Zheng, P. Zhao, H. Bao, W. Fang, N. Xu, Toxicol. In Vitro (2011), doi:10.1016/j.tiv.2011.03.023.
[2] A.R. Setty, L.H. Sigal, Semin. Arthritis Rheum. 34 (2005) 773.
[3] T. Chou, P. Mei, Chin. J. Phys. 10 (1936) 527.
[4] T.G. Cotter, Nat. Rev. Cancer 9 (2009) 501.
[5] M. Kiaei, K. Kipiani, S. Petri, J. Chen, N.Y. Calingasan, M.F. Beal, Neurodegener. Dis. 2 (2005) 246.
[6] D.Y. Kim, J.W. Park, D. Jeoung, J.Y. Ro, Eur. J. Pharmacol. 612 (2009) 98.
[7] H.J. Yang, D. Chen, Q.Z.C. Cui, X. Yuan, Q.P. Dou, Cancer Res. 66 (2006) 4758.
[8] Y. Xia, W.Y. Wang, Y.W. Zhang, J.P. He, W.Y. Gao, H.Q. Duan, Chin. Tradit. Herb. Drugs 36 (2005) 1154.
[9] Q. Xu, M. Huang, M.C. Jin, Q.L. Ren, Chromatographia 66 (2007) 735.
[10] T.M. Pizzolato, M.J.L. de Alda, D. Barceló, Trends Anal. Chem. 26 (2007) 609.
[11] X.H. Chen, X.K. Ou Yang, M.C. Jin, Phys. Test. Chem. Anal. (Part B: Chem. Anal.) 46 (2010) 948.
[12] C.M. Xiong, J.L. Ruan, Y.L. Cai, Y. Tang, J. Pharm. Biomed. Anal. 49 (2009) 572.
[13] M. Rezaee, Y. Assadi, M.R.M. Hosseinia, E. Aghaee, F. Ahmadi, S. Berijani, J. Chromatogr. A 1116 (2006) 1.
[14] H.X. Chen, H. Chen, J. Ying, J.L. Huang, L. Liao, Anal. Chim. Acta 632 (2009) 80.
[15] D. Nagaraju, S.D. Huang, J. Chromatogr. A 1161 (2007) 89.
[16] M. Rezaee, Y. Yamini, S. Shariati, A. Esrafili, M. Shamsipur, J. Chromatogr. A 1216 (2009) 1511.
[17] M.A. Farajzadeh, M. Bahram, J.A. Jonsson, Anal. Chim. Acta 591 (2007) 69.
[18] Q.X. Zhou, L. Pang, J.P. Xiao, J. Chromatogr. A 1216 (2009) 6680.
[19] Q. Zhou, X. Zhang, J. Xiao, J. Chromatogr. A 1216 (2009) 4361.
[20] Q.X. Zhou, H.H. Bai, G.H. Xie, J.P. Xiao, J. Chromatogr. A 1177 (2008) 43.
[21] H.F. Zhang, Y.P. Shi, Talanta 82 (2010) 1010.
[22] L. Xu, H.K. Lee, J. Chromatogr. A 1216 (2009) 5483.
[23] M.T. Pena, M.C. Casais, M.C. Mejuto, R. Cela, J. Chromatogr. A 1216 (2009) 6356.