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Journal of Chromatography B, 842 (2006) 22–27
JOURNAL OF CHROMATOGRAPHY B
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Simultaneous determination of geniposide, baicalin, cholic acid and hyodeoxycholic acid in rat serum for the pharmacokinetic investigations by high performance liquid chromatography–tandem mass spectrometry

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Received 17 October 2005; accepted 2 May 2006
Available online 5 June 2006

Abstract

A simple, rapid, and specific analytical method for simultaneous determination of geniposide, baicalin, cholic acid and hyodeoxycholic acid in 50 μL samples of rat serum was developed by high performance liquid chromatography–tandem mass spectrometry. The quantification of the target compounds was determined by multiple reaction monitoring (MRM) mode using electrospray ionization (ESI). The correlation coefficients of the calibration curves were better than 0.997. The intra- and inter-day accuracy, precision, and linear range had been investigated in detail. This method was subsequently applied to pharmacokinetic studies of geniposide, baicalin, cholic acid and hyodeoxycholic acid in rats successfully.

Keywords: HPLC–MS–MS; Geniposide; Baicalin; Cholic acid; Hyodeoxycholic acid; Pharmacokinetics

1. Introduction

Geniposide, baicalin, cholic acid and hyodeoxycholic acid (Fig. 1) are the four main active components of Qingkailing injection, which is an important multiherb remedy in Traditional Chinese Medicine (TCM). As described in the Chinese pharmacopoeia [1], it is a combination of eight herbs, including cholic acid, hyodeoxycholic acid, *Cornu bubali* (Shuiniujiao), baicalin, *Concha margaritifera* (Zhenzhumu), *Fructus gardeniae* (Zhizi), *Radix isatidis* (Banlangen) and *Flos lonicerae japonicae* (Jinyinhua). Qingkailing injection possesses various pharmacological effects such as antifebrile, antiinflammation, sedative, anticonvulsion, antiinfection, and vasodilatation. As a popular medicine, it has been widely used clinically.

To investigate the synergistic interaction among the bioactive components and the pharmacological effects of Qingkailing injection, simultaneous determination of geniposide, baicalin, cholic acid and hyodeoxycholic acid in biological fluids for pharmacokinetic investigation is required.

Several methods have been previously described for the determination of each of these analytes in serum separately [2–11]. Among these reports, reversed-phase HPLC was the most frequently used technique [4–11]. However, HPLC is limited because cholic acid and hyodeoxycholic acid do not possess any chromophore necessary for UV detection, the chromatographic separation of these two compounds had to be traced by a shorter UV wavelength (192 nm). At this short wavelength, the specificity was poor, and the sensitivity could also be affected by using methanol/acetonitrile–water gradients. Using HPLC with ELSD detection, Cao et al. [6] performed pharmacokinetic investigations of cholic acid and hyodeoxycholic acid in Qingkailing injection. This method needed long chromatographic run time of 50 min, time-consuming sample pretreatments and large amount of samples. Recently, high performance liquid chromatography–tandem mass spectrometry (LC–MS–MS) technique was applied to analyze hyodeoxycholic acid [3].

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doi:10.1016/j.jchromb.2006.05.001

