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Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 867–873
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Micelle-mediated extraction and cloud point preconcentration for the analysis of aesculin and aesculetin in *Cortex fraxini* by HPLC

Zhihong Shi *, Xiaomin Zhu, Hongyi Zhang

College of Chemistry and Environmental Science, Hebei University, Baoding 071002, China

Received 17 October 2006; received in revised form 28 March 2007; accepted 31 March 2007
Available online 6 April 2007

Abstract

In this paper, a micelle-mediated extraction and cloud point preconcentration method was developed for the determination of less hydrophobic compounds aesculin and aesculetin in *Cortex fraxini* by HPLC. Non-ionic surfactant oligoethylene glycol monoalkyl ether (Genapol X-080) was employed as the extraction solvent. Various experimental conditions were investigated to optimize the extraction process. Under optimum conditions, i.e. 5% Genapol X-080 (w/v), pH 1.0, liquid/solid ratio of 400:1 (ml/g), ultrasonic-assisted extraction for 30 min, the extraction yield reached the highest value. For the preconcentration of aesculin and aesculetin by cloud point extraction (CPE), the solution was incubated in a thermostatic water bath at 55 °C for 30 min, and 20% NaCl (w/v) was added to the solution to facilitate the phase separation and increase the preconcentration factor during the CPE process. Compared with methanol, which was used in Chinese Pharmacopoeia (2005 edition) for the extraction of *C. fraxini*, the extraction efficiency of 5% Genapol X-080 reached higher value.

Keywords: Micelle-mediated extraction; Cloud point preconcentration; Genapol X-080; *Cortex fraxini*; Aesculin; Aesculetin

1. Introduction

Cortex fraxini (Chinese name Qin-pi), which comes from the dry tegument of different categories of *fraxini chinensis Roxb*, has been widely used in China for over 2000 years [1]. It has the therapeutic effect of clearing away pathogenic heat and removing the toxin, drying dampness, nourishing the liver and improving vision. It is also effective in the treatment of diarrhoea, cough and some gynecopathy. Furthermore, data indicate that *C. fraxini* has some anti-cancer effect [2]. Aesculin and aesculetin are the main effective constituents of *C. fraxini*. These two compounds have coumarin as their parent structure (as shown in Fig. 1).

Methods commonly used for the determination of aesculin and aesculetin in *C. fraxini* are high performance liquid chromatography (HPLC) [3], high performance capillary electrophoresis (HPCE) [1,2,4,5]. In Chinese Pharmacopoeia (2005 edition) [6], aesculin and aesculetin are selected to be determined by HPLC for the quality control of *C. fraxini*. Before HPLC analysis, powders of *C. fraxini* are refluxed with methanol for 1 h to extract aesculin and aesculetin. This conventional extraction method not only use large volume of toxic organic solvent but also need a long time for the extraction. Some simple and environmental-friendly method should be established.

The micelle-mediated extraction and cloud point preconcentration method offers a convenient alternative to the conventional extraction systems. It bases on the surfactant's special properties: one is good capacity to solubilize solutes of different character and nature, the other is their cloud point behavior. When the temperature rises above the cloud-point temperature, the solution separates into two phases: the small volume of surfactant-rich phase and the large volume of aqueous phase. The small volume of the surfactant-rich phase allows us to preconcentrate the analytes [7–12]. This methodology offers the advantages of safety, low cost, ability to concentrate solutes, easy disposal of surfactant, and low toxicity compared with classical organic solvents, etc. [13]. Up till now, the majority of cloud point extraction (CPE) applications have been reported to deal with analytes

* Corresponding author. Tel.: +86 312 5079357; fax: +86 312 5079525.
E-mail address: shizhihong@mail.hbu.edu.cn (Z. Shi).

