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Simultaneous separation and determination of four phenylethanoid glycosides in rat plasma sample after oral administration of *Cistanche salsa* extract by microemulsion liquid chromatography

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Abstract

A simple, rapid and specific method was developed to separate as well as to determine the four phenylethanoid glycosides (PhGs) (echinacoside, tubuloside B, acteoside and isoacteoside) in rat plasma after oral administration of *Cistanche salsa* extract by reversed phase high performance liquid chromatography using a microemulsion as the mobile phase. The separations were performed on a Zorbax Extend-C18 column at 25°C. Photodiode-array detector was conducted at 322 nm and with a flow rate of 0.8 mL min⁻¹. The optimized microemulsion mobile phase consisted of 0.3% triethylamine in 20 mM phosphoric acid at pH 6.0, 0.8% (v/v) ethyl acetate as oil phase, 1.5% (v/v) Genapol X-080 as surfactant, 2.5% (v/v) n-propanol as co-surfactant. Under the optimal conditions, the calibration curve for four PhGs was linear in the range of 10-1000 ng mL⁻¹ with the correlation coefficients greater than 0.9994. The intra-day and inter-day precision (RSD) were below 8.64 % and the limits of detection (LOD) for the four PhGs were 0.4-1.3 ng mL⁻¹ (S/N=3). The microemulsion liquid chromatography (MELC) method was successfully
applied to separate and determine the four PhGs in rat plasma after oral administration of
*Cistanche salsa* extract.

**Keywords**: *Cistanche salsa*; Phenylethanoid glycosides; Microemulsion liquid chromatography; Rat plasma

1. Introduction

The stem of *Cistanche salsa*, a parasitic plant native to northwest China, is a kind of traditional Chinese herbal medicine and used for the treatment of kidney deficiency, female infertility, morbid leucorrhea, neurataxia as well as senile constipation [1]. The phytochemical investigations showed that Echinacoside (ECH), Tubuloside B (TUBB), Acteoside (ACT) and Isoacteoside (ISO) are the four main Phenylethanoid glycosides of *Cistanche salsa* extract, which were regard as the main active and marker components [2].

Quantification and pharmacokinetics studies on constituents of Traditional Chinese Medicine in plasma are required to offer suitable references in clinical application. Several methods, such as high-performance liquid chromatography (HPLC) [3-10] have been reported for the analysis of the four PhGs in a variety of sample matrices. However, to our knowledge, there has been no publication on the simultaneous separation and determination of the four PhGs in plasma sample.

In HPLC analysis it has always been important to minimize the sample preparation in order to eliminate all the possible errors and losses. A direct injection of biological sample is preferred whenever possible. However, the presence of organic solvents in the conventional mobile phase in the concentration above 5% tend to precipitate proteins and makes direct HPLC analysis impossible. At the same time, the conventional HPLC methods may cause an environmental problem because of the large amounts of organic solvents needed. For overcoming these analytical problems in liquid chromatography, the microemulsion as mobile phase was employed in past few years.

Microemulsion is liquid disperse systems containing organic solvent (oil), water, surfactant and co-surfactant [11]. It can be formed spontaneously, constituting thermodynamically stable dynamic structures with the appearance of clear transparent liquids of relatively low viscosity. Many substances of hydrophilic and hydrophobic character can solve in microemulsion. In comparison to conventional hydro-organic mobile phase, the phases used in microemulsion liquid
chromatography are less flammable, inexpensive, non-toxic and biodegradable. Both hydrophilic and hydrophobic samples are often easily dissolved in these phases. Solutes with different polarities can be separated in a single run owing to the interactions with the stationary and mobile phase components, then the separations being highly reproducible [12]. Microemulsion is classified as either oil-in-water (O/W) or water-in-oil (W/O), where the O/W microemulsion is the preferred for HPLC [13]. Recently, it has been proved that the use of microemulsions as mobile phases in HPLC is feasible and provides selectivity and separation efficiency comparable to or greater than those of conventional HPLC systems [13–18]. The advantage of the microemulsion systems which has also been proved for MELC is short retention time and low cost as it avoids procedures such as protein precipitation used in advance of a conventional HPLC analysis [19].

