A Green Method Using a Micellar System for Determination of Andrographolide and Dehydroandrographolide in Human Plasma

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A method based on cloud point extraction (CPE) coupled with high-performance liquid chromatography separation and ultraviolet (UV) detection was developed to determine andrographolide and dehydroandrographolide in human plasma. The nonionic surfactant Triton X-114 was chosen as the extraction medium. Variable parameters affecting the CPE efficiency were evaluated and optimized, such as concentrations of Triton X-114 and NaCl, pH, equilibration temperature and equilibration time. A Zorbax SB C18 column (250 × 4.6 mm i.d., 5 μm) was used for separation of the two analytes at 30°C. The UV detection was performed at 254 nm. Under the optimum conditions, the limits of detection of andrographolide and dehydroandrographolide are 0.032 and 0.019 μg/mL, respectively. The intra-day and inter-day precisions expressed as relative standard deviation ranged from 3.2 to 7.3% and from 2.9 and 8.6%. The recoveries of andrographolide and dehydroandrographolide were in the range of 76.8–98.6% at three fortified concentrations of 0.1, 0.5 and 1.0 μg/mL. This method was efficient, environmentally friendly, rapid and inexpensive for the extraction and determination of andrographolide and dehydroandrographolide in human plasma.

Introduction

Andrographis paniculata Nees (A. paniculata), also known commonly as “King of Bitters,” is a member of the plant family Acanthaceae and an ancient medicinal herb for the treatment of gastric disorders, infectious diseases and common colds (1). The primary medicinal constituents of A. paniculata are andrographolide (Figure 1A) and dehydroandrographolide (Figure 1B). The two components have many types of bioactivity, such as anti-inflammatory (2), anti-microbial (3), anti-platelet aggregation (4), hepatoprotective (5, 6), anti-cancer (7) and anti-HIV (8) activities. Moreover, the reproductive toxicity and anti-fertility properties of andrographolide and dehydroandrographolide are receiving increasing attention (9, 10). Due to the extensive applications of these two medicines, simple, green and sensitive methods for the determination of andrographolide and dehydroandrographolide in human plasma have long been desired.

So far, several analytical methods have been reported for the quantification of andrographolide and dehydroandrographolide, including spectrophotometry (11), high-performance liquid chromatography (HPLC) (12, 13, 14), high-performance thin layer chromatography (15), HPLC–mass spectrometry (16, 17, 18), microemulsion electrokinetic chromatography (19), micellar electrokinetic capillary chromatography (20) and capillary electrophoresis (21). This paper presents a method using an HPLC–ultraviolet (UV) technique, the required instruments for which can easily be obtained and conveniently operated in laboratories, to determine these two lactones in human plasma.

Extraction of the two lactones from biological fluids before analysis is a critical step, and the most routine approach is the precipitation of plasma proteins using a kind of organic reagent followed by centrifugation. Afterward, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are used for concentration and clean-up of the analytes (22, 12). These sample preparation techniques are rather laborious and time consuming, and certain large volumes of toxic and hazardous organic solvents are employed. The cloud point extraction (CPE) technique not only minimizes the use of organic solvents, but also combines the extraction and preconcentration in a single step.

It is well known that surfactants are amphiphilic molecules, the head of which is hydrophilic and the tail is hydrophobic. The surfactant molecules can associate in aqueous solutions to form molecular aggregates called micelles. The minimum concentration of surfactant required for this phenomenon to occur is called the critical micellar concentration (23). One important property of micelles is that when the temperature rises above cloud point temperature, the system is separated into two isotropic phases. One is the surfactant-rich phase and the other is the aqueous phase; the surfactant-rich phase can be separated by centrifugation. This phenomenon is reversible and, upon cooling, a single phase is obtained again (24, 25).

Recently, CPE has successfully been used for the selective extraction of various compounds from plasma samples, such as flurbiprofen (26), arbidol (27), osthole (28), venlafaxine (29), ampolosin (30) and polycyclic aromatic, hydrocarbons, polychlorinated and dibenzo-p-dioxins (31). However, reports about its applications on how to extract active components of herb medicine from plasma have been limited. This paper reports a new method for the determination of andrographolide and dehydroandrographolide in human plasma by CPE using Triton X-114 as the surfactant coupled with HPLC determination, which demonstrates the feasibility of CPE for clinical studies.

