

Research Article

Simultaneous Analysis of Honokiol and Magnolol in Rat Serum by HPLC with Fluorescence Detection after Solid-phase Extraction for Pharmacokinetic Studies

Yasuhiko Higashi*

Department of Analytical Chemistry, Faculty of
Pharmaceutical Sciences, Hokuriku University, Japan***Corresponding author:** Yasuhiko Higashi,
Department of Analytical Chemistry, Faculty of
Pharmaceutical Sciences, Hokuriku University,
Kanagawa-machi, Japan**Received:** April 03, 2015; **Accepted:** April 22, 2015;**Published:** April 24, 2015**Abstract**

The author and co-workers previously reported an HPLC method coupled with fluorescence detection (FL) for determination of honokiol and magnolol, and showed that *Hange-koboku-to* dried extract granules (1.00 g) contained honokiol (3.30 mg) and magnolol (5.40 mg). In this study, the author modified the previous method to make it suitable for simultaneous HPLC-FL analysis of honokiol and magnolol in rat serum. Solid-phase extraction with a Bond Elut-C₁₈ cartridge was carried out for pretreatment of rat serum. 1,1-Bis(4-hydroxyphenyl)cyclohexane was utilized as an internal standard. The mobile phase was prepared by the addition of methanol (750 mL) to a solution of trifluoroacetic acid (0.1 v/v%) in 250 mL of water. The regression equations for honokiol and magnolol in rat serum showed good linearity in the range of 0.1 to 2.5 µg/mL with lower limits of detection (signal-to-noise ratio of 3) of 0.011 and 0.014 µg/mL, respectively. The coefficient of variation of the assay and the extraction ratios of honokiol and magnolol by the pretreatment were satisfactory. Magnolol was determined in rat serum after a single *p.o.* administration of the granules (18 mg/kg as honokiol and 30 mg/kg as magnolol) to rats and its pharmacokinetic parameters were estimated. The level of honokiol in the serum was less than the lower limit of quantification.

Keywords: HPLC; Fluorescence detection; Magnolol; Honokiol; Solid-phase extraction; Pharmacokinetic study

Abbreviations

HPLC: High-performance Liquid Chromatography; FL: Fluorescence Detection; UV: Ultraviolet Absorption Detector; MS: Mass Spectrometry; CZE: Capillary Zone Electrophoresis; ECD: Electrochemical Detection; LIF: Laser-induced Fluorescence; IS: Internal Standard; *p.o.*: Per Orally; AUC: Area Under the Serum Concentration-time Curve; C_{max}: Peak Serum Concentration; T_{max}: Time to Reach C_{max}

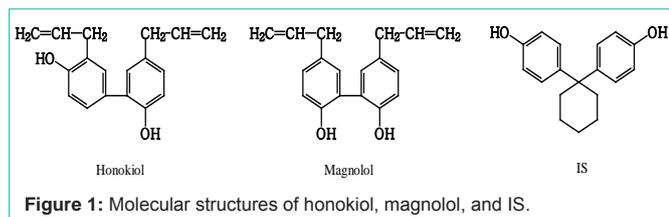
Introduction

The bark of *Magnoliae Cortex* has been used in traditional Chinese medicine for the treatment of thrombotic stroke, typhoid fever, and headache [1]. Two constituents of *Magnoliae Cortex*, honokiol and magnolol, were reported to produce sedation, ataxia, muscle relaxation and loss of the righting reflex in rats [2], and they also improved learning and memory impairment [3]. Honokiol activates extracellular signal-regulated kinases to promote neurite outgrowth [4], while magnolol has anti-inflammatory and analgesic effects, and ameliorates diabetic nephropathy [5,6].