In the present work, we investigated the possibility of separation and determination of the four PhGs in rat plasma after oral administration of *Cistanche salsa* extract by reversed phase high performance liquid chromatography using a microemulsion as the mobile phase. Moreover, the effect of operating parameters on the separation and determination performance was studied.

2. Experimental

2.1. Chemicals and reagents

ECH (C_{35}H_{46}O_{20}, MW=786.73), TUBB (C_{32}H_{40}O_{16}, MW= 680.65), ACT (C_{20}H_{30}O_{15}, MW= 624.59), ISO (C_{29}H_{36}O_{15}, MW= 624.59) and *p*-coumaric acid (internal standard) (IS) (C_{9}H_{8}O_{3}, MW=164.16) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their structures are shown in Fig.1. Non-ionic surfactant C_{13}E_{8} (Genapol X-080) was obtained from Sigma (St. Louis, MO, USA) and used without further purification. Various concentrations (v/v) of aqueous surfactant solutions were prepared by weighing certain amounts of the surfactant and by directly dissolving the surfactant in distilled water. Heptanes, ethyl acetate, *n*-octanol, toluene, *n*-hexane and *n*-propanol were obtained from Merck (Darmstadt, Hessen, Germany). Phosphoric acid and triethylamine (Beijing Chemical Factory, AR Beijing, China) were prepared before the experiment. All other reagents used in this work were of analytical grade. Distilled water (Millipore, Bedford, MA, USA) was used throughout the study.

2.2. Preparation of standard solutions
Stock solutions (50 μg mL\(^{-1}\)) of the four PhGs and IS solutions (10.0 μg mL\(^{-1}\)) were got by dissolving suitable amounts of each pure substance in microemulsion mobile phase and kept stable for 2 months when stored at 4°C in the refrigerator (assessed by HPLC).

2.3. Preparation of Cistanche salsa extract

The crude drug of Cistanche salsa was purchased from local drug stores. Fifty grams of crude drug of Cistanche salsa were extracted twice by refluxing with 75% ethanol for 1.5 h, and the extract solution was filtered. The filtered extract was then concentrated under reduced pressure and lyophilized to give an extract (4.26 g), which was stored at 4°C before use.

In order to calculate the administered dose of four PhGs, their contents in Cistanche salsa extract were quantitatively determined. The extract of Cistanche salsa was dissolved in microemulsion mobile phase and diluted to the concentration of 225 ng mL\(^{-1}\). Then 10 μL of this solution was injected into HPLC system for analysis. The contents of four PhGs (ECH, TUBB, ACT and ISO) in the extract of Cistanche salsa were determined to be 12.94, 1.23, 6.75 and 3.47 %, respectively.

2.4. Application of the method

Sprague–Dawley male rats (200±20 g) were purchased from the Experimental Animal Center of Fourth Military Medical University (Xi’an, China). The animals were pathogen-free and housed in an environmentally controlled breeding room (temperature maintained at 24±1°C and a 12:12 h light–dark cycle) for at least 1 week before experimentation. Standard laboratory food and water were available at all times except that food was withdrawn 18 h prior to initiation of the experiments. Before drug administration, the animals were fasted overnight. All animal procedures were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People’s Republic of China.

Aqueous solution of Cistanche salsa extract was orally administrated to six rats at a dose of 50 mg kg\(^{-1}\). Blood samples were collected in clean heparinized glass tubes after dosing.

2.5. Preparation of biological samples

Rats were anesthetized by pentobarbital sodium and blood was collected from abdominal artery in clean heparinized glass tubes. The blank plasma was separated by immediate centrifugation at
4000 rpm for 10 min and stored at -20°C until required. The frozen plasma was thawed at room temperature (22-25°C) before use.

About 0.1 mL of rat plasma sample and 20 μL of IS solution (50 ng mL⁻¹) were added to a 1.5 mL capped centrifugation tube. To each of these plasma samples in glass tubes, 0.5 mL of microemulsion mobile phase was added. The obtained solution was vortex mixed (CAY-1, Beijing Chang’an Instrumental Factory, China) for 5 min and centrifuged (Anke TCL-16G, Shanghai, China) (13,000 rpm for 5 min) at room temperature. A volume of 10 μL of the supernatant was injected into the HPLC for analysis.