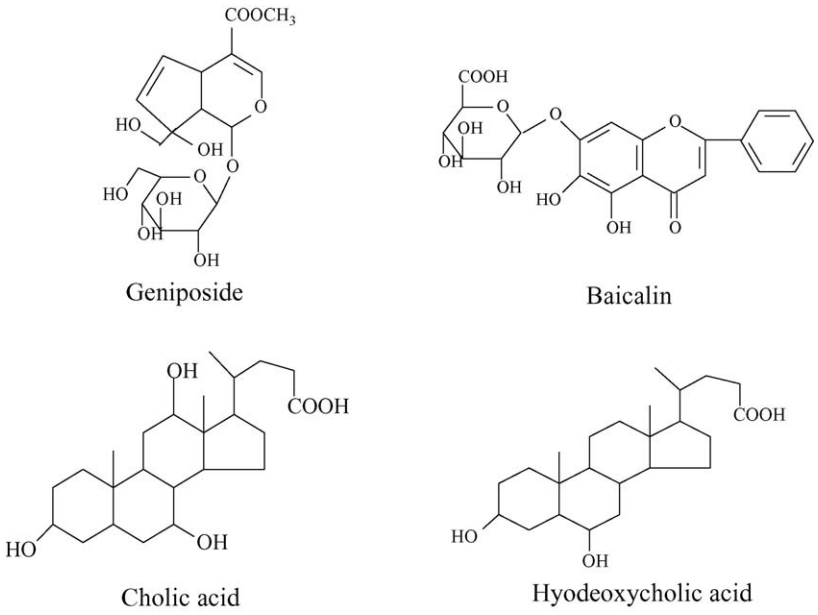

Fig. 1. Chemical structures of geniposide, baicalin, cholic acid and hyodeoxycholic acid.

To our knowledge, there has not been any method reported for the simultaneous estimation of these four active constituents for pharmacokinetic investigations. Being a common analytical tool for various compounds, LC–MS–MS has advantage over HPLC, for it can detect the compound which has no chromophore necessary for UV detection. Therefore, the present research developed a LC–MS–MS method for simultaneous analysis of geniposide, baicalin, cholic acid and hyodeoxycholic acid, requiring only 50 μL sample volume. This method was subsequently applied to pharmacokinetic studies in rats following the 4 mL/kg Qingkailing injection.

2. Experimental

2.1. Chemicals and reagents

Geniposide (100%) and baicalin (100%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Cholic acid (98%) and hyodeoxycholic acid (98%) were purchased from Sigma (St. Louis, MO, USA).

HPLC grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Analytical grade ammonium acetate was purchased from Beijing Chemical Reagents Company (Beijing, China). Distilled water was purified using a MilliQ system (Millipore Corp. Bedford, MA, USA). The Qingkailing injection (content: geniposide, 0.17 mg/mL; baicalin, 4.32 mg/mL; cholic acid, 1.97 mg/mL; hyodeoxycholic acid, 0.86 mg/mL) was from the Pharmaceutical Factory of Beijing University of TCM (Beijing, China).

2.2. LC–MS–MS

An Applied Biosystems (Toronto, Canada) API 3000 triple-quadrupole tandem mass spectrometer, equipped with a Turbo Ionspray interface and an Agilent 1100 binary HPLC system, was used for LC–MS–MS analysis. The data were processed using Macquan software (PE Sciex). A Waters X-Terra^{TM} RP C18 column (150 mm × 3.9 mm I.D., 5 μm particle size) was applied for the separation. The temperature of the column was set at 30 °C. The flow-rate was 500 μL/min, mobile phase component A was 3 mM CH_3COONH_4 (aq) and B was 3 mM CH_3COONH_4 acetonitrile, and the injection volume was 20 μL. The column was eluted with a linear gradient of 5–75% B over 0–8 min, 75–95% B over 8–10 min, and the composition was maintained 95% B for 2 min, then returned to 5% B at 12 min immediately. The LC effluent was split by the ratio of 4:1 using a valved three-way split, so that approximately 100 μL/min was introduced into the source of the mass spectrometry. The negative-ion mode of MS–MS was adopted and MRM was used for the specific detection of geniposide, baicaline, cholic acid and hyodeoxycholic acid. The ionspray voltage of −3500 V and ion source temperature of 350 °C were applied. The nebulizer gas (air) and curtain gas (nitrogen) were set at a value of 8 and 11 (instrument units), respectively. The flow-rate of drying gas (nitrogen) was 2 L/min. The dwell time was 200 ms. The optimized quad 2 rod offset (RO2) voltage, the quad 3 rod offset (RO3) voltage, stubbies 3 (ST3) voltage, orifice potential (OR) and ring focus voltage (RNG) are shown in Table 1. The collision gas (nitrogen) was set at 6 (instrument units), and the optimized collision energy (Q0-RO2) of 12 eV was used for geniposide, 30 eV for baicalin, 48 eV for cholic acid and 44 eV for hyodeoxycholic acid.

