J Pharm Anal Vol 1, No 2, 108 - 112 (2011)

ORIGINAL ARTICLES

Sensitive determination of 4-O-methylhonokiol in rabbit plasma by high performance liquid chromatography and application to its pharmacokinetic investigation

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Abstract: A novel high performance liquid chromatographic method was developed for the determination of 4-O-methylhonokiol in rabbit plasma and was applied to its pharmacokinetic investigation. Plasma samples were treated by one-fold volume of methanol and acetonitrile to remove the interference proteins. A reverse phase column of SHIM-PACK VP-ODS (150 mm × 4.6 mm, 5.0 μm) was used to separate 4-O-methylhonokiol in the plasma samples. The detection limit of 4-O-methylhonokiol was 0.2 μg/L and the linear range was 0.012 - 1.536 mg/L. The good extraction recoveries were obtained for the spiked samples (84.7%, 89.3% and 87.7% for low, middle and high concentrations of added standards, respectively). The relative standard deviation of intra-day and inter-day precisions was in the range from 0.6% to 13.5%. The pharmacokinetic study of 4-O-methylhonokiol was made and the results from the plasma-concentration curve of 4-O-methylhonokiol showed a two-apartment open model. This work developed a sensitive, stable and rapid HPLC method for the determination of 4-O-methylhonokiol and the developed method has been successfully applied to a pharmacokinetic study of 4-O-methylhonokiol.

Keywords: 4-O-methylhonokiol; Cortex Magnoliae Officinalis; high performance liquid chromatography; pharmacokinetic

1 Introduction

Cortex Magnoliae Officinalis (i.e. Magnolia Bark), a traditional Chinese medicine (TCM), is the dried bark of the trunk or root of *Magnolia officinalis* Rehd. etWils. or *Magnolia officinalis* Rehd. etWils. var. biloba Rehd. etWils., which belongs to the Magnoliaceae family. It has been frequently used as an important ingredient in many traditional prescriptions [1,2] and some concentrated composite herbal preparations that contain Cortex Magnoliae Officinalis in their prescriptions are widely used in oriental countries because it can alleviate gastric and abdominal distension and other digestive discomforts, reduce the symptom of cough and asthma due to the accumulation of phlegm in the lung, and treat syndromes caused by emotional distress, digestive disturbance, and emotional turmoil [1]. Its antibacterial properties have been recognized and may be responsible for its ability to alleviate digestive discomforts due to some intestinal bacterial infections [2]. Several compounds were identified as the bioactive ingredients of Cortex Magnoliae Officinalis, including honokiol, magnolol and 4-O-methylhonokiol (Figure 1). Previous reports have shown that honokiol and magnolol have a broad range of physiological activities such as anti-inflammatory [3], anti-oxidant [4], anti-tumor [5], anti-bacteria [6], anti-arrhythmia [7], and anti-platelet [8]. The total content of honokiol and magnolol is an important parameter for evaluating the quality of Cortex Magnoliae Officinalis. Pharmacopoeia of P.R. China requires the total content of honokiol and magnolol in Cortex Magnoliae Officinalis to be no less than 2.0% [1]. As another bioactive ingredient, the content of 4-O-methylhonokiol is within the range of 1.0% - 2.5% in Cortex Magnoliae Officinalis. The literature showed that 4-O-methylhonokiol could be developed as an anti-inflammatory drug [9-11]. Previous study showed that 4-O-methylhonokiol (1 mg/ear) suppressed TPA-induced ear edema, which was accompanied by the suppression on nuclear factor (NF)-κB, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) expression, and was equivalent to the indomethacin (0.5 mg/ear). Moreover, no significant toxicity during a 28-day oral treatment (1 mg/kg) was observed. Currently, there have been reports about the determination of magnolol and honokiol with the concentration of the drugs measured *in vivo* by high-performance liquid chromatography (HPLC) at home [12,13]. However, no reports are found for the determination of 4-O-methylhonokiol and its pharmacokinetic study. This work was designed to develop a novel method for the determination of 4-O-methylhonokiol and its pharmacokinetic study in rabbit plasma by HPLC.