2.6. Protein precipitation in the conventional HPLC analysis

Twenty microliters of internal standard stocking solution (50 ng mL⁻¹) were added to a 2 mL tube and the methanol was under the reduced pressure at room temperature. Then 0.1 mL plasma was added. After vortexing for 1 min, 1.5 mL of acetonitrile was added into the plasma sample, then the mixed solution was vortexed for 5 min and centrifuged for 5 min at 13,000 rpm. 1.0 mL of the supernatant was moved into another vial and evaporated under slight N₂ stream at 40°C. The residue was dissolved in 0.5 mL of methanol and filtered through a 0.45 μm filter. 10 μL of the filter liquid was injected into the HPLC system for analysis.

2.7. Chromatographic conditions

The analyses were completed using an Agilent 1100 series HPLC system (Palo Alto, CA, USA), including a quaternary pump, a photodiode array detector (G1315B), a column oven and a Rheodyne 7225i injector (10 μL). The analytical column was a Zorbax Extend-C₁₈ (100 mm×4.6 mm i.d., 3.5 μm particle diameter) column connected to Agilent Zorbax Extend-C₁₈ guard column (12.5 mm × 4.6 mm i.d., 3.5 μm particle diameter). The column temperature was controlled at 25°C. The microemulsion mobile phases were prepared by mixing all the microemulsion components and treating them in an ultrasonic bath for 20 min. Then it was filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA). A computer equipped with an Agilent Chemstation program for LC systems accomplished acquiring and processing chromatographic data. Peak area was evaluated as the analytical measurement.
Before analysis, the column was conditioned by flowing the mobile phase through the system
for 20 min at 0.8 mL min$^{-1}$. During the separation, the mobile phase flow rate was kept at 0.8 mL
min$^{-1}$ and peaks were detected at 332 nm.

2.8. Method validation

2.8.1. Calibration curve

By spiking the appropriate stock solution containing the IS at a constant concentration to 0.1
mL of blank plasma, six effective concentrations $10, 50, 100, 500, 750$ and $1000$ ng mL$^{-1}$ for
ECH, TUBB, ACT and ISO were obtained separately. The quality control (QC) samples were
prepared in blank plasma at the concentrations of $10, 100, 1000$ ng mL$^{-1}$ containing the IS at a
constant concentration, respectively. 10 µL of the spiked plasma samples (standards and quality
controls) were injected into the HPLC. The procedure was carried out in triplicate for each
concentration. The obtained analyte/IS peak area ratios were plotted against the corresponding
concentrations of the four PhGs and the calibration curves were set up by the least-squares method.
The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated
according to Chinese Pharmacopoeia [20] guidelines. The analyte concentrations gave rise to
peaks whose heights were 10 and 3 times the baseline noise, respectively.

2.8.2. Extraction recovery (absolute recovery)

By assaying the samples at three QC levels, absolute recoveries of ECH, TUBB, ACT and ISO
were determined. The analyte/IS peak area ratios were compared to those obtained from the direct
injection of the compounds which were dissolved in the mobile phase of the blank plasma at the
same theoretical concentrations. The extraction recovery values were calculated as follows:

$$\left(\frac{\text{analyte} / \text{IS peak area ratio}}{\text{corresponding standard}}\right)_{\text{spiked blank}} \times 100\%$$

2.8.3. Precision and accuracy

The precision, including intra-day and inter-day precision expressed as % relative standard
deviation (RSD) values, was assessed by assaying the samples at three QC levels. The intra-day
variance was determined by assaying the spiked samples five times during one day with the inter-
day variance assayed five consecutive days. The accuracy was evaluated by mean recovery and expressed as \((\text{mean measured concentration}) / (\text{spiked concentration}) \times 100\%\) and \% RSD values.

2.8.4. Selectivity

Blank plasma and drug plasma samples from rats were injected into the HPLC. The resulting chromatograms were checked for possible interference from endogenous substances and metabolites of ECH, TUBB, ACT and ISO. The acceptance criterion was no interfering peak in the place of an analyte peak.