In connection with pharmacokinetic studies of honokiol and magnolol and quality evaluation of *Magnoliae Cortex*, several methods are available for determination of honokiol and/or magnolol, including HPLC-ultraviolet absorption detector (UV) [7-11], HPLC-mass spectrometry (MS) [12,13], capillary zone electrophoresis (CZE) with UV, electrochemical detection (ECD) or laser-induced

fluorescence (LIF) detection [14-16], and high-capacity high-speed counter-current chromatography [17]. Further, a recently reported µHPLC-ECD method exhibited very high sensitivity (0.13 pg for honokiol and magnolol) for determining honokiol and magnolol contents in *Magnolia Bark* [18]. An HPLC method with fluorescence detection (FL), using honokiol as an internal standard (IS), has been described for magnolol determination, and it was applied for a pharmacokinetic study of magnolol in rats [19]. Pretreatment of plasma samples was performed by precipitation with acetonitrile [19]. However, injection of such a sample leads to degradation of the analytical column. In addition, the tissue distribution and bioavailability of magnolol were investigated in Sprague-Dawley rats by HPLC-UV with the lower limit of detection of 0.06 µg/mL [20]. But, this procedure required liquid-liquid extraction of serum samples with ethyl acetate [20], which is unsuitable for routine application. We considered that pretreatment using solid-phase extraction would be advantageous.

Hange-koboku-to dried extract granules (3.75 g) consist of a mixture of a half extract (1.25 g) from five medical herbs (Japanese Pharmacopoeia requirements: *Pinelliae Tuber*, 3.0 g; *Polia*, 2.5 g; *Magnoliae Cortex*, 1.5 g; *Perillae Herba*, 1.0 g; *Zingiberis Rhizoma*, 0.5 g) and three additives (2.5 g, magnesium stearate, lactose hydrate, sucrose fatty acid ester). Recently, the author and co-workers previously developed HPLC-FL method to show that *Hange-koboku-to* dried extract granules (1.00 g) contain honokiol (3.30 mg) and magnolol (5.40 mg) [21]. In the present study, the author developed



a modification of that method for simultaneous HPLC-FL analysis of honokiol and magnolol in rat serum, using solid-phase extraction for pretreatment of rat serum. 1,1-Bis(4-hydroxyphenyl)cyclohexane as an IS was used, since it resembles honokiol and magnolol in chemical structure and it was reported to be well separated from peaks of honokiol and magnolol [21]. The developed method was used for pharmacokinetic studies in rats after a single *p.o.* administration of *Hange-koboku-to* dried extract granules.

Material and Methods

Reagents

Honokiol and magnolol were purchased from Nacalai tesque (Kyoto, Japan). 1,1-Bis(4-hydroxyphenyl)cyclohexane was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Their chemical structures are shown in Figure 1. *Hange-koboku-to* dried extract granules (Lot No. BD4131, Tsumura & Co., Tokyo) were obtained from a pharmaceutical market. Methanol and general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). A Milli-Q water purification system (Millipore Corp., Bedford, MA, U.S.A.) was used to obtain water for HPLC use.

Equipment

The HPLC system comprised a model L-6200 pump (Hitachi), a Rheodyne injection valve (Cotati, CA, U.S.A.) with a 100- μ L loop and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan). The fluorometer was operated at an excitation wavelength of 275 and an emission wavelength of 315 nm up to 6.7 min, then switched to excitation and emission wavelengths of 304 and 340, respectively, until 10.1 min. After 10.1 min, the emission wavelength was changed to 405 nm. The columns (Nacalai tesque) were 150 mm \times 4.6 mm i.d. C₁₈-MS-II (Nacalai tesque) with 5 μ m particles. Quantification of the peaks was performed with a Chromatopac Model C-R3A integrator (Shimadzu). The mobile phase was prepared by the addition of methanol (750 mL) to a solution of trifluoroacetic acid (0.1 v/v %) in 250 mL of water. The samples were eluted from the column at room temperature at a flow rate of 1.0 mL/min.