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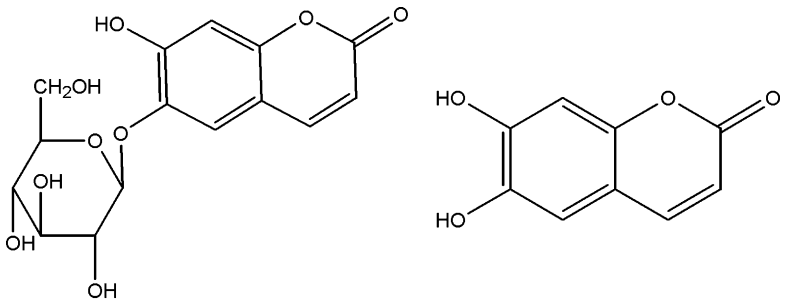
doi:10.1016/j.jpba.2007.03.035



aesculin aesculetin

Fig. 1. Molecular structures of aesculin and aesculetin.

present in aqueous samples, CPE has rarely been reported to be used for the extraction and preconcentration of chemical constituents from solid materials, especially from plants or herbal materials. Fang [14] and Choi [15] have reported the extraction of ginsenosides from Chinese herbal medicine with Triton X-100 as extractant. In our previous work, we have studied the feasibility of employing non-ionic surfactant solution as an alternative and effective solvent for the extraction of tanshinones from Salvia miltiorrhiza [16] and isoflavone daidzein from Puerariae radix [17].

In this paper, the application potential of the micelle-mediated extraction and cloud point preconcentration method has been further evaluated by employing non-ionic surfactant oligoethylene glycol monoalkyl ether (Genapol X-080) for the extraction and preconcentration of less hydrophobic compounds aesculin and aesculetin from *C. fraxini*. The extraction and preconcentration method established in this work includes two steps: the first step is to extract aesculin and aesculetin from solid herbal materials into aqueous surfactant solution; the second step is to preconcentrate them by phase separation based on the cloud-point phenomenon of the surfactant. To optimize the micelle-mediated extraction step, the influences of pH and concentration of the surfactant solution, granularity of the plant powder, liquid/solid ratio and ultrasonic assisted extraction time were all investigated. For the preconcentration of aesculin and aesculetin by cloud point extraction (CPE), the effects of the equilibration time and temperature as well as the amount of electrolyte on the recovery of the analytes were all studied.

2. Experimental

2.1. Plant materials

C. fraxini was purchased from local pharmaceutical store (Baoding, China), which had already been cut into pieces. The dried plant materials were pulverized and sieved to produce samples with particle sizes of >420 μm, 250–420 μm, 150–178 μm, 74–104 μm and <74 μm.

2.2. Chemicals and reagents

Authentic standards of aesculin and aesculetin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Non-ionic surfactant oligoethylene glycol monoalkyl ether (Genapol X-080) was obtained from Fluka (USA) and used as received without further purification. Various concentrations (w/v) of aqueous surfactant solutions were prepared by weighing appropriate amounts of the surfactant and by directly dissolving the surfactant in double-distilled water. All other reagents used in this work were of analytical grade.

2.3. Apparatus

All analyses were performed on a Shimadzu LC-6A liquid chromatograph (Shimadu, Japan) equipped with a solvent delivery pump, Shimadzu SPD-M6A photodiode array UV–VIS detector and a 7125-injection valve with 20 μl loop. The chromtographic data were recorded and processed with the Shimadzu SPD-M6A software. Supelcosil LC-18-DB chromatographic column (150 mm × 4.6 mm i.d., 5 μm) was used and the column temperature was controlled at 30 °C.

A versatile plant pulverizer (Foshan, Guangdong, China) was used to make the plant materials into powder. A KQ-250 ultrasonic generator from the Kunshan Company (Jiangsu, China) was used to extract the effective compounds from the samples. Cloud-point extraction was carried out in a water bath with a thermostat.

Sieves (Zhejiang, China) were used to sieve the *C. fraxini* powder.

High-speed centrifuge was employed to centrifuge the sample solutions (Model 800, Shanghai, China).

A votex mixer (WH-861 tai cang) was used to blend the solution adequately.