2.8.5. Stability

To evaluate sample stability after freeze–thaw cycles and at room temperature, five replicates of QC samples at each of 10, 100 and 1000 ng mL\(^{-1}\) concentrations were subjected to three freeze–thaw (−20 to 25°C) cycles or were stored at room temperature (approximately 22-25°C) for 24 h in volumetric flask, respectively. Long-term stability was studied by assaying samples that had been stored at −20°C for a certain period of time (15 day) and assessed by comparing the mean concentration of the stored QC samples with the mean concentration of those prepared freshly. ECH, TUBB, ACT and ISO were considered stable under storage conditions if the assay percent recovery was found to be 85-115% of the nominal initial concentration [21].

3. Results and discussion

In this study, a microemulsion liquid chromatography was applied for the separation and determination of the four PhGs. Some important parameters affecting the separation and determination selectivity of the MELC system, such as surfactant, co-surfactant, oil phase and pH, were investigated and optimized.

3.1. Optimization of MELC conditions

3.1.1. Selection of surfactant

Previous studies using microemulsion as the mobile phase for HPLC has employed SDS as a surfactant. However, this kind of mobile phase cannot separate highly hydrophilic compounds that have very similar chemical properties [22]. So, non-ionic surfactant Genapol X-080 was chosen as the microemulsion surfactant in this work. Genapol X-080 is a polyoxyethylene glycol mono ether-type surfactant that has eight oxyethylene units and tridecyl alkyl moieties (MW=552). Because possessing no aromatic moiety, Genapol X-080 does not absorb above 210 nm, it will not
interfere with the determination of ECH, TUBB, ACT and ISO. Moreover, it is relatively cheap, available and non-toxic.

3.1.2. Concentration of surfactant

The adsorbed surfactant on the stationary phase of HPLC could have a direct impact on the retention of solutes and their partition with the stationary phase [23]. Different concentrations of Genapol X-080 were investigated (0.5%-2.5% v/v) (see Fig. 2). It was found that the retention of ECH, TUBB, ACT and ISO decreased with increasing the concentration of Genapol X-080 from 0.5% to 1.5%. This shows that Genapol X-080 may have modified the stationary surface and therefore reduce the retention time of the four PhGs. In this study, when the concentration of Genapol X-080 was below 0.5%, the microemulsion was hard to form or unstable. However, further increasing concentration to 2.5% had a very small effect on the retention time of the four PhGs. Therefore, 1.5% (v/v) Genapol X-080 was chosen as the optimal surfactant concentration for further studies.

3.1.3. Selection of the co-surfactant

The formation of microemulsion is encouraged by the addition of a co-solubiliser [24]. Short-chain alcohols, such as n-propanol, are completely miscible with water and influence mobile-phase hydrophobicity, which may affect retention. Different concentrations of co-surfactant n-propanol were studied in the range of 1.0–3.0 v/v (see Fig. 3). The result showed that retention time of ECH, TUBB, ACT and ISO decreased with increasing the concentration of n-propanol between 1.0 and 2.5% v/v. Nevertheless, a further increase of n-propanol concentration had shown no marked effect on the retention time. Therefore, 2.5% (v/v) n-propanol was used as co-surfactant in subsequent experiments.

3.1.4. Selection of the oil phase
In MELC, the type and concentration of oil have an obvious effect on the retention time of analytes [25]. Five types of organic solvent (heptanes, ethyl acetate, n-octanol, toluene and n-hexane) were investigated in this study. But it indicated that the types of oil such as heptanes, n-octanol, toluene and n-hexane were not forming microemulsion in the presence of Genapol X-080. So ethyl acetate was chosen as the oil type. Different concentrations of oil (ethyl acetate) were studied in the range of 0–1% v/v (see Fig. 4). When the concentration of oil was zero, the mobile phase would contain micelles. The micellar mobile phase gave longer retention times compared to microemulsion mobile phase. The addition of oil decreases the retention time of the four PhGs. This is due to the fact that microemulsion has a stronger elution capacity than that of the micellar solution [26]. A slight decrease in retention of analytes was observed with increasing the oil content above 0.8%. It is possible that the hydrophilic compounds such as ECH, TUBB, ACT and ISO have a high affinity for the continuous phase of the microemulsion. Therefore they are not partitioned as fully in the oil droplet. At last, 0.8% (v/v) ethyl acetate was used as oil phase.