2.3. Calibration procedure

2.3.1. Stock solutions, calibration standards, and quality control samples

Stock solutions of 100 μg/mL geniposide, baicalin, cholic acid and hyodeoxycholic acid were prepared in volumetric

Table 1
Optimized parameters for MRM analysis of geniposide, baicalin, cholic acid and hyodeoxycholic acid

Compound	Transition	RO2 (V)	RO3 (V)	ST3 (V)	OR (V)	RNG (V)
Geniposide	386.9/224.9	22	24	36	−46	−180
Baicalin	444.9/268.9	40	42	54	−36	−130
Cholic acid	407.1/407.1	58	60	78	−90	−110
Hyodeoxycholic acid	391.1/391.1	54	56	78	−101	−200

flasks in methanol individually. A series of standard working solutions with the concentrations of 50, 100, 200, 500, 1000, 1500, 3000 ng/mL for geniposide, 1000, 1500, 3000, 5000, 10,000, 20,000, 30,000 ng/mL for baicalin, 100, 200, 500, 1000, 1500, 5000, 10,000 ng/mL for cholic acid and 100, 200, 500, 1000, 1500, 3000, 5000 ng/mL for hyodeoxycholic acid were obtained by further dilution of each standard stock solution with methanol. All standard working solutions were evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL of drug-free rat serum to prepare the calibration standards. The serum samples were treated with 200 μL methanol, mixed and centrifuged for 10 min at 13,000 rpm at 4 °C.

Quality control (QC) samples were prepared in the same way as calibration standards with blank serum, and the concentrations of each analyte were at the low, middle and upper limits of quantification (see Table 2 for individual standard concentrations).

2.3.2. Calibration curves

External calibration method was used for the quantitative analysis. Calibration curves were obtained by the plots of the peak-area versus the concentration of the calibration standards. Since cholic acid and hyodeoxycholic acid are endogenous, and both have a signal in drug-free rat serum, for preparing the standard curves with serum, the background response of cholic acid and hyodeoxycholic acid in blank serum was subtracted. The concentrations of the unknown samples were determined by using the equations of linear regression obtained from the calibration curves. For cholic acid and hyodeoxycholic acid, the average background response in blank serum samples was subtracted from the real sample for quantification in pharmacokinetic studies.

2.4. Method validation

Intra-day accuracy and precision (each $n = 5$) were evaluated by analysis of QC samples at different times on the same 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 error (RE), and precision was determined by the coefficients of variation (CV). Signals 3 and 10 times higher than the peak noise height were regarded as the limit of detection (LOD) and quantification (LOQ) for geniposide and baicalin. Because cholic acid and hyodeoxycholic acid are endogenous in serum, LOD and LOQ for them were calculated using the following formula: $\mathrm{LOD} = 3.3\,\sigma/S$; $\mathrm{LOQ} = 10\,\sigma/S$. Where σ = the standard deviation of the analytical background response, S = the slope of the calibration curve [12].

The extraction recovery was determined by comparing the peak areas obtained from QC samples with the un-extracted standard working solutions at the same concentration in the same solvent.

The compound stability for 0, 4, 8, 16, and 24 h at room temperature in serum was evaluated by repeated analysis at the medium concentration of QC samples.