2.4. Procedures for the extraction and preconcentration of *C. fraxini*

2.4.1. Extraction procedure

Powders of *C. fraxini* were accurately weighed and placed in a 10 ml centrifuge tube; Genapol X-080 solution of various concentrations (pH 1.0) was added. To extract the *C. fraxini*, the tube was capped and blended adequately, then placed in the ultrasonic cleaning bath to assist extraction, the *C. fraxini* extracts were centrifuged for 10 min; the supernatant was filtered through 0.45 μm membrane and then injected into the HPLC system for analysis.

2.4.2. Preconcentration procedure

To study the preconcentration of the extracted *C. fraxini* by phase separation of the aqueous surfactant solution, an appropriate amount of sodium chloride was added to the sample solution and votex-dissolved for 2 min. The sample solution was then kept in a thermostatic water bath at appropriate temperature until the solution completely separated into two distinct phases—the upper phase was the small volume of surfactant-rich phase and the lower phase was the large volume of aqueous phase. After centrifugation for 10 min, the aqueous phase was sucked out using a syringe with long pinhead, and the sticky surfactant-rich phase was left in the tube. Methanol was added to lower the viscosity of the surfactant-rich phase. After filtration through a 0.45 μm nylon membrane, 10 μl of the solution was injected into the HPLC system for analysis.

2.5. HPLC analysis

The HPLC mobile phase was a mixture of methanol–0.1% phosphoric acid (30:70, v/v). The flow rate was 0.9 ml min^{-1}; the detection wavelength was set at 357 nm. Peaks in the chromatograms were identified by comparing the retention times and UV spectra with those of the authentic aesculin and aesculetin. Peak area was used for quantification. To avoid the potential influence of Genapol X-080 to the separation of aesculin and aesculetin, the column was flushed with methanol to completely elute Genapol X-080 after each day's work.

3. Results and discussion

3.1. Optimization of the micelle-mediated extraction conditions

To optimize the micelle-mediated extraction of aesculin and aesculetin from the solid herbal materials, a number of experiments under different conditions were performed. The type of surfactant, pH and concentration of the surfactant solution, granularity of the plant powder, liquid/solid ratio and extraction time were all investigated.

3.1.1. Selection of the surfactant

Non-ionic surfactant Genapol X-080 is a polyoxyethylene glycol mono ether-type surfactant that has eight oxyethylene units and tridecyl alkyl moieties [$HO(CH_2CH_2O)_8(CH_2)_{13}H$], its molecular weight is 553, critical micellar concentration (CMC) = 0.05 mmol/l (0.028 g/l), and HLB = 13. Several research groups have successfully used Genapol X-080 in the extraction procedures [8,18,19]. Compared with Triton X series surfactant, Genapol X-080 does not absorb above 210 nm, thus it will not interfere with the determination of aesculin and aesculetin. So, Genapol X-080 was chosen as the CPE surfactant in this paper.

3.1.2. Effect of acidity of solution and the surfactant concentration on the extraction efficiency

It can be judged from the structure of aesculin that it is a kind of glycoside, which is easy to hydrolyze to produce aesculetin and glucose. We have studied the effect of the acidity of the solution on the stability of the *C. fraxini* extract. Experimental results showed that the transformation of aesculin to aesculetin could be suppressed when the pH of the solution was less than 1.0. So the surfactant solution was ajusted to pH 1.0 with hydrochloric acid.