Received 2 November 2010; Accepted 21 February 2011

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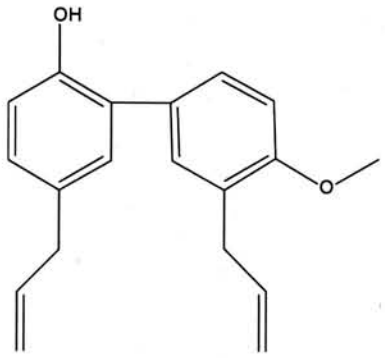

Figure 1 Chemical structure of 4-O-methylhonokiol

2 Experimental

2.1 Chemicals and reagents

Methanol and acetonitrile were of HPLC grade and purchased from Fisher Scientific (Springfield, NJ); Silica gel G (00-200 mesh) was supplied by Qingdao Haiyang Chemical Co., Ltd. (China); Cortex Magnoliae Officinalis was purchased from Xi'an Medical Company (China) and identified by Professor Guang-De Yang (Department of Pharmacognosy, Xi'an Jiaotong University, Xi'an, China).

2.2 Instruments and experiment conditions

The LC analyses were accomplished using an LC-10Avp (Shimadzu, Japan) HPLC system consisting of an LC-10ATvp secondary pump system, a DGU-12A on-line degasser, a CTO-10ASvp thermostatted column compartment, and an SPD-M10Avp diode array detector. CLASS-VP software was used to control the LC components and to process ultraviolet data. A reverse phase column of SHIM-PACK VP-ODS (150 mm × 4.6 mm, 5.0 μm) was used. The mobile phase consisted of methanol and water (85 : 15, v/v). A flow-rate of 0.8 mL/min provided a retention time of 6.6 min for 4-O-methylhonokiol, whereas the runtime was 13.0 min for the chromatographic analysis of plasma samples. Chromatograms were recorded at 294 nm using the variable wavelength detector.

2.3 Preparation of 4-O-methylhonokiol standard from Magnoliae Officinalis

The bark of Magnoliae Officinalis was dried in the shade at room temperature and stored in a dark, cold room until use. The air-dried bark of Magnoliae Officinalis (150 g) was cut into pieces and extracted twice with four-fold of 90% (v/v) ethanol and water mixture for 4 h and 2 h respectively. The filtered solution was concentrated under reduced pressure and 2% NaOH solution was added to obtain the extraction consisting of honokiol, magnolol and 4-O-methylhonokiol. The contents of the three compounds in the extraction were measured as 25.3%, 48.5% and 15.9%. After filtering through the 400-mesh filter cloth, 2.0 M hydrochloric acid was added to the resulting mixture to adjust pH value of 1 − 2. Then, the stage extraction was done using n-butanol, ethyl acetate and petroleum ether through ethyl acetate layer. The suspension was dissolved in chloroform and eluted with chloroform. The front layer was evaporated to dryness, and the residue was separated on silica gel using chloroform as the elute solvent to obtain a crude fraction that included 4-O-methylhonokiol. This fraction was repeatedly purified by silica gel chromatography using n-hexane and ethyl acetate as the solvents to obtain 4-O-methylhonokiol standard. Its content was measured as 95%. 4-O-methylhonokiol was identified by ^{1}H-NMR (400 MHz, $CDCl_3$): δ mg/L: 3.35 (2H, d, aryl-CH_2-), 3.44 (2H, d, aryl-CH_2-), 3.90 (3H, s, -OMe), 5.05 − 5.20 (4H, m, 2×-C=CH_2), 5.94 − 6.07 (2H, m, 2×-CH=C), 6.91 (1H, d, Ar-H), 6.98 (1H, d, Ar-H), 7.05 − 7.08 (2H, m, ArH), 7.24 − 7.31 (2H, m, ArH). And 4-O-methylhonokiol was identified by ^{13}C-NMR (400 MHz, $CDCl_3$): δ mg/L: 34.2(C-7), 39.3 (C-7′), 55.5 (OMe), 110.9 (C-3′), 115.4 (C-4′), 115.5 (C-9), 115.7 (C-9′), 127.8 (C-1′), 127.8 (C-6), 128.7 (C-3), 129.0 (C-1), 129.7 (C-5), 130.1 (C-6′), 130.4 (C-2), 132.11 (C-5′), 136.4 (C-8), 137.7 (C-8′), 151.8 (C-2′), 157.0 (C-4). All the results were in agreement with previously published data [14,15]. 4-O-methylhonokiol was found to exhibit a potent anti-inflammatory effect in different experimental models [16].