Experimental

Material

Reagents and samples

Analytical grade andrographolide and dehydroandrographolide were purchased from National Institute for the Control of
Figure 1. Chemical structures: andrographolide (A); dehydroandrographolide (B).

Pharmaceutical and Biological Products (Beijing, China). The stock standard solutions of andrographolide and dehydroandrographolide (0.5 mg/mL) were prepared by dissolving an appropriate amount of these compounds in 50% methanol aqueous solution (v/v). The solution was stored in a refrigerator at 4 °C and found to be stable for one month. The working standard solution was prepared daily by diluting the stock standard solution with water.

The nonionic surfactant Triton X-114 was purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride, sodium hydroxide and hydrochloric acid were of analytical grade and purchased from Beijing Chemical (Beijing, China). Chromatographic grade methanol, acetonitrile and formic acid were obtained from Fisher (Pittsburgh, PA). High purity water with resistivity of 18.2 MΩ·cm was obtained from a Milli-Q water system (Millipore, Billerica, MA). Blank human plasma samples were obtained from Blood Station of Changchun (China). Human blood from healthy volunteers was obtained from China-Japan Friendship Hospital (Changchun, China). The plasma was separated from blood by centrifugation at 3,000 rpm for 10 min and was stored at −4°C until it was used. Values of pKa parameters for these compounds were calculated using ACD/pKa DB (ACD lab).

Apparatus
Chromatographic analysis was performed on an Agilent 1100 liquid chromatograph (Palo Alto, CA) that was equipped with a quaternary pump, a heated column compartment, a UV detector, an LC workstation and a 7725 injection valve. A Zorbax SB C18 column (250 × 4.6 mm i.d., 5 μm) was used as an analytical column (Palo Alto, CA). A DK-98-IIA thermostatic bath (TaiSite, Tianjin, China) was used to implement the CPE. A SH-36 vortex mixer (Zhenghui, Shanghai, China) was used to mix the CPE solution. A SC-3610 centrifuge (Keda, Beijing, China) was used to accelerate the phase separation process.

Calibration standards and quality control samples
The solutions used for calibration were prepared by addition of the working standard solutions into blank human plasma, giving final concentration series of 0.1–2.5 μg/mL. Quality control (QC) samples containing low (0.1 μg/mL), middle (0.5 μg/mL) and high (1.0 μg/mL) concentrations of standards were prepared by the same procedure as the calibration standards.

Methods
CPE procedure
CPE was used to analyze plasma in previously reported references (27, 31). Based on this, the method was modified to extract andrographolide and dehydroandrographolide in human plasma. A 0.5 mL sample of human plasma was added to a 2.0 mL capped centrifugal tube with 1 mL of aqueous solution of Triton X-114 at the concentration of 5% (v/v) and 0.045 g sodium chloride. The mixture was stirred in the vortex for approximately 3 min and then incubated in the thermostatic bath at 60°C for 15 min. The phase separation was then accelerated by centrifugation at 4,000 rpm for 5 min. After removing the aqueous phase, hydrophobic proteins were removed from the surfactant-rich phase by precipitation with 120 μL of methanol, vortex-mixed and centrifuged at 12,000 rpm for 10 min. The resulting supernatant was diluted to 0.25 mL and 20 μL of diluent solution was injected into the HPLC system for analysis.

HPLC–UV analysis
The separation and determination of andrographolide and dehydroandrographolide were conducted by using an HPLC–UV system. A mobile phase of methanol–acetonitrile–0.5% formic acid aqueous solution (10: 17: 43, v/v/v) was used. The flow rate was 1.0 mL/min. The column temperature was 30°C. The analytes were monitored at the wavelength of 254 nm.

Method validation
Specificity
A blank plasma sample and a spiked plasma sample, which were prepared by spiking 0.2 μg/mL andrographolide and dehydroandrographolide to a blank plasma sample, were analyzed by the proposed method to investigate the existence of interference from endogenous compounds from the blank human plasma.

