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Column-switching high-performance liquid chromatographic assay for determination of asiaticoside in rat plasma and bile with ultraviolet absorbance detection

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Abstract

A column-switching high-performance liquid chromatography (HPLC) method is described for the determination of asiaticoside in rat plasma and bile using column-switching and ultraviolet (UV) absorbance detection. Plasma was simply deproteinated with acetonitrile prior to injection and bile was directly injected onto the HPLC system consisting of a clean-up column, a concentrating column, and an analytical column, which were connected with two six-port switching valves. Detection of asiaticoside was accurate and repeatable, with a limit of quantification of 0.125 μg/ml in plasma and 1 μg/ml in bile. The calibration curves were linear in a concentration range of 0.125–2.5 μg/ml and 1–20 μg/ml for asiaticoside in rat plasma and bile, respectively. This method has been successfully applied to determine the level of asiaticoside in rat plasma and bile samples from pharmacokinetics and biliary excretion studies.

Keywords: Asiaticoside

1. Introduction

Asiaticoside, one of the principle terpenoids in *Centella asiatica* belonging to the *Umbelliferae* family, is known to stimulate collagen synthesis in fibroblast [1,2]. This compound is clinically used as an agent for wound healing in combination with madecassic acid and asiatic acid [3]. Asiaticoside is presumed to be converted in vivo to asiatic acid by hydrolytic cleavage of the sugar moiety and the metabolic product is responsible for the therapeutic effects [4,5].

The analytical method of asiaticoside has been developed using HPLC system with a photodiode array detector [6]. But this method was not suitable for the determination of asiaticoside in biological fluids. We had tried to develop the method to analyze asiaticoside in plasma and bile using single-column HPLC system coupled with various sample pretreatment methods. Since asiaticoside is monitored at 210 nm, asiaticoside was hardly separated from interfering peaks in bile and consequently the detection limit was too high. Therefore, a more sensitive and convenient method was required to determine

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asiaticoside at lower levels for pharmacokinetics and disposition studies of this compound.

In this paper, we report on the development of a column-switching HPLC method for the detection of low levels of asiaticoside in rat plasma and bile using UV absorbance detection. This method was very simple and time saving, and was successfully applied for a blood kinetic and biliary excretion study in rat, in which the level of asiaticoside was determined after intravenous (i.v.) injection of asiaticoside.

2. Experimental

2.1. Chemicals and materials

Asiaticoside (Fig. 1) extracted from *Centella asiatica* was supplied from Dong-Gook Pharmaceuticals (Seoul, Korea). Acetonitrile (HPLC grade) was obtained from J.T. Baker (Mallinckrodt-Baker, Inc., Phillipsburg, NJ, USA). Sodium phosphate was purchased from Junsei Chemical (Tokyo, Japan). Water was deionized and filtered through a Milli-Q water system (Millipore, Bedford, MA, USA).

2.2. Standard solutions and spiked samples

Asiaticoside was dissolved in methanol (1.0 mg/ml) as a stock solution and stored at −20°C and it was further diluted in methanol. Spiked plasma samples were prepared by addition of the stock solution of asiaticoside to blank rat plasma giving final concentrations of 0.125, 0.25, 0.5, 1.25, and 2.5 μg/ml. Spiked bile samples were prepared by addition of the stock solution of asiaticoside to bile to obtain concentrations of 1, 2, 5, 10, and 20 μg/ml.

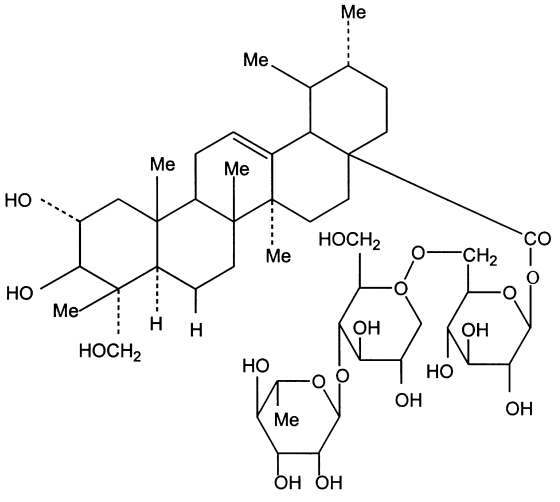

Fig. 1. Chemical structure of asiaticoside.