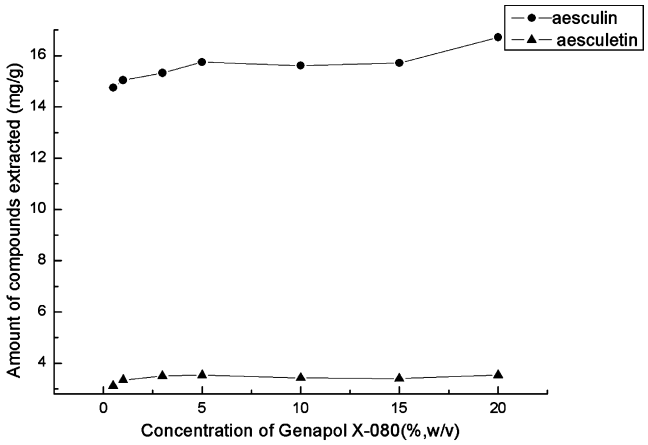


Fig. 2. Effect of concentration of Genapol X-080 (%, w/v) on the extraction efficiency of aesculin and aesculetin. Other extraction conditions: granularity: less than 74 μm; liquid/solid ratios: 100 (ml/g); ultrasonic-assisted extraction time: 30 min.

The ability of the aqueous non-ionic Genapol X-080 solution in extracting aesculin and aesculetin may be related to the solubility-enhancement effect of the surfactant micelles. It can be seen from Fig. 2 that the amount of extracted aesculin and aesculetin increases when the surfactant concentration increases from 0.5% to 5% (w/v) and then tends to remain fairly constant in the surfactant concentration range of 5–15%. When the surfactant concentration rises to 20%, the amounts of aesculin and aesculetin extracted are both ascending, but the solution will become too sticky to be dealt with. Considering the extraction efficiency and maneuverability, 5% (w/v) was chosen as the optimum surfactant concentration for further studies.

3.1.3. Effect of granularity of the plant powder on the extraction efficiency of C. fraxini

Granularity is another important factor influencing the extraction efficiency. In our experiment, a series of granularity (>420 μm, 250–420 μm, 150–178 μm, 74–104 μm and <74 μm) were compared. The result shows that the extraction efficiency reaches the highest value when plant powders of 74–104 μm were used. So plant powders of 74–104 μm were chosen for further studies.

3.1.4. Effect of liquid/solid ratio on the extraction efficiency of aesculin and aesculetin

Liquid/solid ratio is the ratio between the volume of solvent to the amount of crude material. It is one of the factors influencing the extraction efficiency of aesculin and aesculetin. As shown in Fig. 3, when the liquid/solid ratio is 400:1 (8 ml/0.02 g), extraction efficiency reaches the highest value. If less amount of *C. fraxini* was used, the reproducibility of the extraction would be

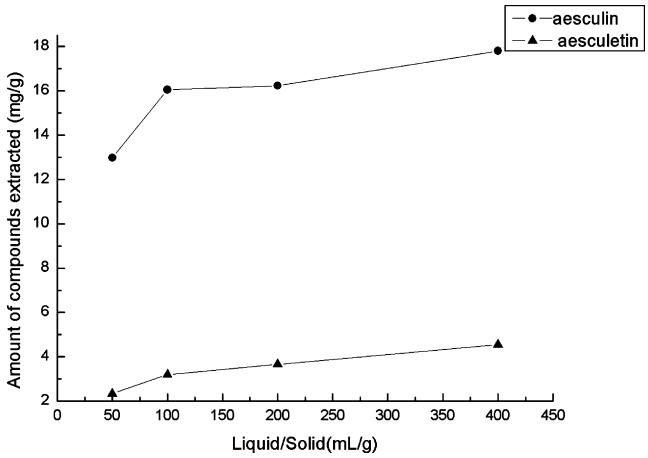


Fig. 3. Effect of liquid/solid ratio on the extraction efficiency of aesculin and aesculetin. Other conditions: concentration of Genapol X-080: 5% (m/v), granularity: 74–104 μm, ultrasonic-assisted extraction time: 30 min.

poor. So the liquid/solid ratio of 400:1 (ml/g) was employed in the following experiments.

3.1.5. Effect of ultrasonic assisted extraction time on the extraction efficiency of aesculin and aesculetin

The effect of ultrasonic assisted extraction time on the extraction efficiency of aesculin and aesculetin was studied by varying the extraction time from 5 to 60 min. The results indicated that the amount of extracted aesculin and aesculetin increased with the increase of extraction time and the extraction efficiency reached the highest value for aesculin at 30 min. As for aesculetin, the extraction efficiency kept the highest value between 20 and 30 min. Thus 30 min was selected as the best extraction time.