Calibration curve
Calibration curves were obtained by plotting the peak area of the studied analytes in the spiked sample versus the theoretical concentration of the analytes added to drug-free human plasma. The curves were constructed from three replicate measurements of six concentrations over a range of 0.1–2.5 μg/mL. The data were subjected to least-squares regression analysis, to provide information on the linearity of the method.

Precision and recovery
Precision was evaluated by measuring intra-day and inter-day relative standard deviations (RSDs). The intra-day precisions were performed by analyzing a human plasma sample six times in one day at three fortified concentrations of 0.1, 0.5 and 1.0 μg/mL. The inter-day precisions were performed over six days by analyzing spiked plasma samples at three fortified concentrations of 0.1, 0.5 and 1.0 μg/mL.
The recoveries were calculated by comparing mean peak areas of three spiked samples with peak areas of the same concentration from calibration curves. The mean recoveries of the analyses from human plasma were determined by the recoveries at the same levels.

**Sensitivity**
The limit of detection (LOD) is considered to be the minimum analyte concentration that can be confidently identified by the method. The LOD in plasma was defined as the concentration providing a signal-to-noise ratio of three.

**Stability**
Short-term temperature stability was studied as follows: the plasma samples spiked at three concentration levels were kept at 18°C for 24 h. The samples were analyzed by the proposed method every four hours.

Freeze and thaw stability was studied as follows: the plasma samples spiked at three concentration levels were stored at −20°C for 24 h and thawed at room temperature. After completion of three freezing and thawing cycles, the samples were analyzed by the proposed method.

Long-term stability was studied as follows: the spiked samples at three concentration levels were stored at −18°C for one month. After one month, the recoveries of the samples were analyzed by the proposed method.

**Results and Discussion**

**Optimization of the CPE conditions**
Triton X-114 was chosen as the CPE surfactant for its low cloud point temperature of 23–30°C and low UV absorbance (24). Several parameters that can influence the extraction efficiency, such as the Triton X-114 concentration, pH, sodium chloride concentration, equilibration temperature and time, were investigated in these experiments. All the experiments were performed in triplicate.

**Effect of Triton X-114 concentration**
It is well known that surfactant concentration above the critical micellar concentration is required to achieve high recovery. However, the ratio between the volume of preconcentrated aqueous solution and the surfactant-rich phase volume increases with the decrease of the surfactant concentration (23). This shows that the smaller the surfactant concentration, the higher the preconcentration factor.

The concentration of Triton X-114 used for extraction compounds from biological samples was 0.4 to 6% (27, 28, 33–36). Therefore, the effect of Triton X-114 concentration from 2.0 to 6.0% on the recoveries of andrographolide and dehydroandrographolide was investigated (Figure 2). The recoveries of the analytes increased with the increase of surfactant concentration from 2.0 to 3.0%, but remained constant when the surfactant concentration was higher than 3.0%. A small concentration of Triton X-114 was not enough to completely extract the analytes. However, when a large concentration of surfactant was used, the lower concentration factor was obtained due to the increase of the volume of surfactant-rich phase. In this work, 3.0% (v/v) was chosen as the optimum surfactant concentration for further studies.

**Effect of pH**
The pH of the solution is an important factor during the CPE process. The ionic form of the analyte does not interact with the micellar aggregate as strongly as its neutral form; therefore, fewer of the analytes are extracted (37). Thus, pH should be adjusted to ensure that the neutral molecular form of the analyte is present before performing the CPE step. The effect of pH that was adjusted with 0.1 mol/L hydrochloric acid or sodium hydroxide on the recoveries of andrographolide and dehydroandrographolide was studied over the pH range 2.0–10.0 (Figure 3). The experimental result indicated that the...
recoveries were satisfactory over the pH range 5.0–8.0. The pKa of andrographolide and dehydroandrographolide are 12.3 and 15.1, respectively. This indicates that the two substances remain neutral when the pH is lower than 10. Therefore, the lower recoveries of the compounds when pH is above 9 are most likely due to the hydrolysis of the lactone ring under basic condition (38). Low recovery of andrographolide when pH is less than 4 is most likely due to the dehydration of allyl alcohol to form a diene species under the experimental condition. Moreover, the actual pH of plasma analyzed in this study is approximately 7.0. In the following experiments, the pH was not adjusted, and its value is approximately 7.0.