3.1.5. Microemulsion pH

In MELC the retention of ionisable species is also affected by the pH of the micellar mobile phase [27]. As a microemulsion is a modification of a micellar system where a lipophilic organic solvent has been dissolved in the micelles, it was important to investigate the effect of the pH of the microemulsion eluent on the separation and retention of ECH, TUBB, ACT and ISO. The pH of the mobile phase was changed in the interval from 2 to 6 using increasing amounts of triethylamine (0.3% v/v) in phosphoric acid (20 mM) (see Fig. 5). The result showed us that retention time of ECH, TUBB, ACT and ISO decreased with increasing the pH value. Maybe the four PhGs were weak acid compounds and the ionization of the four PhGs increased at higher pH values. Thus, they become less hydrophobic and retention times decrease due to decreased retention on the stationary phase. This is the same effect as it would be seen if the mobile phase
pH was altered in conventional HPLC. In this study, when the pH value was higher than 6.0, the four PhGs did hard to separate and thus the accuracy and reproducibility are not satisfactory. Therefore, a pH value of 6.0 seemed to be optimal for the separation and detection of the four PhGs in a short run.

Based on the experiments discussed above, the optimum conditions were as follows: a mobile phase containing 0.3% triethylamine in 20 mM phosphoric acid at pH 6.0, 0.8% (v/v) ethyl acetate as oil phase, 1.5% (v/v) Genapol X-080 as surfactant, 2.5% (v/v) n-propanol as co-surfactant. Microemulsion mobile phase was stable for at least 2 months at room temperature.

3.2. Method validation

Good linearities with correlation coefficients (r) of 0.9996 (ECH), 0.9995 (TUBB), 0.9994 (ACT) and 0.9994 (ISO) were obtained over the concentration range of 10–1000 ng mL$^{-1}$. The limit of detection (LOD, S/N=3) was estimated to be 0.4 ng mL$^{-1}$ for ECH, 1.1 ng mL$^{-1}$ for TUBB, 0.8 ng mL$^{-1}$ for ACT and 1.2 ng mL$^{-1}$ for ISO in rat plasma, respectively. The limits of quantification (LOQ, S/N=10) were found to be 1.2 ng mL$^{-1}$ for ECH, 3.2 ng mL$^{-1}$ for TUBB, 3.1 ng mL$^{-1}$ for ACT and 3.5 ng mL$^{-1}$ for ISO in rat plasma.

The intra-day and inter-day precision and accuracy values of the assay method are shown in Table 1. All of the intra-day precision was less than 8.04 % and inter-day precision were lower than 8.56 % at the five concentrations evaluated. The intra-day and inter-day accuracies were found to be within 100.6 and 104.6 % for the four PhGs over the concentrations studied.

Extraction yield (absolute recovery) was also evaluated by assaying the QC samples. The results of these assays are listed in Table 1. Mean extraction yields were always higher than 92.1 % for the four PhGs.

The results indicated that the four PhGs were stable for at least 15 days in rat plasma stored at $-20^\circ$C. Extracted plasma samples were also found to be stable for at least 24 h when the samples were kept at room temperature (22-25$^\circ$C) and the final concentration were 93.2–97.6% of the initial values. The four PhGs were also stable following three freeze-thaw cycles. The coefficient of variation was 1.95–7.29%.

Blank rat plasma samples were evaluated for selectivity and no endogenous peaks interfered with the quantification of the four PhGs.
3.3. **Comparison with conventional HPLC analysis**

To prove the validity of the MELC method the results obtained by the proposed method were compared with those by conventional HPLC analysis. A conventional HPLC gradient mobile phase of A (0.1% phosphoric acid and 0.04% triethylamine) and B (methanol) was employed. The linear gradient was as follows: 10–30% B over 0–20 min, then 30–40% B over 20–35 min, next 40–45% B over 35–45 min and lastly returned to 10% B at 45 min immediately.

Compared with conventional HPLC analysis, the MELC method has drastic shorter separation and determination time under identical experimental conditions. The effect of the MELC method is clearly demonstrated in Fig 6.

