Short communication

Analysis of magnolol and honokiol in biological fluids by
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Abstract

Capillary zone electrophoresis (CZE) method was used for analysis of magnolol and honokiol. Under the optimized condition, CZE with UV absorption detection provided that the limit of detection was at μM level. To enhance detection sensitivity of magnolol and honokiol, CZE separation system was coupled with a laser-induced fluorescence (LIF) detector for the first time. The limits of detection of magnolol and honokiol were 12 nM (3.20 ng ml^{-1}) and 18 nM (4.79 ng ml^{-1}), respectively, showing that the CZE-LIF system provides greater than 100-fold sensitivity improvements than does the CZE-UV system. The developed method was applied to analyze magnolol and honokiol in spiked human plasma samples, microsome incubation samples as a preliminary demonstration of its potential in pharmacokinetic studies.

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1. Introduction

The stem bark of *Magnolia officinalis* is one of the most important traditional herbal medicine in China and Japan for the treatment of thrombotic stroke, typhoid fever, depression, and headache [1,2]. Magnolol and honokiol were claimed to be the major pharmacologically active components in *Magnolia officinalis*. They possess activities against microbial [3], platelet aggregation [4], oxidation [5], anxiety [6], depression [7] and cancer [8]. Recent studies demonstrated that these compounds might be potential therapeutic agents for neurodegenerative diseases [9], acute promyelocytic leukemia [10] as well as

atherosclerosis [11]. In view of many in-progress researches associated with magnolol and honokiol, an efficient and sensitive analytical method is required to facilitate these studies.

Magnolol and honokiol in *Magnolia officinalis*, rats and human serum have been analyzed widely by HPLC methods [12–15]. Other studies also used supercritical fluid chromatography [16], high speed counter current chromatography [17] and liquid column chromatography–mass spectrometry [18] to quantitatively determine the magnolol and honokiol in *Magnolia officinalis*.

Capillary electrophoresis (CE) has been applied in the analysis of magnolol and honokiol in *Magnolia officinalis* [19–21]. However, because of a small injection volume and a short optical path-length of CE, low concentration sensitivity of CE-UV hampers its applicability to analyze magnolol and honokiol in biological fluids. The primary goal of our research was to develop a sensitive and efficient electrophoretic method to facilitate the pharmacokinetic study of magnolol and honokiol. To achieve this goal, both capillary zone electrophoresis (CZE) systems with UV absorption and laser-induced fluorescence (LIF) detection were developed to improve the sensitivity. We used

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spiked human plasma samples, microsome incubation samples to demonstrate the practicability of the methods developed in this study. To the best of our knowledge, this is the first time using CZE-LIF to analyze magnolol and honokiol in biological fluids.

2. Experimental

2.1. Chemicals

Magnolol and honokiol were purchased from Wako (Osaka, Japan). Disodium tetraborate was purchased from Merck (Darmstadt, Germany). Sodium hydroxide was purchased from Fluka (Buchs, Switzerland). NADPH was purchased from Sigma (St. Louis, MO, USA).

2.2. Apparatus

The CZE experiments with UV detection were carried out in a system consisting of a Prince programmable injector from Lauer Labs (Emmen, The Netherlands) and a 30 kV high-voltage supply, connected to a UV-C absorbance detector from Dynamax (Rainin, Emeryville, CA, USA). A fused-silica capillary from Polymicro Technologies (Phoenix, AZ, USA) was used. The electropherograms were recorded using an EZChrom (Scientific Software, San Ramon, CA, USA) chromatographic data system.

For CZE experiments using LIF detection, the basic design of the separation system was previously described [22]. In brief, a high-voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, USA) was used to drive electrophoresis. A diode-pumped solid state nanolaser (JDS Uniphase, Manteca, CA, USA) having a 5-mW output at 266 nm was used to induce intrinsic fluorescence. The intrinsic fluorescence was collected using a 10× objective (numeric aperture 0.25) before reaching the photomultiplier tube (R928 from Hamamatsu, Shizuoka-Ken, Japan). The amplified current was transferred directly through a 10-k Ω resistor to a 24-bit A/D interface at 5 Hz (Borwin, JMBS Developments, Le Fontanil, France).

2.3. Electrophoretic conditions

CZE-UV experiments were performed on a 75 cm (60 cm effective length) $\times$ 50 μ m I.D. fused-silica capillary. CZE-LIF experiments were performed on a 40 cm (30 cm effective length) $\times$ 50 μ m I.D. fused-silica capillary. The new capillary was pre-conditioned prior to use by flushing with 1.0 M sodium hydroxide, 0.2 M sodium hydroxide, de-ionized water, and running buffer each for 10 min. At the beginning of each experiment, the capillary was washed with 0.2 M sodium hydroxide, then followed by running buffer each for 5 min.