Table 2
Intra-day accuracy and precision values for geniposide, baicalin, cholic acid and hyodeoxycholic acid from the assay QC standards ($n = 5$)

Compound	Spiked concentration (ng/mL)	Measured concentration (mean ± S.D.) (ng/mL)	RE (%)	CV (%)	Extraction recoveries (%)
Geniposide	120	132.2 ± 9.60	10.2	7.3	88.3
	450	453.7 ± 22.6	0.8	5.0	92.1
	1400	1518.0 ± 61.1	8.4	4.0	87.5
Baicalin	1400	1429.7 ± 93.8	2.1	6.6	80.7
	8000	7589.2 ± 123	−5.1	1.6	84.8
	20000	19377.3 ± 395	−3.1	2.0	85.2
Cholic acid	120	122.6 ± 17.7	2.2	14.4	83.9
	1800	1826.9 ± 88.6	1.5	4.8	87.4
	5500	5425.5 ± 567	−1.4	10.5	86.5
Hyodeoxycholic acid	120	125.0 ± 7.70	4.2	6.2	96.2
	1800	1902.7 ± 51.3	5.7	2.7	95.6
	4000	3796.3 ± 242	−5.1	6.4	96.2

2.5. Assay application

Male Wistar rats (approximately 200 g), used in the pharmacokinetics study, were obtained from Beijing Vital River Animal Tech. Ltd. (Beijing, China). A total of 70 rats were acclimatized for 7 days and then divided into 10 dose groups for 10 different time points to collect serum. At 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4 h after the administration of 4 mL/kg of Qingkailing injection via intraperitoneal (ip) injection (equivalent to 0.68, 17.28, 7.88 and 3.44 mg/kg of geniposide, baicalin, cholic acid and hyodeoxycholic acid, respectively), the rats in relevant time-point groups were killed and serum samples were collected. The samples were maintained at −80 °C. Thawed at room temperature prior to analysis, a 50 μL serum was treated with 100 μL methanol to precipitate proteins. After vortexing, centrifugation at 13,000 rpm for 10 min at 4 °C, the supernatant was ready for injection.

3. Results and discussion

3.1. ESI mass spectra and LC–MS–MS conditions

The analytes were easily cleaved to generate negative product ions, so the negative-ion mode was applied for analysis. The transitions for MRM analysis of geniposide and baicalin were selected at *m/z* 386.9/224.9 and *m/z* 444.9/268.9, respectively. Because these two transitions were specific and the intensities of these two product ions were the strongest. It was found that the cholic acid and hyodeoxycholic acid were so stable that none of their fragment ions had satisfied abundance while good intensity of the parent ion at Q3 can still be achieved even under high collision energy. Transitions of parent ion to parent ion at relatively high collision energies were employed for sensitive detection for cholic acid and hyodeoxycholic acid since higher collision energy may have an advantage in enhancing the selectivity.

Endogenous interferences and ion suppression were the significant problems using the isocratic mobile phase to elute for high throughput analysis, so the gradient conditions were optimized. The standard solutions were added to the blank serum for specific evaluation. Under the optimized HPLC conditions, good LC–MS–MS chromatographic separation was achieved in the assay of the serum sample (Fig. 2).

As shown in Fig. 2, the retention times of geniposide, baicalin, cholic acid and hyodeoxycholic acid were approximately 7.2, 7.7, 9.5, and 10.2 min, respectively.

3.2. Calibration curves

The linear regression analysis was constructed by plotting the peak-area of analytes versus analyte concentration (ng/mL) in serum samples. The regression equation of these curves and their correlation coefficients (r) were calculated as follows: geniposide, $y = 43.22x - 99.87$, $r = 0.9996$; baicalin, $y = 1.41x - 266.00$, $r = 0.9976$; cholic acid, $y = 190.52x + 31243.36$, $r = 0.9996$; hyodeoxycholic acid $y = 161.73x + 8858.26$, $r = 0.9976$. The error for back-calculated concentration of each calibration point was within 10% except for the lowest concentration with an error of below 20%.

The LOD and LOQ were 2 and 10 ng/mL for geniposide, 100 and 800 ng/mL for baicalin, 4.27 and 12.94 ng/mL for cholic acid, 5.42 and 16.43 ng/mL for hyodeoxycholic acid, respectively.