Calibration curves for honokiol and magnolol in rat serum and solid-phase extraction

Standard honokiol and magnolol solutions (each 50 μ g/mL) were prepared in methanol and stored at -18°C. Equal volumes of the two solutions were mixed and diluted with water to obtain a series of working solutions (0, 0.1, 0.25, 0.5, 1, and 2.5 μ g/mL). Aliquots of 100 μ L of blank rat serum were spiked with 100 μ L of standard mixture (0, 0.1, 0.25, 0.5, 1, and 2.5 μ g/mL), 100 μ L of 1,1-bis(4-hydroxyphenyl)cyclohexane as an IS (2 μ g/mL), and 20 μ L of 85% *o*-phosphoric acid. The samples were vortexed vigorously for 30 s and loaded onto Bond Elut-C₁₈ cartridges (VARIAN, 100 mg, 1 mL) conditioned with 1 mL of methanol and 1 mL of water. After addition of 1 mL of 5% methanol, compounds of interest were eluted with 1 mL of methanol.

After evaporation under vacuum with a concentrator (TC-8, TAITEC Corp., Japan), residues were reconstituted by addition of 300 μ L of mobile phase, and 100 μ L samples were directly injected onto the HPLC system.

Extraction ratio of honokiol and magnolol

The two standard solutions (0.1 and 1 μ g/mL, 100 μ L) and IS solution (2 μ g/mL, 100 μ L) were mixed with water (100 μ L, control) or rat serum (100 μ L). After pretreatment of rat serum as described above, extraction ratios were calculated as percentage ratios of honokiol or magnolol peak areas to the IS peak area in serum sample with respect to the ratios in the control.

Animal study

Male Wistar rats (9-10 weeks, 255-272 g of weight volume) were obtained from Sankyo Laboratory Animals (Toyama, Japan) and treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of Hokuriku University. *Hange-koboku-to* dried extract granules (1.5 g/head, *i.e.*, 18 mg/kg as honokiol and 30 mg/kg as magnolol) were *p.o.* administered to rats after having been suspended in water (3 mL). Rats were fasted for 12 h prior to the administration, while water was freely available. Under light anesthesia with diethyl ether, blood samples (0.3 mL) were withdrawn from the jugular vein at the designated time intervals (0.5, 1, 2, 4, 6, 8, and 12 h) *via* a separate venous puncture. Blood samples were allowed to clot, then centrifuged (3,000 \times g, 10 min) to obtain the serum. Drug-free pooled serum was similarly obtained from rats.

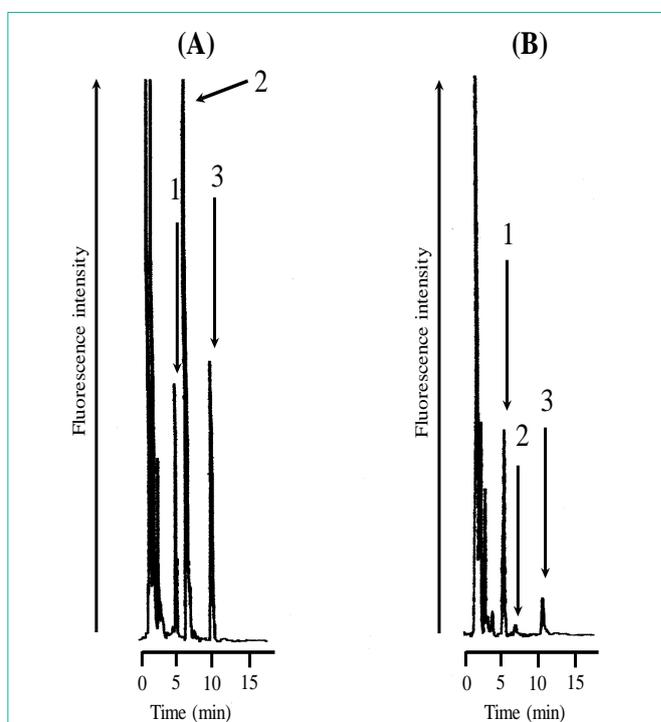


Figure 2: Typical chromatograms of rat serum spiked with standard (A) and rat serum after a single *p.o.* administration of *Hange-koboku-to* dried extract granules to rats (B). Chromatograms: (A), rat serum spiked with 2 μ g/mL of IS and 0.5 μ g/mL of honokiol and magnolol; (B), rat serum sample at 2 h after administration spiked with IS. Peaks: 1, IS; 2, honokiol; 3, magnolol. *Hange-koboku-to* dried extract granules were administered to rats (1.5 g/head, *i.e.*, 18 mg/kg as honokiol and 30 mg/kg and magnolol).