3.4. **Application of method**

The described method has been successfully employed to determine the four PhGs in rat plasma samples. Under these optimum conditions, the chromatographic peaks were neat, symmetric and well separated (Fig.7). The concentration of the four PhGs from *Cistanche salsa* extract in rat plasma were determined to be 668.35 ng mL$^{-1}$ (1 h) for ECH, 62.95 ng mL$^{-1}$ (1 h) for TUBB, 364.64 ng mL$^{-1}$ (1 h) for ACT and 179.17 ng mL$^{-1}$ (1 h) for ISO after a single oral dose of 50 mg kg$^{-1}$, respectively.

4. **Conclusion**

The HPLC method, using microemulsion as mobile phase, for the analysis of ECH, TUBB, ACT and ISO in plasma after oral administration of *Cistanche salsa* extract was developed, validated and applied smoothly. The method makes the direct analysis of biological samples possible only after proper dilution with microemulsion mobile phase. The use of a microemulsion mobile phase enabled analysis of plasma samples without prior extraction, thus the possibility of errors and losses was excluded during sample processing. The proposed method proved to be simpler, easier and less time-consuming comparing to previously reported conventional HPLC methods.

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References


Figure Legends

Fig.1. Chemical structures of ECH, TUBB, ACT, ISO and IS

Fig.2. Effect of Genapol X-080 concentration (% v/v) in the microemulsion upon peak retention. Other extraction conditions: 0.3% triethylamine in 20 mM phosphoric acid at pH 6.0, 0.8% (v/v) ethyl acetate as oil phase, 2.5% (v/v) n-propanol as co-surfactant.

Fig.3. Effect of cosurfactant concentration (% v/v) in the microemulsion upon peak retention. Other extraction conditions: 0.3% triethylamine in 20 mM phosphoric acid at pH 6.0, 0.8% (v/v) ethyl acetate as oil phase, 1.5% (v/v) Genapol X-080 as surfactant.

Fig.4. Effect of oil concentration in the microemulsion upon peak retention. Other extraction conditions: 0.3% triethylamine in 20 mM phosphoric acid at pH 6.0, 1.5% (v/v) Genapol X-080 as surfactant, 2.5% (v/v) n-propanol as co-surfactant.

Fig.5. Effect of pH in the microemulsion upon peak retention. Other extraction conditions: 0.8% (v/v) ethyl acetate as oil phase, 1.5% (v/v) Genapol X-080 as surfactant, 2.5% (v/v) n-propanol as co-surfactant.

Fig.6. Chromatogram of rat plasma spiked with the four PhGs at 50 ng mL\(^{-1}\) level was extracted by (A) Conventional HPLC, (B) MELC

Fig.7. Typical chromatogram for the separation of ECH, TUBB, ACT and ISO (50 ng mL\(^{-1}\)) using micromulsion mobile phase: (A) a blank rat plasma sample; (B) a blank rat plasma sample spiked with the four PhGs and IS; (C) rat plasma sample 1 h after orally administration (50 mg kg\(^{-1}\)).
● An HPLC using microemulsion as the mobile phase has been developed.
● The MELC were investigated detailedly.
● The first report MELC for the analysis of the four phenylethanoid glycosides in plasma sample.
Fig. 1.

Fig. 2.

Concentration of each analyte, 50ng mL\(^{-1}\); Injection volume, 10\(\mu\)L
Fig. 3.

Co-surfactant concentration (%)

Concentration of each analytes, 50ng mL\(^{-1}\); Injection volume, 10\(\mu\)L

Fig. 4.

Oil concentration (%)

Concentration of each analytes, 50ng mL\(^{-1}\); Injection volume, 10\(\mu\)L
Fig. 5.

Concentration of each analytes, 50ng mL⁻¹; Injection volume, 10μL.

Fig. 6.
Fig. 7.
Table 1. Precision, accuracy and recovery for the analysis of ECH, TUBB, ACT and ISO in plasma sample (n=5)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Assay</th>
<th>Spiked concentration (ng mL$^{-1}$)</th>
<th>Concentration found (mean ± SD) (ng mL$^{-1}$)</th>
<th>Precision (RSD%)</th>
<th>Accuracy (RE%)</th>
<th>Recovery (%)</th>
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