3.2. Comparison of the extraction efficiency of Genapol X-080 with conventional extraction solvent-methanol

Compared with methanol, 5% Genapol X-080 has higher extraction efficiency under identical experimental conditions. With Genapol X-080 as extractant, determined content of aesculin is 17.80 mg/g and aesculetin is 4.55 mg/g, while determined content of aesculin is 16.85 mg/g and aesculetin is 3.40 mg/g employing methanol as extractant.

3.3. Optimization of the preconcentration conditions

As we have mentioned, the cloud point extraction of aesculin and aesculetin from *C. fraxini* includes two steps: the first step is to extract the aesculin and aesculetin from solid herbal materials into aqueous surfactant solution; the second step is to preconcentrate them by phase separation based on the cloud-point phenomenon of the surfactant. Now that the first step has been finished, we will study on the cloud point preconcentration of the aesculin and aesculetin.

From literature [13,14] and our previous work [16], we know that adding electrolyte into the solution and heating the solution above the cloud-point temperature for some time will facilitate the separation of the two phases. In this part, we will study on the effect of the equilibration time and temperature as well as the amount of electrolyte on the recovery of the aesculin and aesculetin during the cloud-point extraction step. The recovery of aesculin and aesculetin was calculated using the following equation:

$$\text{extraction recovery} = \frac{\text{amount of analyte determined after CPE}}{\text{amount of analyte determined before CPE}} \times 100\%$$

Table 1
Cloud point temperature of Genapol X-080

Concentration of Genapol X-080 (%, g/ml)	Cloud point temperature (°C)
0.3	39.5
1	34.5
2	29.5
3	26.0
4	25.5
5	26.0
10	30.5
20	46.5

3.3.1. Cloud-point temperature of the surfactant

The cloud-point temperature of Genapol X-080 at different concentraton was determined by observing the temperature required for the onset of turbidity upon heating a 10.0 ml aqueous solution of the surfactant in a colorimetric tube that had been placed in a controlled temperature water bath, the slope of the temperature change of the bath was about 1 °C per 10 min. The results are summarized in Table 1. The effect of the salt concentration on the cloud-point temperature of 1% Genapol X-080 (w/v) was studied by adding different amount of sodium chloride into the surfactant solution. The results are shown in Table 2. It can be seen from Table 2 that the cloud point temperature of Genapol X-080 decreases with the increase of the concentration of sodium chloride. Although turbidity could be observed at lower temperature when the concentration of sodium chloride was increased, relatively higher temperature was still needed to facilitate the complete phase separation process.

3.3.2. The effect of the amount of sodium chloride on the recovery of the aesculin and aesculetin after CPE

It has been reported that the addition of an inert salt can facilitate the separation of the two phases for some non-ionic surfactant systems, since it increases the density of the bulk

Table 2
Cloud point temperature of Genapol X-080 with different concentration of sodium chloride in its 1% surfactant solution

Concentration of sodium chloride (mol/l)	Cloud point temperature (°C)
0.01	34.0
0.1	33.5
0.2	30.5
0.4	27.0
1.0	19.0

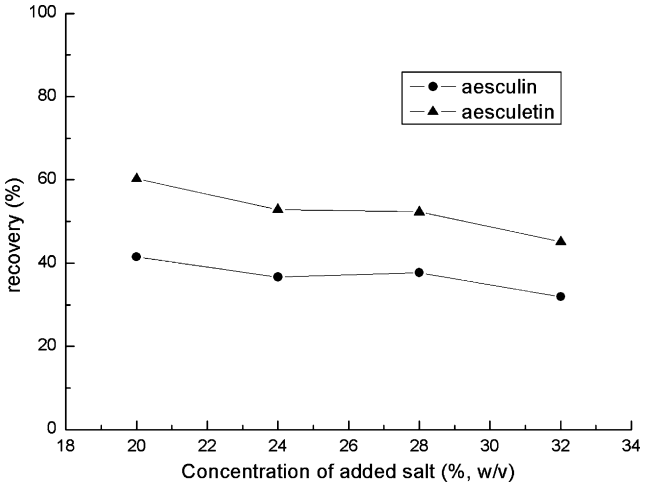


Fig. 4. The effect of the amount of sodium chloride on the recovery of aesculin and aesculetin after CPE . Other conditions: equilibration temperature: 55 °C; equilibration time: 30 min.