The background electrolyte (BGE) solution was 45 mM sodium tetraborate, pH 9.6 (by titrating with 1 M NaOH). Operation conditions for CZE-UV were set as follows: voltage, 30 kV; injection time, 4.8 s (hydrodynamic, 50 mbar); detection wavelength, 254 nm. Operation conditions for CZE-LIF were set as

follows: voltage, 12 kV; excitation wavelength, 266 nm. The sample was hydrodynamically injected by raising the sample solution to create a 25 cm height difference relative to the buffer reservoir for 10 s. All of the experiments were run at room temperature (23 ± 1 °C).

All the solutions were filtered through a 0.45 μ m filter (Millipore, Bedford, MA, USA) before use.

2.4. Preparation of standard solution

Stock solutions of magnolol and honokiol with concentrations of 5 mM and 2 mM were separately prepared in ethanol. These solutions were diluted with ethanol to give the working solution. The concentrations of magnolol and honokiol in the working solution were 1.25 mM and 0.25 mM, respectively. Propyl paraben was added as an internal standard, and its final concentration was 0.2 mM.

2.5. Sample preparation

2.5.1. Plasma sample

Blood samples were picked up from healthy volunteers and immediately transferred to a heparinized microcentrifuge tube. Treatment of blood samples was according to the published method [14]. Magnolol, honokiol and internal standard were spiked to the blood sample then centrifuged at 3000 rpm for 10 min. The resulting plasma sample was then vortex-mixed with acetonitrile (ACN). After 15 min, the mixture was centrifuged at 3000 rpm for 5 min to remove proteins. The supernatant was then separately transferred into a 1.5-ml tube containing 50–60 mg sodium chloride. The suspension was vortex-mixed briefly and incubated at room temperature for 20 min and was vortex-mixed again. Finally, the plasma sample was centrifuged at 3000 rpm for 5 min. A portion of the supernatant was subjected to CZE-LIF analysis.

2.5.2. Microsome incubation sample

The incubation mixture (final volume 0.5 ml) contained 0.75 mg rat liver microsomes, 0.2 mM NADPH, 1.65 mM MgCl₂, 0.1 M sodium phosphate buffer, pH 7.4, and 5 mM magnolol, which was shaken (100 oscillations min⁻¹) for 5 min at 37 °C [23,24]. Then the substrate was added to the mixture to start the reaction. After 30 min of incubation with shaking at 37 °C, the reaction was stopped by adding 0.2 ml of methanol and the mixture was centrifuged at 39,000 rpm for 25 min at 4 °C. Part of the supernatant (100 μ l) was mixed with 100 μ l ACN. After 15 min, the mixture was centrifuged at 3000 rpm for 5 min to separate precipitated proteins. The supernatant was then transferred into a 1.5-ml tube containing 50–60 mg sodium chloride. The suspension was vortex-mixed briefly and incubated at room temperature for 20 min and vortex-mixing again. Finally, the mixture was centrifuged at 3000 rpm for 5 min, and then was subjected to CZE-LIF analysis.

All sample solutions were filtered through a 0.45- μ m filter (Millipore, Bedford, MA, USA) before analysis.

3. Results and discussion

3.1. Method development

Chemical structures of magnolol and honokiol are shown in Fig. 1. The pK_{a1} and pK_{a2} values of magnolol are 7.01 and 10.64, and those for honokiol are 9.92 and 10.71 [21]. Since their first pK_a values are quite different, CZE method was used in this study. The pH values over the range of 8.0–10.0 of the solutions were chosen to preserve greater differences in the charged densities of magnolol and honokiol. When the pH value was below 8.8, honokiol remains in its neutral form and co-migrated with electroosmotic flow (EOF). With increasing the pH level, the degree of ionization increased and thus the peak efficiency increased as a result of reduced analyte adsorption. At pH 9.6, the peak efficiency of honokiol was at its maximum. Further increases in the pH value encountered poor reproducibility and low peak efficiencies. Therefore, pH 9.6 was considered as optimum separation pH. Sodium tetraborate at concentrations between 30 mM and 50 mM with pH 9.6 were tested in this study. Peak efficiency was found to increase with the increase of ionic strength. When sodium tetraborate was at the concentration higher than 50 mM, poor reproducibility occurred. Therefore, 45 mM sodium tetraborate was chosen for the separation. Under the optimum separation conditions, the analysis was completed within 7 min as shown in Fig. 2.

3.2. Method validation

Relative standard deviation (RSD) of the repeatability ($n=10$) and intermediate precision (inter-day, $n=3$) of reten-

Fig. 2. Electropherogram of magnolol and honokiol under optimum separation conditions using UV detection. Separation conditions: fused-silica capillary, 75 cm $\times$ 50 μ m I.D., 60 cm detection length; 45 mM sodium tetraborate, pH 9.6; applied voltage, 30 kV; temperature, ambient ($23 \pm 1^\circ\text{C}$); injection, 50 mbar, 4.8 s. Peak: (1) honokiol; (2) magnolol.

tion times of magnolol and honokiol were within 1.51% and 1.94%, respectively. The precision of peak area ratios of magnolol and honokiol to internal standard, propyl paraben, were tested at the 1.25 mM and 0.25 mM. Repeatability ($n=10$) and intermediate precision (inter-day, $n=3$) were within 2.76% and 6.46% RSD, respectively.