2.3. Plasma and bile collection

Male Sprague-Dawley rats (200–220 g) were used in this experiment. For the pharmacokinetic study, the femoral artery and vein were cannulated with PE-50 and PE-10 tubing two days before the experiment. The rats were fasted overnight before use and were given a single 10 mg/kg i.v. bolus of asiaticoside. Heparinized samples of blood (0.4 ml) were collected at 0, 1, 3, 5, 10, 20, 30, and 60 min postdose. Plasma was obtained by centrifugation of blood at 1000 *g* for 10 min and was stored at −20°C until analysis. For the biliary excretion study, bile duct and femoral vein were catheterized using PE-10 tubing under pentobarbital anesthesia (40 mg/kg). Bile was collected at 1 h intervals for 24 h. Obtained bile was weighed and stored at −20°C until analysis.

2.4. Instrumentation

The HPLC system consisted of a Shisheido HPLC system (NANOSPACE, Shisheido, Tokyo, Japan) with SI-1/2001 pump, an automatic six-port switching valve (SI-2012), an autosampler (SI-1/2003) coupled to the degasser (SI-1/2009) and UV–Vis detector (SI-1/2002). The columns used in this experiment were a Capcell Pak MF Ph-1 (150×4.6 mm, 5 μm, Shisheido) as column A for clean-up, a Capcell Pak C_{18} UG120 (35×2.0 mm, 5 μm, Shisheido) as column B for concentration of analyte, and a Capcell Pak C_{18} UG120 (250×1.5 mm, 5 μm, Shisheido) as column C for analysis. Column temperature was maintained constant at 40°C using thermostatically controlled column oven (SI-1/2004). Quantitative evaluation of chromatograms was performed at 210 nm using chromatogram integration software SM-C (Shisheido, Tokyo, Japan). The mobile phases used were 10 m*M* sodium phosphate (pH 6.86) (buffer A) and 50% acetonitrile

in deionized water (v/v) (buffer B). The samples were kept in the autosampler at 10°C until injected.

2.5. Chromatographic conditions

A schematic diagram of the HPLC system is shown in Fig. 2. Sample clean-up and chromatographic separation were performed as following.

2.5.1. Step 1

Plasma samples (200 μl) were vortex-mixed after the addition of five volumes of acetonitrile and centrifuged at 1000 *g* for 5 min. The supernatant was dried under nitrogen stream and the residue was dissolved in 50 μl of 50% aqueous methanol and 30 μl was injected onto the clean-up column. Bile samples were filtered with PVDF syringe filter (13 mm, 0.2 μm, Millipore, Bedford, MA, USA) and 20 μl of filtered sample was injected onto the clean-up column. At the time of sample injection, the column-switching valves 1 and 2 were placed in position I. Proteins and other interfering compounds were washed to waste by isocratic elution using washing solvent consisting of A–B (82:18, v/v) for plasma and A–B (87:13, v/v) for bile at a flow-rate of 1.0 ml/min.

2.5.2. Step 2

The switching valve 1 was shifted to position II at 4.9 min for plasma and at 7.7 min for bile, thus eluting fractions containing the target compound from the precolumn to the concentration column by isocratic elution using washing solvent. A flow-rate was reduced to 0.5 ml/min at this step.

2.5.3. Step 3

The six-port valve 1 was shifted to position I and valve 2 was switched to position II at the time of 9.9 min for plasma and 11.7 min for bile, and the enriched compounds were eluted in the back-flush mode from the concentration column into the analytical column. The eluted compounds were separated on the analytical column by mobile phase consisting of A–B (46:54, v/v) for plasma and A–B (44:56, v/v) for bile at a flow-rate of 0.1 ml/min. The eluate was monitored at 210 nm.