2.4 Preparation of the plasma samples

The fresh blood was collected from the heart of rabbit and centrifuged at 4000 rpm for 10 min. 0.5 mL of plasma was mixed with 0.5 mL methanol and 0.5 mL acetonitrile to precipitate protein. The solution was vortex-mixed for 2 min and centrifuged for 10 min at 4000 rpm. An aliquot of 1.0 mL supernatant was oven dried at 40 ℃ under the stream of N_2. The residue was dissolved in mobile phase consisting of 0.5 mL methanol-water (85 : 15, v/v) and vortex-mixed for 1.0 min. Twenty microlitres of the resulting mixture was injected into HPLC system.

2.5 Preparation of standards and quality control samples

Stock solution of 1.0 mg/mL 4-O-methylhonokiol in methanol was prepared in volumetric flasks. Calibration standard solution of 12.0 mg/L 4-O-methylhonokiol was prepared by dilution and stored at 4 ℃. The working solutions of calibration were prepared by diluting the standard solution with blank plasma to get the concentration of 0.012, 0.024, 0.048, 0.096, 0.192, 0.384, 0.768 and 1.536 mg/L. The preparation of quality control (QC) samples with the concentration of 0.02, 0.17 and 1.20 mg/L was the same as the calibration standards.

2.6 Method validation

2.6.1 Selectivity

Selectivity was studied by comparing chromatograms of six

different batches of blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with 4-O-methylhonokiol and plasma sample after oral administration of the extracts.

2.6.2 Calibration and detection limits

The feasibility of the method was verified by plotting calibration curves. The concentrations of the unknown samples were determined using the equation of linear regression obtained from the calibration curves. Limit of detection (LOD) and limit of quantification (LOQ) for 4-O-methylhonokiol were determined at the signal-to-noise ratios(S/N) of 3 and 10, respectively.

2.6.3 Precision and accuracy

Intra-day accuracy and precision (each $n = 5$) were evaluated by analysis of QC samples at different time within one day. Inter-day accuracy and precision (each $n = 6$) were determined by repeated analyses of QC samples twice per day at three concentration levels over three consecutive days. The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy of the method was determined by the relative standard deviation (CV).

2.6.4 Extraction recovery and matrix effect

The extraction recovery of 4-O-methylhonokiol was determined by comparing the peak areas obtained from blank plasma samples spiked with the known amount of the analytes before extraction with those from post-extraction blank plasma samples spiked at corresponding concentrations. In order to evaluate the matrix effect on the ionization of the analyte, 4-O-methylhonokiol of three different concentration levels was added to the extract of 1.0 mL of blank plasma, evaporated and reconstituted with 0.25 mL of mobile phase. According to the guidance of USFDA [17], this procedure was repeated for five replicates at three concentrations of 0.02, 0.17 and 1.20 mg/L. The corresponding peak areas (A) were compared with those of 4-O-methylhonokiol standard solutions evaporated directly and reconstituted with the same mobile phase (B). The ratio (A/B × 100%) was used to evaluate the matrix effect. The matrix effect of the internal standard was also evaluated by the same method.

2.6.5 Stability

Stability experiments were performed to evaluate the analyte stability in the stock solutions and in the plasma samples under different conditions, simulating the same conditions which would occur during the study of sample analysis. The long-term stability was evaluated by injecting the QC plasma samples kept at low temperature (−70 ℃) for 60 d. The short-term stability of 4-O-methylhonokiol was assessed by analyzing the QC plasma samples kept at ambient temperature (30 ℃) for 24 h prior to be processed, analyzed and compared with the nominal values. In order to estimate the stability of 4-O-methylhonokiol in the prepared sample, five aliquots of QC samples at low, mid and high concentration were kept in an auto-sampler maintained at 4 ℃ for about 4 h in the prepared samples.