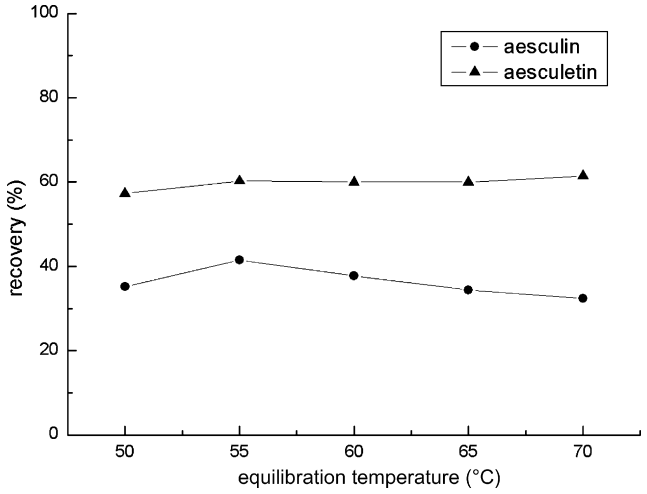


Fig. 5. The effect of equilibration temperature on the recovery of aesculin and aesculetin after CPE. Other conditions: amount of salt: 20% (w/v); equilibration time: 30 min.

aqueous phase [13]. In this paper, sodium chloride was chosen as the modifier. The study of the influence of the ionic strength on the extraction recovery was carried out by varying the concentration of NaCl between 20% and 32% (w/v), and by keeping the equilibration temperature at 55 °C for 30 min. The result shows that the addition of NaCl facilitates the separation between the surfactant-rich phase and the aqueous phase. With the increase of the salt concentration, the micelle size and the aggregation number are increased and the critical micellar concentration remains constant. In addition, analytes may become less soluble in the solution at higher salt concentrations and thus contribute to higher recoveries. That is, the inert salt increases the extraction recovery by decreasing the solubility of the organic species in the aqueous phase. The result obtained from Fig. 4 indicates that the CPE at salt concentration of 20% (w/v) gives the optimum extraction recovery. When the concentration was less than 20% (w/v), the phase separation time will be too long. So 20% (w/v) NaCl should be chosen for the effective extraction.

3.3.3. The effect of equilibration temperature on the recovery of aesculin and aesculetin after CPE

The dependence of the recovery of aesculin and aesculetin on the equilibration temperature is shown in Fig. 5. When the incubation temperature increases from 50 to 55 °C, the extraction recovery of each compound increases. Between 55 and 70 °C, the recovery of aesculin declines and the recovery of aesculetin keeps constant. Considering the extracted compounds are remedies and lower temperature is beneficial in maintaining their biological activities, 55 °C was selected for further studies.

3.3.4. The effect of equilibration time on the recovery of aesculin and aesculetin

The influence of equilibration time on the recovery of the aesculin and aesculetin was studied by varying the equilibration time between 10 and 50 min, and by keeping the equilibration temperature at 55 °C and the salt concentration at 20%. The extraction recovery depends on the time that the analytes need to interact with the micelles and get into their cores [20]. As shown in Fig. 6, the recovery for aesculin increases when the incubation time increases from 10 to 30 min. Between 30 and 40 min, the recovery remains fairly constant. After 40 min, the extraction recovery declines. Aesculetin has the highest recovery between 20 and 30 min. So, 30 min was chosen as the optimum equilibration time for the cloud-point preconcentration procedure.