2.6. Calibration and calculations

Calibration curves for asiaticoside in plasma and bile were generated by plotting the peak area versus those nominal concentrations in the standard plasma

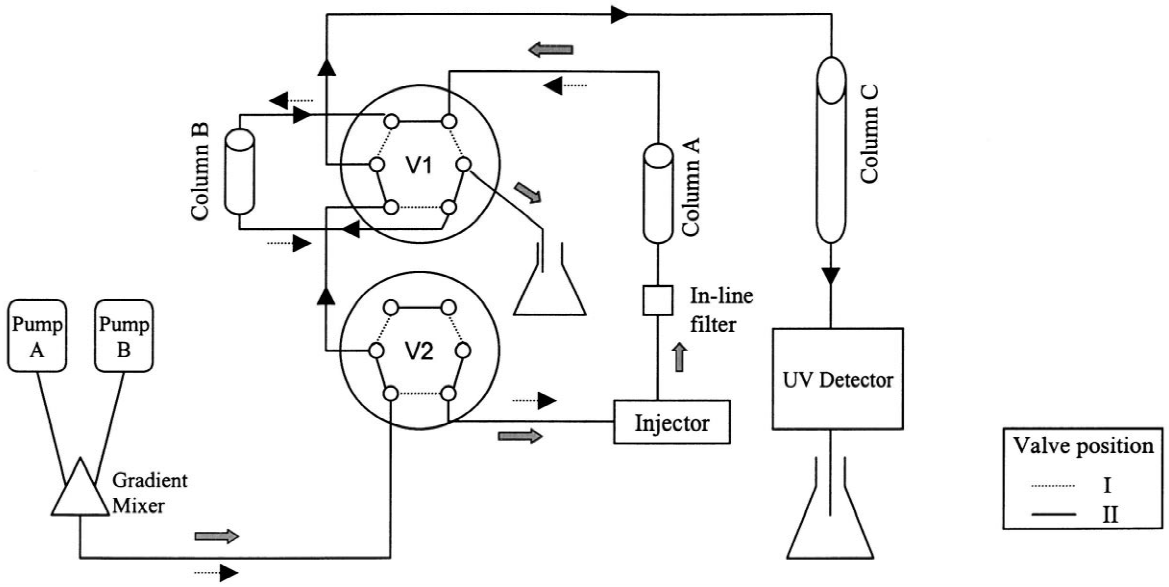

Fig. 2. Schematic diagram of the column-switching HPLC system. The arrows indicate the direction of the flow depending on the valve position: bold line, V1/V2=I/I (washing); dashed line, V1/V2=II/I (concentration); and the arrow in line, V1/V2=I/II (analysis).

or bile by the $1/X$ weighted least-square linear regression:

$$Y = aX + b$$

where Y, a, X, and b are the peak area, the slope, the concentration, and the Y-intercept, respectively. The concentrations of the asiaticoside in samples containing unknown amounts of asiaticoside were calculated on the basis of this regression lines.

2.7. Recovery, coefficient of variation, and accuracy

Recovery was determined by injecting a standard solution of asaticoside directly onto the HPLC column and by comparing its peak area with those produced by plasma and bile samples.

Intra-day coefficient of variation (C.V.) and accuracy of the quantitation were evaluated by the analysis of 6 samples of plasma or bile spiked at the same concentrations as the calibration standards. The C.V. and accuracy for inter-day assay were assessed at the same concentration, repeated for 6 different days.

3. Results and discussion

3.1. Chromatography

Terpenoids including asiaticoside do not possess chromophore and consequently are difficult to be analyzed in biological fluids. Although the analysis of asiatic acid by gas chromatography–mass spectrometry [4] or HPLC [5] has been reported, no analytical method was developed to determine asiaticoside in plasma or bile. Since asiaticoside is not extractable by liquid–liquid extraction and monitored at short ultraviolet wavelength, column-switching HPLC method has been developed to obtain sufficient separation of the asiaticoside from interfering compounds in plasma and bile. Column-switching techniques have been reported to be useful for rapid analysis of drugs in biological fluids [7,8].