Table 1: Method comparisons for simultaneous determination of honokiol and magnolol in rat serum.

Analytical methods	Quantification limit ($\mu\text{g/mL}$)		Detection limit ($\mu\text{g/mL}$)		References
	Honokiol	Magnolol	Honokiol	Magnolol	
HPLC-FL (Absolute amount)	0.037 (1.2 ng)	0.047 (1.5 ng)	0.011 (0.37 ng)	0.014 (0.43 ng)	Present method
HPLC-FL (Absolute amount)	0.021 (1.1 ng)	0.028 (1.4 ng)	0.0047 (0.24 ng)	0.0061 (0.32 ng)	[21]
HPLC-FL	Not described		Not described		[19]
mHPLC-ECD (Absolute amount)	(0.67 pg)	(0.67 pg)	(0.13 pg)	(0.13 pg)	[18]
CZE-LIF	Not described		Not described		[15]
CZE-UV	Not described		Not described		[15]
CE-ECD	0.266	0.266	0.101	0.136	[16]
HPLC-MS	0.0025	0.0025	Not described		[12]
HPLC-MS	0.0005	0.0002	0.0002	0.0001	[13]
HPLC-UV	Not described	0.13	Not described	0.06	[20]
HPLC-UV	0.013	0.025	Not described		[11]
HPLC-UV	0.32	0.88	0.16	0.44	[10]
HPLC-UV	0.81	1.03	0.25	0.23	[7]

Pharmacokinetic and Statistical Analysis

The area under the serum concentration-time curve from zero to 8 h (AUC_{0-8}) was calculated using the linear trapezoidal rule. The peak serum concentration (C_{\max}) and the time to reach C_{\max} (T_{\max}) were determined from the actual data obtained after a single *p.o.* administration. Data are expressed as the mean \pm S.D. ($n=5$).

Results and Discussion

Figure 2 shows typical chromatograms of a rat serum sample spiked with standard honokiol and magnolol (each 0.5 $\mu\text{g/mL}$) and IS (2 $\mu\text{g/mL}$) (A) and a rat serum sample at 2 h after a single *p.o.* administration of the granules to rats (B). The retention times of IS, honokiol, and magnolol were 5.6, 7.2, and 10.7 min, respectively.

Standard curves were constructed by plotting integrated peak areas vs. honokiol and magnolol concentration. Linear relationships were obtained for honokiol ($y = 7.333x + 0.040$, in the concentration range from 0.1 to 2.5 $\mu\text{g/mL}$, $r^2 = 0.9940$) and for magnolol ($y = 2.953x + 0.019$, in the concentration range from 0.1 to 2.5 $\mu\text{g/mL}$, $r^2 = 0.9930$).