From the above discussion, it could be seen that under the optimum cloud point preconcentration conditions (incubated in a thermostatic water bath at 55 °C for 30 min, with 20% NaCl added into the solution), the extraction recoveries for aesculin and aesculetin were $41.47 \pm 1.32\%$ and $60.29 \pm 1.59\%$ ($n = 5$), respectively. The low recovery was attributed to the hydrophilicity of aesculin and aesculetin. Their log P values were calculated to be -0.79 for aesculin and 1.05 for aesculetin. As hydrophilic compounds, aesculin and aesculetin are not easy to be com-

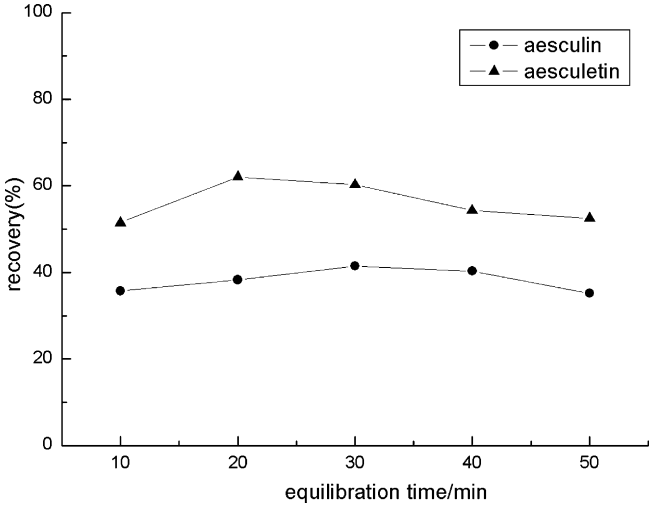


Fig. 6. The effect of equilibration time on the recovery of aesculin and aesculetin after CPE. Other conditions: the salt amount: 20% (w/v); equilibration temperature: 55 °C.

pletely extracted into the surfactant-rich phase. Compared with aesculin, aesculetin is more hydrophobic, so it is much easier to be extracted into the upper surfactant-rich phase during the cloud-point extraction procedure. That is why the recovery of aesculetin (60.29 ± 1.59%) is much higher than that of aesculin (41.47 ± 1.32%).

3.3.5. Preconcentration factor of the CPE method

In this paper, the preconcentration factor (C_F) was calculated as the ratio of the volume of the original sample solution to that of the obtained surfactant-rich phase. For the convenient measurement of the volume of the surfactant-rich phase, larger volume of 5% Genapol X-080 solution was used and the following experiment was carried out: 0.020 g *C. fraxini* was accurately weighed and placed into a 50 ml centrifuge tube, 25 ml 5% Genapol X-080 solution was added. Under the optimized cloud point extraction and preconcentration conditions, a surfactant-rich volume of 2 ml was obtained and the preconcentration factor (C_F) is 12.5. To reduce the viscosity of the surfactant-rich phase, methanol was added into the tube, the final volume of the analyte is 5 ml, which leads to a final preconcentration factor of 5.

3.4. HPLC profiles of the extracted aesculin and aesculetin

Typical HPLC profiles of authentic aesculin and aesculetin as well as the extracted and preconcentrated aesculin and aesculetin are shown in Fig. 7. In Fig. 7, A is the chromatogram of aesculin and aesculetin extracted from *C. fraxini* prior to cloud point preconcentration, B is the chromatogram of aesculin and aesculetin after cloud point preconcentration and C is the chromarogram of authentic aesculin and aesculetin. The preconcentration effect of cloud point extraction is clearly demonstrated in Fig. 7A and B.