**Effect of sodium chloride concentration**

The addition of salt can facilitate the phase separation process for some nonionic surfactant systems, because it increases the density of the bulk aqueous phase (37, 38). When the concentration of salt is increased, the micelle size and aggregation number are increased, but the critical micellar concentration remains constant (39). In addition, non-polar analytes may become less soluble in the solution at higher salt concentrations, and thus contribute to higher recovery (23).

Sodium chloride is commonly used in CPE to change the ionic strength of micellar systems. The concentration range of sodium chloride used in literature is between 0.06 and 5% (28, 29, 40–42). To study the influence of the electrolyte, different concentrations of sodium chloride, ranging from 1 to 5% (m/v), were added into the solution (Figure 4). It was observed that the recoveries of andrographolide and dehydroandrographolide increased with the increase of the sodium chloride concentration from 1 to 3%, and no significant difference was observed above 3%. When the concentration is higher than 3%, the surfactant-rich phase is on the surface of the solution, which makes it difficult to collect, and the accuracy and reproducibility were not satisfactory. Therefore, the sodium chloride concentration of 3% was chosen in this study.

**Effect of equilibrium temperature**

It is known that two phases are formed when aqueous solutions of nonionic surfactant are heated above the cloud-point temperature (32). As the equilibration temperature increases, the volumes of the surfactant-rich phase decrease because hydrogen bonds are disrupted and dehydration occurs (40). The amount of water in a surfactant-rich phase also decreases.

Theoretically, the optimal extraction occurs when the equilibration temperature is 15–20°C higher than the cloud point temperature of the surfactant (43). The equilibration temperature applied in literature was 45—70°C (40, 43, 44). As shown in Figure 5, the recoveries of andrographolide and dehydroandrographolide increased with an increase of the temperature from 40 to 60°C. No significant difference in the recoveries was observed when the temperature ranged from 60 to 80°C. The high temperature (above 80°C) could cause protein denaturation, which would have a negative influence on the subsequent determination. Based on these results, 60°C was selected as the equilibrium temperature.

**Effect of equilibrium time**

The recovery depends on the time that the analytes have to interact with the micelles and get into their core (45). The recoveries of andrographolide and dehydroandrographolide increased with prolonged equilibrium time. Fifteen minutes was sufficient for the highest extraction recovery, which ensured that the analytes completely moved into the micellar core. Therefore, a short equilibrium time of 15 min was selected for equilibrium.

The procedure was then accelerated by centrifugation at 4,000 rpm for 10 min, which was enough to achieve complete phase separation.

Afterward, the surfactant-rich phase was directly injected into the HPLC system for analysis. However, some interfering peaks were found in the chromatogram. The methanol was added into the surfactant-rich phase to remove hydrophobic proteins and dilute the surfactant. Different amounts of methanol ranging from 50 to 200 μL were evaluated. When 120 μL...
methanol was added to the system, the chromatograms of the analytes showed that the interfering peak disappeared. The clean-up procedure was not very satisfactory when less than 120 μL of methanol was used. However, when a large amount of methanol was used, the final extraction volume was too large, which would decrease the sensitivity of the method. Therefore, in this work, 120 μL of methanol was applied.

**Method validation**

**Specificity**

Figure 6 shows the typical chromatograms of a blank plasma sample (Figure 6A) and a blank plasma sample spiked with 0.2 μg/mL andrographolide and dehydroandrographolide (Figure 6B), respectively. No significant interference of endogenous substances was detected from the blank human plasma with the two analytes. Therefore, the proposed method was selective for the determination of andrographolide and dehydroandrographolide in human plasma.