2.7 Application in pharmacokinetic study

New Zealand healthy rabbits (2.0 − 2.4 kg) were obtained from the Animal Center of Xi'an Jiaotong University. The rabbits were kept in standard animal holding room at a temperature of (23 ± 2)℃ and a relative humidity of (60 ± 10)%. Water and food were allowed ad libitum. The animals were acclimatized to the facilities for 7 days and then fasted with free access to water for 12 h prior to each experiment. The ethics of animal experiments were in accordance with the approval of the Department of Health Guidelines in Care and Use of Animals.

The rabbits were given 4-O-methylhonokiol (single oral dose of 0.6 mg/kg). Heparinized heart blood samples of 2.0 mL were collected just prior to dosing and then at 15 min, 35 min, 1 h, 2 h, 5 h, 6 h, 7 h, 8 h and 24 h after administration. All the plasma samples were immediately separated by centrifugation at 4000 rpm for 10 min, then transferred into suitably labeled tubes and stored at −20 ℃ for further use. Plasma drug concentration-time curves of 4-O-methylhonokiol were plotted by DAS 2.0 software.

2.8 Data analysis

The Microsoft Excel Program Drug and Statistics 2.0 (T.C.M. Shanghai, China) were employed to process and calculate the pharmacokinetic parameters. The parameters of the area under curve (AUC), the maximum plasma concentration (C_{max}) and the corresponding time (t_{max}), and the half-life of absorption ($t_{1/2\alpha}$) were used to decrypt the pharmacokinetic properties of 4-O-methylhonokiol. Statistical analysis of the biological data was performed by the Student's t-test. All results are expressed as arithmetic mean ± standard deviation (CV).

3 Results and discussion

3.1 Optimization of chromatographic separation

The selection of mobile phase components was critical to the separation of 4-O-methylhonokiol. In the experiment, different ratios (10 : 90, 15 : 85, 30 : 70, 50 : 50 and 70 : 30, v/v) of water/methanol were used as mobile phases to optimize the separation effect. The ratio of 15 : 85 of water/methanol was selected as the mobile phase in view of retention time and peak shape of drug. In this work, ammonium formate was employed to improve the resolution. It was found that a mixture of 10 − 30 mM ammonium formate buffer-water/methanol could preferably improve peak shape and was finally not adopted as the mobile phase.

3.2 Method validation

3.2.1 Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma with those of the corresponding spiked plasma. As shown in Figure 2, no interference from endogenous substance was observed at the retention time of 4-O-methylhonokiol.

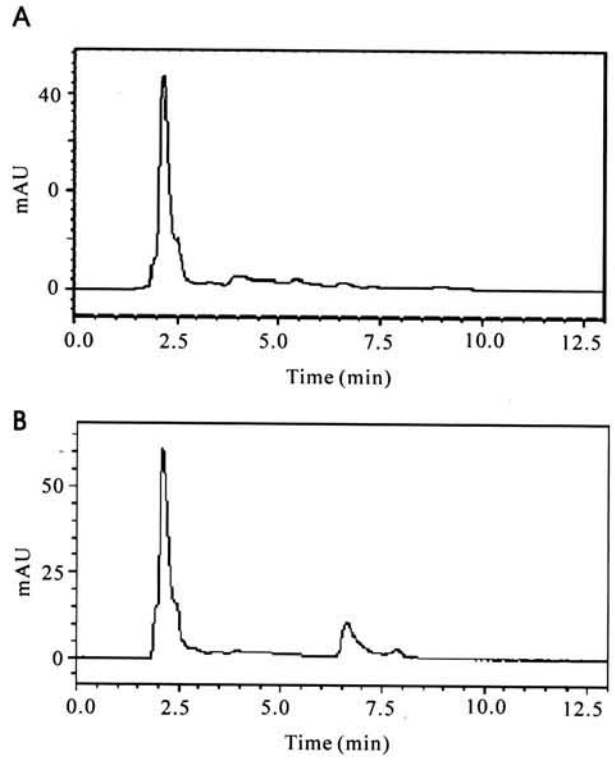

Figure 2 Representative chromatograms of plasma samples. **A**, blank plasma samples; **B**, plasmas sample after oral administration of 4-O-methylhonokiol.