The Capcell pak MF column, used as the precolumn in this study, has a property of a mixture of hydrophilic and hydrophobic phases coating on silicone polymer-coated silica beads [9,10] and therefore minimized interference by the biological hydrophilic components such as proteins or bilirubin. In using 9% (plasma) or 6.5% (bile) acetonitrile in 10 m*M* phosphate buffer (pH 6.86) as washing solvents, the majority of endogenous components were relatively unretained while asiaticoside exhibited strong retention on Capcell Pak MF Ph-1 precolumn. The initial clean-up was done by washing the precolumn at a flow-rate of 1.0 ml/min with washing solvents and the fractions containing asiaticoside were enriched on the concentration column by switching the valve position. The analyte was subsequently transferred to the analytical column by reversing the flow and further separation was achieved. Since the analytical column's diameter was 1.5 mm, the flow-rate was reduced to 0.1 ml/min during analysis. Fig. 3 shows the chromatograms of blank rat plasma (A), rat plasma containing 1 μg/ml of asiaticoside (B), blank rat bile (C), and rat bile containing 5 μg/ml of asiaticoside (D). There were no interfering peaks from endogenous compounds in plasma and bile near the retention time of asiaticoside.

3.2. Stability of standard solution and recovery

Stability of asiaticoside in stock solution was confirmed to be satisfactory during the development of the analytical method: the remaining percentage was 99.2% after 90 days under refrigeration. Table 1 describes the recoveries of asiaticoside from rat plasma and bile. The recovery was 92.0±10.4%–102.9±8.7% for plasma and 92.6±8.7%–110.5±9.8% for bile in the concentration range of calibration curve.

3.3. Calibration curve, coefficient of variation, and accuracy

The standard calibration curve for spiked rat plasma containing asiaticoside was linear over the range of 0.125 to 2.5 μg/ml with correlation coefficient greater than 0.996. The typical calibration curve was given by the equation, $y = 217435x + 5996$, where y indicates the peak area and x represents the concentration of asiaticoside in μg/ml. The calibration curve for spiked rat bile was linear between 1 and 20 μg/ml with a correlation coefficient greater than 0.999. The equation of the cali-