The lower limits of quantification for honokiol and magnolol were 0.037 $\mu\text{g/mL}$ (absolute amount of 1.2 ng) and 0.047 $\mu\text{g/mL}$ (absolute amount of 1.5 ng), respectively (signal-to-noise ratio of 10:1). The linearity from quantification limit to 0.1 $\mu\text{g/mL}$ is as follows. Linear relationships were obtained for honokiol ($y = 7.640x + 0.008$, in the concentration range from 0.037 to 0.1 $\mu\text{g/mL}$, $r^2 = 0.9893$) and for magnolol ($y = 3.135x + 0.005$, in the concentration range from 0.047 to 0.1 $\mu\text{g/mL}$, $r^2 = 0.9890$). The lower limits of detection for honokiol and magnolol were 0.011 $\mu\text{g/mL}$ (absolute amount of 0.37 ng) and 0.014 $\mu\text{g/mL}$ (absolute amount of 0.44 ng), respectively (signal-to-noise ratio of 3:1). Table 1 summarizes the lower limits of detection and quantification of methods previously reported for honokiol and magnolol assay. The $\mu\text{HPLC-ECD}$ method of Kotani *et al.* is one of the most sensitive, with detection limits for honokiol and magnolol of 0.13 pg [18]. The sensitivity of the method presented here is slightly poorer than that of our previous method [21], because of minor

Table 2: Extraction ratios of honokiol and magnolol in rat serum after pretreatment with Bond Elut- C_{18} cartridges.

Compounds		Concentration in rat serum	
		0.1 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$
Honokiol	Mean \pm S.D. (% , n=6)	96.1 \pm 8.5	101.5 \pm 7.8
	R.S.D. (%)	8.8	7.7
Magnolol	Mean \pm S.D. (% , n=6)	95.2 \pm 8.8	98.5 \pm 8.4
	R.S.D. (%)	9.2	8.5

interference in blank rat serum and slight loss of analyte during pretreatment of rat serum with solid-phase extraction. While the detection limits of honokiol and magnolol in the present assay were 4.3- to 31-fold improved compared with several results [7,10,16,20], it was 55- to 140-fold inferior previous report [13].

Judging from these data, it is considered that present assay will be relatively superior in terms of sensitivity.

The solid-phase extraction ratios of six solutions containing honokiol and magnolol (each 0.1 and 1 $\mu\text{g/mL}$ in rat serum) were calculated by comparison with standard solutions (Table 2). The mean ratios at 0.1 and 1 $\mu\text{g/mL}$ of honokiol were 96.1 and 101.5%, respectively. The mean ratios at 0.1 and 1 $\mu\text{g/mL}$ of magnolol were 95.2 and 98.5, respectively. The values of relative standard deviation (R.S.D.) were less than 9.2%. Previous methods have utilized the denatured protein precipitation by acetonitrile for pretreatment of biological samples instead of solid-phase extraction [19,20]. These results suggest that the tested Bond Elut- C_{18} cartridge is simple and appropriate for solid-phase extraction of honokiol and magnolol from rat serum.

Precision and accuracy for intra-day and inter-day assays of honokiol and magnolol are shown in Table 3. In the intra- and inter-day assays, the range of standard deviation of the mean for honokiol and magnolol was within 2.7 to 10.1%. The recoveries of honokiol and magnolol were within 88.1 to 109.0%. The assay exhibited satisfactory precision and accuracy.

Table 3: Intra- and inter-day assay reproducibility of honokiol and magnolol in rat serum.

Compounds		Concentration ($\mu\text{g/mL}$)	Found ($\mu\text{g/mL}$), Mean \pm S.D. (n=6)	C.V. (%)	Recovery values (%)
Honokiol	Intra-day assay	0.1	0.0910 \pm 0.0070	7.7	91.0
		1.0	1.04 \pm 0.05	4.8	104.0
		2.5	2.57 \pm 0.07	2.7	102.8
	Inter-day assay	0.1	0.109 \pm 0.011	10.1	109.0
		1.0	1.02 \pm 0.07	6.9	102.0
		2.5	2.44 \pm 0.07	2.9	97.6
Magnolol	Intra-day assay	0.1	0.0881 \pm 0.0078	8.9	88.1
		1.0	1.03 \pm 0.03	2.9	103.0
		2.5	2.72 \pm 0.15	5.5	108.8
	Inter-day assay	0.1	0.102 \pm 0.010	9.8	102.0
		1.0	0.975 \pm 0.082	8.4	97.5
		2.5	2.59 \pm 0.19	7.3	103.6