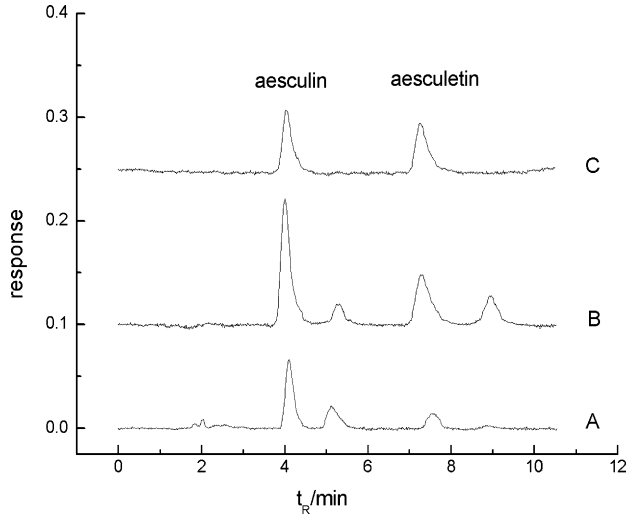


Fig. 7. HPLC chromatograms of authentic aesculin and aesculetin as well as extracts from *C. fraxini*. Chromatographic conditions: mobile phase was a mixture of methanol–0.1% phosphoric acid (30:70, v/v); the flow rate was 0.9 ml min^{-1}; the detection wavelength was set at 357 nm. (A) The chromatogram of aesculin and aesculetin extracted from *C. fraxini* prior to cloud point preconcentration, (B) the chromatogram of aesculin and aesculetin after cloud point preconcentration, (C) the chromarogram of authentic aesculin and aesculetin.

Possessing no aromatic moiety, Genapol X-080 does not absorb above 210 nm, as shown in Fig. 7A and B, it does not interfere with the determination of aesculin and aesculetin.

3.5. Method validation

Peak area was used for the quantification of the extracted aesculin and aesculetin. Calibration graphs were obtained by plotting the peak area (y) versus concentration (x). Calibration curves were linear from 0.0048 to 0.4820 mg/ml for aesculin ($Y = 0.1775 + 325.2195X$, $r = 0.9997$, $n = 7$), from 0.0029 to 0.2874 mg/ml for aesculetin ($Y = -1.0305 + 613.1158X$, $r = 0.9996$, $n = 7$).

The repeatability of the HPLC profile was determined by injecting the same processed sample five times on the same day. The RSD of peak area was 0.67% for aesculin and 0.74% for aesculetin ($n = 5$).

The reproducibility of the established CPE method was determined by processing five replicates of samples. The RSD of the extracted amount of analyte was 2.53% for aesculin and 4.07% for aesculetin before CPE. The RSD of the extracted amount of analyte was 3.16% for aesculin and 2.77% for aesculetin after CPE preconcentration.

4. Conclusions

The results obtained in this study indicate that non-ionic surfactant Genapol X-080 solution is an effective alternative for the extraction of less hydrophobic compounds—aesculin and aesculetin from *C. fraxini*. Compared with commonly used organic solvent-methanol, 5% Genapol X-080 solution has the highest extraction efficiency. The present work further demonstrates that micelle-mediated extraction is a potentially powerful tool for the extraction of active ingredients from herbal medicines. From the analytical point of view, it provides the possibility of extracting and preconcentrating analytes of different polarities in a simple procedure. As Genapol X-080 does not absorb above 210 nm, the extracts can be directly injected into HPLC for analysis without removal of the surfactant. This method is also valuable in the large-scale extraction and purification of active ingredients from herbal materials. Non-ionic surfactant can be removed via hydrophobic adsorption of the surfactant with polystyrene resins [21] The resins can be added batchwise to the preparation and removed, together with the bound surfactants, simply by centrifugation.

As for the preconcentration effect of aesculin and aesculetin by cloud-point extraction, the extraction recovery was much lower than tanshinones which show strong hydrophobicity [16]. This phenomenon indicates that Genapol X-080 is more suitable for the cloud-point preconcentration of hydrophobic compounds than for hydrophilic ones.

Acknowledgements

Financial support from the National Natural Science Foundation of China (20575016) and the Natural Science Foundation of Hebei Province China (B2006000953) are gratefully acknowl-

edged. The authors also thank the financial support from 'the introduction of talents' programme of Hebei University.

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