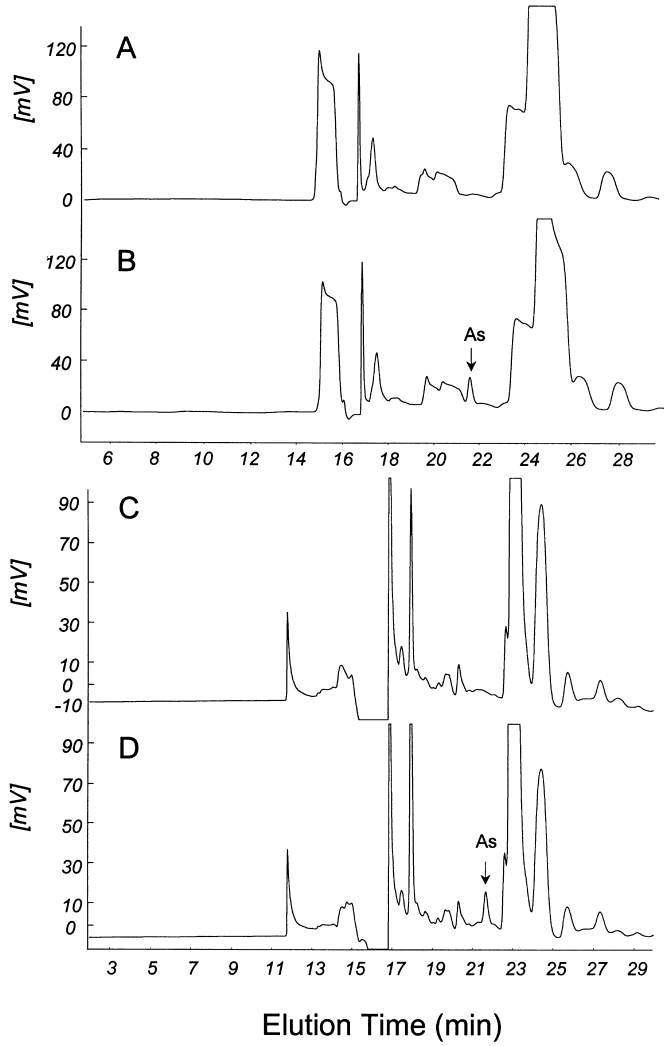

Fig. 3. HPLC chromatograms of blank rat plasma (A), rat plasma spiked with asiaticoside (1 μg/ml) (B), blank rat bile (C), and rat bile spiked with asiaticoside (5 μg/ml) (D).

Table 1
Recovery of asiaticoside from rat plasma and bile ($n=6$)

Concentration (μg/ml)		Recovery (%) (mean±SD)	
Plasma	Bile	Plasma	Bile
0.125	1	102.9±8.7	92.6±8.7
0.25	2	92.0±10.4	96.1±9.4
0.5	5	105.1±3.2	110.5±9.8
1.25	10	98.1±10.9	108.3±5.0
2.5	20	92.4±4.0	107.2±5.1

bration curve of bile was $y = 41513x - 3085$. The intra and inter-days variations of the asiaticoside determinations in plasma and bile are summarized in Table 2. The intra-day coefficients of variation were less than 15.1%, and the intra-day accuracies were between 83.2% and 106.6% within the concentration range of the calibration curves in plasma and bile. The inter-day coefficients of variation did not exceed 14.4%, and its accuracy was between 86.4% and 104.1%. The limit of quantitation for asiaticoside was set to 0.125 μg/ml in plasma and 1 μg/ml in bile, which is the lowest concentration of the analyte

Table 2
Intra-day and inter-day coefficient of variation and accuracy for determination of asiaticoside in rat plasma and bile ($n=6$)

Theoretical concentration (μg/ml)	Intra-day			Inter-day		
	Concentration found (mean±SD) (μg/ml)	C.V.	Accuracy	Concentration found (mean±SD) (μg/ml)	C.V.	Accuracy
Plasma						
0.125	0.104±0.014	13.5	83.2	0.108±0.016	14.4	86.4
0.25	0.214±0.032	15.1	85.6	0.235±0.027	11.4	94.0
0.5	0.533±0.012	2.3	106.6	0.508±0.036	7.1	101.6
1.25	1.288±0.129	10.0	103.0	1.297±0.095	7.3	103.8
2.5	2.461±0.088	3.6	98.4	2.492±0.084	3.4	99.7
Bile						
1	1.039±0.110	10.6	103.9	0.999±0.163	12.3	99.9
2	1.918±0.172	9.0	95.9	2.070±0.269	13.6	103.5
5	5.234±0.451	8.6	104.7	5.203±0.547	9.7	104.1
10	10.122±0.463	4.6	101.2	9.728±0.520	5.0	97.3
20	19.886±0.934	4.7	99.4	19.340±0.732	3.8	96.7

that can be measured with a coefficient of variation and an accuracy less than 20%.

3.4. Application

The present method was applied for the analysis of plasma and bile samples collected from rats dosed with asiaticoside. Mean plasma concentration-time curve of asiaticoside after i.v. injection of asiaticoside at a single dose of 10 mg/kg is shown in Fig. 4. Area under curve (AUC) and elimination half-life ($T_{1/2}$) of asiaticoside was determined to 25.53±4.56 μg·h/ml and 0.26±0.10 h, respectively. The excretion of asiaticoside into bile after i.v. injection at a single dose of 10 mg/kg is shown in Fig. 5. The amount of asiaticoside excreted in bile over 24 h was 81.4±1.6% of administered dose. This method had adequate sensitivity for the pharmacokinetics and biliary excretion of asiaticoside after i.v. injection to rats.