The present method was used to analyze serum samples after a single *p.o.* administration of *Hange-koboku-to* dried extract granules to rats (1.5 g/head, *i.e.*, 18 mg/kg as honokiol and 30 mg/kg as magnolol, $n=5$). As shown in Figure 3, the serum concentration *vs.* time profile of magnolol was constructed for up to 8 h. The serum level of magnolol was less than lower limit of quantification at 0.5 and 12 h after administration. Therefore, the serum level of magnolol was estimated from 1 to 8 h after administration. The values of $AUC_{0\rightarrow 8}$, C_{\max} , and T_{\max} were $0.686 \pm 0.113 \mu\text{g}\cdot\text{h/mL}$, $0.119 \pm 0.014 \mu\text{g/mL}$, and $1.60 \pm 0.49 \text{ h}$, respectively (mean \pm S.D., $n=5$). Lin et al. reported the disposition kinetics of magnolol in rats orally given a single dose of magnolol (50 mg/kg), obtaining the value of $AUC_{0\rightarrow 8}$ of $228.5 \text{ nmol}\cdot\text{min/mL}$ ($1.01 \mu\text{g}\cdot\text{h/mL}$) [20]. The value of $AUC_{0\rightarrow 8}$ from our data (a single dose of 30 mg/kg of magnolol) was $0.686 \mu\text{g}\cdot\text{h/mL}$. The dose ratio is nearly the same as the ratio of AUC values, supporting the validity of our method. On the other hand, the honokiol concentration from 0 to 12 h after the administration was below the lower limit of quantification. Böhmendorfer *et al.* reported very high hepatic extraction ratio and clearance of honokiol (0.99 ± 0.01 and $35.8 \pm 0.04 \text{ mL/min}$, respectively), and very low availability

(0.007 ± 0.001 , ratio of excreted amount of honokiol to administered amount of honokiol in rat perfused liver) in rat perfused liver [22], indicating that the intestinal absorption of honokiol is poor in rats (*i.e.*, there is a very large first-pass effect). The finding that serum honokiol concentration after oral administration was less than the quantification limit is consistent with their result in rats.

Conclusion

The author has modified previously developed HPLC-FL method to make it suitable for simultaneous determination of honokiol and magnolol in rat plasma after oral administration of *Hange-koboku-to* dried extract granules. A simple pretreatment of rat serum by means of solid-phase extraction with a Bond Elut- C_{18} cartridge was effective. While the extraction ratio is satisfactory and the sensitivity is only moderate in comparison with previous methods, present method is suitable for pharmacokinetic studies of magnolol after administration of *Hange-koboku-to* dried extract granules to rats. However, honokiol concentrations were below the lower limit of quantification in this study. Further work is needed to improve the sensitivity of honokiol detection in order to clarify the honokiol disposition kinetics.

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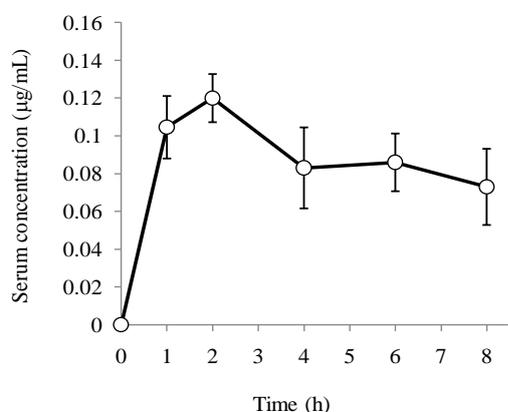


Figure 3: Serum concentration-time curve of magnolol after a single *p.o.* administration of *Hange-koboku-to* dried extract granules to rats. *Hange-koboku-to* dried extract granules were administered to rats (1.5 g/head, *i.e.*, 18 and 30 mg/kg as honokiol and magnolol, respectively). Each point represents the mean \pm S.D. ($n=5$). Honokiol levels in rat serum from 1 to 8 h after the administration were less than the lower limit of quantification.

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