**Calibration curve**

The results of linear regression showed an excellent correlation between peak area and concentration of analytes in the concentration range tested (0.1–2.5 μg/mL). The regression equations and correlation coefficients (R) are:

Andrographolide: \( A = 41.02c + 0.69; R = 0.9994 \)

Dehydroandrographolide: \( A = 64.48c + 0.78; R = 0.9993 \)

where \( A \) represents the peak area and \( c \) represents the concentrations of andrographolide and dehydroandrographolide in μg/mL.

**Sensitivity**

The LOD is estimated as the analyte concentration producing a signal-to-noise ratio of 3:1 are 0.032 and 0.019 μg/mL for andrographolide and dehydroandrographolide, respectively.

![Figure 6. Chromatograms obtained by the analysis of: blank plasma (A); spiked (0.2 μg/mL) plasma sample (B) (peak 1, andrographolide; peak 2, dehydroandrographolide). The extraction conditions are as follows: Triton X-114, 3% (v/v); NaCl, 3% (m/v); pH of solution, 7; equilibrium temperature, 60°C; equilibrium temperature, 15 min.](image-url)

**Precision and recovery**

The precision and recovery of the analyses are shown in Table I. The intra-day and inter-day RSDs range from 3.2 to 7.3% and 2.9 and 8.6%, respectively. In all three fortified levels, recoveries of andrographolide or dehydroandrographolide were in the range of 76.8–98.6%.

**Stability**

No significant difference in concentration for andrographolide and dehydroandrographolide in human plasma was observed during the 24-h period of the assay; the RSDs between the initial and final concentrations of andrographolide and dehydroandrographolide stored at 18°C for 24 h were less than 5.4 and 3.8%, respectively.

Freezing and thawing did not reveal any detrimental effect on the absolute concentrations of the analytes spiked to human plasma. After the completion of three freezing and thawing cycles, the recoveries of andrographolide and dehydroandrographolide still ranged between 88.6 and 95.5%.

After the spiked samples were stored at −18°C for one month, no obvious trends in concentration changes of andrographolide and dehydroandrographolide were observed, and the recoveries of andrographolide and dehydroandrographolide still ranged between 86.4 and 94.7%.

**Method comparison with existing reports**

Many studies have reported the determination of andrographolide and dehydroandrographolide from biological samples, and the most commonly used method begins with the extraction of the two analytes from samples using organic reagent, followed by centrifugation (9–20). Large volumes of toxic and hazardous organic solvents (methanol, ethanol, acetonitrile or ethyl acetate) were used in these processes. Compared with the previously mentioned extraction step, this method is environmentally friendly due to the employment of surfactant.

Reports about the application of surfactants on the extraction of andrographolide and dehydroandrographolide from plasma are relatively uncommon. Among them, Liu et al. analyzed andrographolide and dehydroandrographolide in chicken plasma (46). The LLE method was used for the sample clean-up in this report. The intra-day and inter-day precisions (RSD) and recoveries were 3.2–8.7% and 91.1–98.4%, respectively. The LODs of the two analyses were 0.016 μg/mL. Chen et al. determined andrographolide and dehydroandrographolide in rat plasma by online coupling of SPE with HPLC (12). The intra-day and inter-day precisions (RSD) of the two analytes were in the range of 1.2–6.5% and the recoveries were between 92.0 and 102.1%. The LODs were 0.019 μg/mL for andrographolide and 0.022 μg/mL for dehydroandrographolide. Xu et al. determined andrographolide in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS) (17). The RSDs of intra-day and inter-day precisions were less than 7.2% and the recovery was 85.5–99.9%. The low limit of quantification for the determination of andrographolide was 0.001 μg/mL. Gu et al. analyzed andrographolide in human plasma by HPLC–ESI-MS (18). The limit of quantification was 0.01 μg/mL. The mean extraction recovery of andrographolide was 84.3% and the RSD values were all below 9.3%. In the current method, the LODs of...
andrographolide and dehydroandrographolide were 0.032 and 0.019 mg/mL, respectively. The RSDs of intra-day and inter-day precisions were less than 8.6% and the recoveries were in the range of 76.8–98.6% at three fortified concentrations. Compared with the existing reports, the proposed method provides adequate precision, recovery and LOD. The volumes of organic solvents used in previous methods were larger than the proposed method, although the LC–MS method provided low LODs for determining the analytes. The latter method could simplify the analytical procedure with an online analysis, but the instrument setup is complicated. It is obvious that the current method is demonstrated to be green and effective, with little organic solvent consumption.