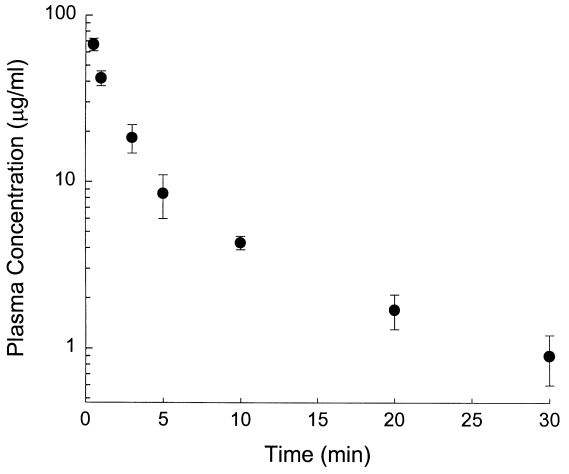

Fig. 4. Mean plasma concentrations of asiaticoside after i.v. injection of asiaticoside at a dose of 10 mg/kg to rats ($n=4$).

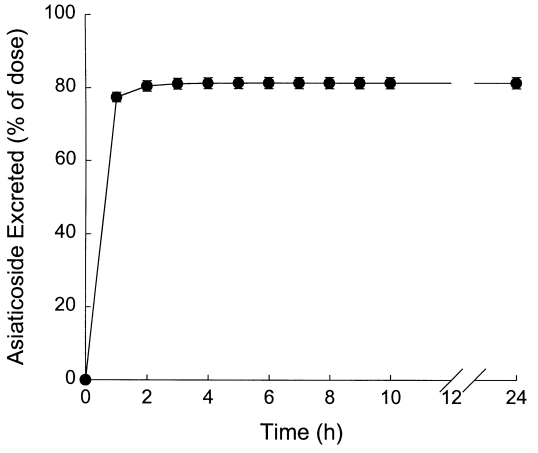

Fig. 5. Biliary excretion of asiaticoside after i.v. injection of asiaticoside at a dose of 10 mg/kg to rats ($n=4$).

4. Conclusions

A selective and sensitive column-switching HPLC method for the determination of asiaticoside in rat plasma and bile was developed. The method seems to be advantageous over other methods reported so far in the literature in terms of peak separation and detection sensitivity. This method was successfully applied to the determination of concentrations of asiaticoside in rat plasma and bile after i.v. injection of asiaticoside.

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References

[1] F.X. Marquart, G. Bellon, P. Gillery, Y. Wegrowski, J.P. Rorel, Connet. Tissue Res. 24 (1990) 107.
[2] F. Bonte, M. Dumas, C. Chaudagne, A. Meybeck, Ann. Pharm. Fr. 53 (1995) 38.
[3] B.M. Hausen, Contact Dermatitis 29 (1993) 175.
[4] W.R. Rush, G.R. Murray, D.J.M. Graham, Eur. J. Drug Metab. Pharmacokinet. 18 (1993) 323.
[5] R. Grimaldi, F. De Ponti, L. D'angelo, M. Caravaggi, G. Guidi, S. Lecchini, G.M. Frigo, A. Crema, J. Ethnopharmacol. 28 (1990) 235.
[6] P. Morganti, A. Fionda, U. Elia, L. Tiberi, J. Chromatogr. Sci. 37 (1999) 51.
[7] B.B. Ba, D. Ducint, M. Fourtillan, M.C. Saux, J. Chromatogr. B 714 (1998) 317.
[8] S.E. Nielsen, L.O. Dragsted, J. Chromatogr. B 713 (1998) 379.
[9] T. Kanda, H. Kursuna, Y. Ohtsu, M. Yamaguchi, J. Chromatogr. A 672 (1994) 51.
[10] D. Song, J.L.-S. Au, J. Chromatogr. B 676 (1996) 165.