3.2.2 Calibration and detection limits

The regression equation of the curves and the correlation coefficients (r) were calculated as $Y = 76820X + 60805$ (Y is the peak area ratio of 4-O-methylhonokiol, and X is the plasma concentration of 4-O-methylhonokiol) and $r = 0.9994$ using weighted least squares linear regression (the weighing factor was $1/C$). Six calibration curves were linear within the concentration range of 0.012 − 1.536 mg/L. The CV of 4-O-methylhonokiol at each level varied from 1.1% to 45.8%. And the relative bias of 4-O-methylhonokiol from the theoretical value varied from −2.3% to 16.7%. As a result, the calibration curves exhibited good linearity within the experimental range. And the above provided an adequate fit to the data (Table 1). The LOD and LOQ of 4-O-methylhonokiol were 0.2 μg/L and 1 μg/L.

3.2.3 Precision and accuracy

The data from QC samples were calculated to estimate the intra-day and inter-day precision and accuracy of the method. The results are presented in Table 2. The intra-day precision for low, mid and high QC levels of 4-O-methylhonokiol was 11.8%, 1.2% and 0.6%, respectively, and that of inter-day analysis was 13.5%, 3.1% and 1.3%, respectively, with an accuracy (RE) within −7.4% to 8.6%. The accuracy deviation values were within 12% of the nominal values. The precision determined at each concentration level did not exceed 15% of the CV, revealing good precision and accuracy for the method.

Table 1 Calibration curve details for the analysis of 4-O-methylhonokiol in rabbit plasma

Calibration curves	Concentration of standard (mg/L)							
	0.012	0.024	0.048	0.096	0.192	0.384	0.768	1.536
First	0.015	0.028	0.055	0.108	0.198	0.395	0.758	1.514
Second	0.013	0.027	0.049	0.109	0.210	0.401	0.745	1.557
Third	0.013	0.029	0.056	0.094	0.196	0.403	0.745	1.533
Fourth	0.013	0.037	0.043	0.121	0.187	0.404	0.735	1.541
Fifth	0.014	0.011	0.069	0.094	0.173	0.392	0.745	1.536
Sixth	0.017	0.010	0.057	0.120	0.191	0.404	0.771	1.560
Mean	0.014	0.024	0.055	0.107	0.193	0.400	0.750	1.540
SD	0.002	0.011	0.009	0.012	0.012	0.005	0.012	0.017
Accuracy (relative bias[a], %)	16.667	0.000	14.583	11.458	0.521	4.167	−2.344	0.391
Precision (CV[b])	14.286	45.833	16.364	11.215	6.218	1.250	1.600	1.104

[a] Relative bias = $\frac{\text{measured value} - \text{prepared value}}{\text{prepared value}} \times 100$

[b] CV(coefficient of variation) = $\frac{\text{SD}}{\text{mean}} \times 100$

3.2.4 Extraction recovery and matrix effect

The extraction recoveries of 4-O-methylhonokiol from rabbit plasma were (84.7 ± 4.9)%, (89.3 ± 2.9)%, and (87.7 ± 4.8)% at concentration levels of 0.02, 0.17 and 1.20 mg/L, respectively. In terms of matrix effect, all the ratios (A/B × 100%) were between 99.1% and 101.3%, which means no matrix effect for 4-O-methylhonokiol in this method.