**Application of the method**

To demonstrate the applicability of the proposed method, it was used to determine andrographolide and dehydroandrographolide in various plasma samples. No andrographolide and dehydroandrographolide at detectable levels were found in these samples. Recovery and RSDs studies were conducted by spiking plasma samples at three different fortified concentrations of 0.1, 0.5 and 1.0 µg/mL. The results are shown in Table II. The recoveries obtained for different plasma samples are not significantly different and are all in the range of 75.8–92.1% at all three fortified levels. The RSDs range from 2.5 to 8.3%. The proposed method was successfully applied to determine andrographolide and dehydroandrographolide in different human plasma obtained from healthy volunteers.

**Conclusions**

The CPE technique has been successfully applied as an effective method for the extraction of andrographolide and dehydroandrographolide from human plasma samples. Coupled with HPLC–UV, the method has been proven to be simple, rapid and reliable for the assay of andrographolide and dehydroandrographolide in biological samples. An important aspect is that the CPE process consumes little organic solvent consumption, which turns it into a low-cost and environmentally friendly technique.

**Acknowledgment**

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**References**


2. Shen, Y.C., Chen, C.F., Chiou, W.F.; Andrographolide prevents oxygen radical production by human neutrophils: Possible mechanism(s) involved in its anti-inflammatory effect; *British Journal of Pharmacology*, (2002); 135: 399–406.


### Table I

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<th>Inter-day precision</th>
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<td>0.1 µg/mL</td>
<td>0.5 µg/mL</td>
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<tr>
<td></td>
<td>Recovery (%)</td>
<td>RSD (%)</td>
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<td>Andrographolide</td>
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<td>7.3</td>
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<td>Dehydroandrographolide</td>
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### Table II

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<td>RSD(%)</td>
<td>Recovery (%)</td>
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24. Vara, L.E.G., Alfaro, B.L.; Separation of membrane proteins according to their hydrophathy by serial phase partitioning with Triton X-114; Analytical Biochemistry, (2009); 387: 280–286.

25. Ma, R., Zhao, J., Du, H.C., Tian, S., Li, L.W.; Removing endotoxin from plasmid samples by Triton X-114 isothermal extraction; Analytical Biochemistry, (2012); 424: 124–126.


29. Saitoh, T., Hinze, W.L.; Concentration of hydrophobic organic compounds and extraction of protein using alkylammoniosulfate zwitterionic surfactant mediated phase separations (CPE); Analytical Chemistry, (1991); 63: 2520–2525.
biphenyls (PCBs) in water; *Fresenius’ Journal of Analytical Chemistry*, (1997); 357: 743–746.

42. Şahin, C.A., Efecinar, M., Satiroglu, N.; Combination of cloud point extraction and flame atomic absorption spectrometry for preconcentration and determination of nickel and manganese ions in water and food samples; *Journal of Hazardous Materials*, (2010) 176: 672–677.


44. Wen, X.D., Deng, Q.W., Ji, S.L., Yang, S.C., Peng, L.; Design of rapidly synergistic cloud point extraction of ultra-trace lead combined with flame atomic absorption spectrometry determination; *Microchemical Journal*, (2012); 100: 31–35.


46. Wang, T., Gao, X., Tong, J., Chen, L.; Determination of formaldehyde in beer based on cloud point extraction using 2,4-dinitrophenylhydrazine as derivative reagent; *Food Chemistry*, (2012); 131: 1577–1582.

47. Santana, C.M., Ferrera, Z.S., Rodríguez, J. J. S.; Use of non-ionic surfactant solutions for the extraction and preconcentration of phenolic compounds in water prior to their HPLC-UV detection; *Analyt*, (2002); 127: 1031–1037.