3.2.1. Limit of detection

When using a UV detector, the LODs of magnolol and honokiol were 4.26 μM ($1.13 \mu\text{g ml}^{-1}$) and 3.75 μM ($0.99 \mu\text{g ml}^{-1}$). This LOD for magnolol is much higher than its concentration in rat plasma samples ($0.06\text{--}0.2 \mu\text{g ml}^{-1}$) [14]. To enhance the sensitivity of the developed method, the CZE separation system was coupled with an LIF detector. The CZE-LIF system was expected to be suitable for the analysis of magnolol and honokiol, both possessing intrinsic fluorescence. When using CE-LIF, the analysis could be completed within 5 min. The LODs of magnolol and honokiol were 12 nM (3.20 ng ml^{-1}) and 18 nM (4.79 ng ml^{-1}), respectively, showing that the CZE-LIF system provides more than 100-fold sensitivity relative to the CZE-UV system. The LODs of magnolol and honokiol using CZE-LIF are much less than their plasma concentrations in rats, and thus it should be practical in pharmacokinetic study. To the best of our knowledge, the CE-LIF system provides the best sensitivity among standard CE methods for the analysis of magnolol and honokiol. Comparing to LC-MS/MS method [18], the present study provided comparable sensitivity and speed. However, the reagent consumption was greatly reduced with CZE-LIF method. Although the LODs of the present method were slightly higher than those obtained by micro-HPLC with electrochemical detection [25], the analytical time of CZE-LIF is greatly reduced (5 min versus 40 min).

Fig. 1. Structures of honokiol, magnolol and propyl paraben (I.S.).

Fig. 3. Electropherograms of magnolol and honokiol in spiked human plasma. (a) blank human plasma; (b) human serum spiked with 100 ng ml^{-1} of magnolol and honokiol. Separation conditions: fused-silica capillary, $40 \text{ cm} \times 50 \text{ }\mu\text{m}$ I.D., 30 cm detection length; 45 mM sodium tetraborate, $\text{pH } 9.6$; applied voltage, 12 kV ; temperature, ambient ($23 \pm 1 \text{ }^\circ\text{C}$); injection, hydrodynamic injection, 10 s . Peak: (1) honokiol; (2) magnolol.

3.3. Application

Before applying CE-LIF method in the analysis of biological fluids, this method was subjected to method validation. The precision of peak area ratios were tested at $10 \text{ }\mu\text{M}$. Repeatability ($n = 10$) and intermediate precision (inter-day, $n = 3$) were within 2.35% and 3.18% RSD, respectively. Linearity of the CZE-LIF method was tested within the range of $0.05\text{--}50 \text{ }\mu\text{M}$. The regression equations are $y = 0.1183x + 0.8854$ ($r^2 = 0.9933$) for magnolol and $y = 0.0305x + 0.0288$ for honokiol ($r^2 = 0.9984$).

To demonstrate the potential application of CE-LIF in pharmacokinetic studies, the concentrations of magnolol and honokiol in spiked human plasma samples were determined. The concentrations ($0.1 \text{ }\mu\text{g ml}^{-1}$) of magnolol and honokiol were selected as average plasma concentration in pharmacokinetic

study. The electropherograms of the human plasma sample and spiked human plasma sample are shown in Fig. 3a and b. Both magnolol and honokiol showed good signal intensity, and were well separated from sample matrix. The recoveries of magnolol and honokiol were 86.7% and 85.5% , respectively, based on the peak area ratios for the two analytes.

Nakazawa et al. had performed the *in vivo* metabolism study of *Magnolia officinalis* [15]. In their study, they have found magnolol and its metabolite dihydroxydihydromagnolol in human urine samples. To demonstrate the potential of our developed method in studying metabolic pathway of magnolol, the microsome incubated samples were analyzed. A representative electropherogram is shown in Fig. 4. Magnolol was slightly overlapped with a small unknown peak, presumably its metabolite, although further study is essential to determine its chemical structure. Nevertheless, the developed method can still be used to quantitate magnolol after metabolizing by rat liver microsome.

4. Conclusion

Both CZE-UV and CZE-LIF methods for the analysis of magnolol and honokiol have been established in this study. With the ability to detect magnolol and honokiol in nanomolar level, the CZE-LIF method has great potential for pharmacokinetic and metabolism studies. Under optimal separation conditions, analysis with good peak efficiencies could be completed within 5 min . The established method was successfully applied to analyze spiked human plasma sample and microsome incubated sample as the preliminary demonstration of its potential.

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Fig. 4. Electropherograms of magnolol after incubation with rat liver microsome. (a) blank incubation medium; (b) 5 mM magnolol incubated with rat liver microsome for 30 min . Separation conditions: 45 mM sodium tetraborate, $\text{pH } 9.6$; applied voltage, 12 kV ; other conditions are as in Fig. 3. Peak: (2) magnolol; (3) unknown.

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