3.2.5 Stability

Table 3 summarizes the results of the freeze-thaw stability, long-term stability, short-term stability, and post-preparative stability of 4-O-methylhonokiol in rabbit plasma at three QC levels. It was found that the concentrations of 4-O-methylhonokiol at three QC levels had no obvious changes after six freeze-thaw cycles. The solutions were also stable at room temperature for 24 h, at 4 ℃ for 4 h, at −70 ℃ for 60 days. These results demonstrated a good stability of 4-O-methylhonokiol over all steps of the determination.

3.3 Application in pharmacokinetic study

The present method was applied to the pharmacokinetic investigation of 4-O-methylhonokiol after administering single oral dose of 0.6 mg/kg. Mean plasma drug concentration-time curve of 4-O-methylhonokiol is shown in Figure 3, and the calculating parameters are listed in Table 4. The results indicated that the plasma profile of 4-O-methylhonokiol was a two-compartment open model.

Table 2 The inter-day and intra-day precision and accuracy of the method for determination of 4-O-methylhonokiol

Nominal concentration (mg/L)	Intra-day			Inter-day		
	Detected concentration (mean ± SD, mg/L)	Mean accuracy (%)	CV (%)	Detected concentration (mean ± SD, mg/L)	Mean accuracy (%)	CV (%)
0.020	0.020 ± 0.002	1.221	11.780	0.018 ± 0.002	−11.485	13.463
0.170	0.169 ± 0.002	−0.688	1.182	0.165 ± 0.005	−3.226	3.146
1.380	1.365 ± 0.009	−1.085	0.628	1.346 ± 0.018	−2.498	1.309

Table 3 Stability of 4-O-methylhonokiol in rabbit plasma at three QC levels (mean ± SD, $n = 5$)

Stability	QC level		
	0.020 mg/L	0.170 mg/L	1.380 mg/L
Freeze-thaw stability(%)	0.020 ± 0.002	0.169 ± 0.010	1.367 ± 0.025
Short-term stability(%)	0.020 ± 0.002	0.165 ± 0.014	1.372 ± 0.013
Long-term stability(%)	0.019 ± 0.001	0.165 ± 0.005	1.346 ± 0.178
Post-preparative stability(%)	0.019 ± 0.002	0.168 ± 0.009	1.365 ± 0.009

Table 4 Pharmacokinetic parameters of 4-O-methylhonokiol after oral administration of 4-O-methylhonokiol (0.6 mg/kg) ($n = 6$)

Parameters	Mean ± SD
C_{max} (mg/L)	1.37 ± 0.23
t_{max} (h)	0.85 ± 0.12
$t_{1/2\alpha}$ (h)	0.35 ± 0.05
CL/F (L/h/kg)	0.17 ± 0.03
Ka(1/h)	3.47 ± 0.58
$AUC_{(0-t)}$ (mg·h/L)	3.46 ± 0.61
$AUC_{(0-\infty)}$ (mg·h/L)	3.56 ± 0.64

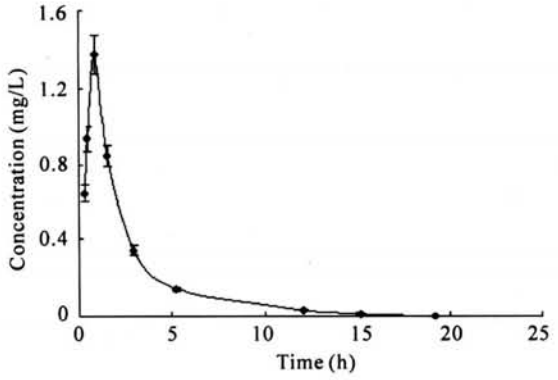

Figure 3 Concentration versus time for 4-O-methylhonokiol in rabbit plasma.

4 Conclusion

A sensitive, selective and rapid HPLC method for the determination of 4-O-methylhonokiol in rabbit plasma was developed. And this method had the properties of reducing ion suppression and offering superior sensitivity with an LOQ of 0.001 mg/L, satisfactory selectivity and short run time of 13.0 min. The proposed method has been successfully applied to a pharmacokinetic study of 4-O-methylhonokiol.

Acknowledgments

This research was financially supported by Xi'an Jiaotong University (No.01380011).

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