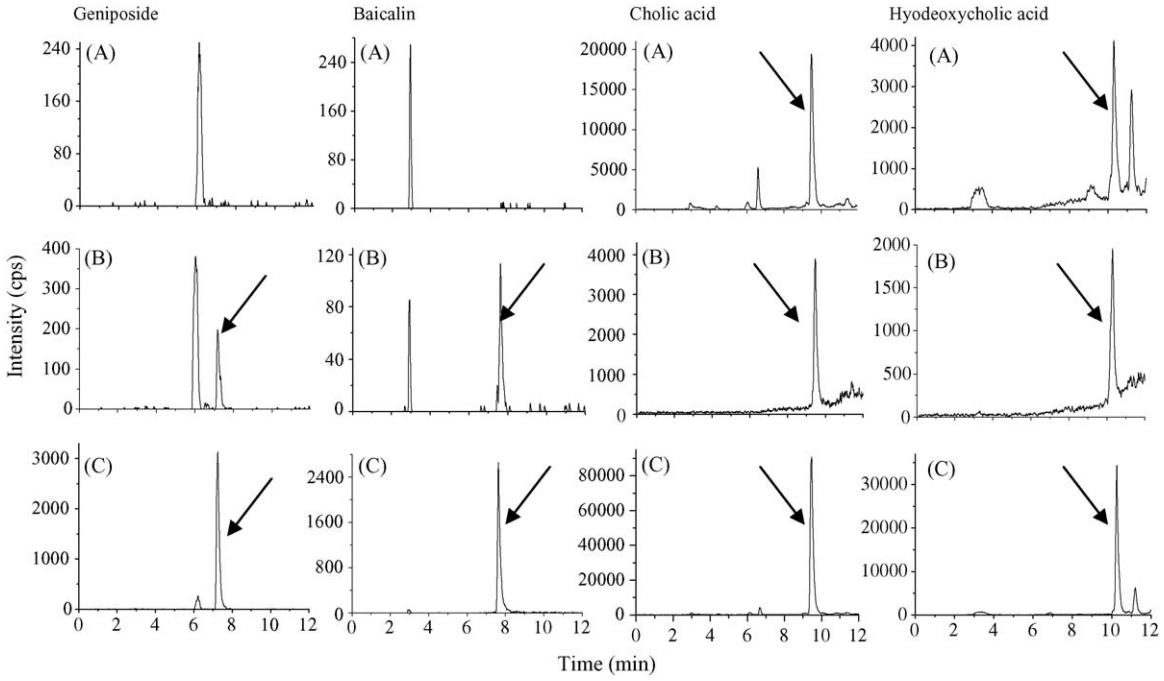

Fig. 2. MRM chromatograms from (A) blank serum, (B) blank serum spicked with geniposide (50 ng/mL) and baicalin (1000 ng/mL), standard solution of cholic acid (50 ng/mL) and hyodeoxycholic acid (50 ng/mL) in methanol, (C) a rat serum sample containing 1010, 18970, 5842 and 2930 ng/mL of geniposide, baicalin, cholic acid and hyodeoxycholic acid, respectively 0.25 h after administration of 4 mL/kg Qingkailing injection via ip.

Table 3
Inter-day accuracy and precision values for geniposide, baicalin, cholic acid and hyodeoxycholic acid from the assay QC standards ($n = 6$)

Compound	Spiked concentration (ng/mL)	Measured concentration (mean ± S.D.) (ng/mL)	RE (%)	CV (%)
Geniposide	120	128.9 ± 7.80	7.4	6.1
	450	462.8 ± 22.3	2.8	4.8
	1400	1419.2 ± 84.8	1.4	6.0
Baicalin	1400	1429.0 ± 66.3	2.1	4.6
	8000	7606.0 ± 106	−4.9	1.4
	20000	19615.1 ± 490	−1.9	2.5
Cholic acid	120	121.2 ± 14.2	1.0	11.7
	1800	1839.5 ± 71.6	2.2	3.9
	5500	5567.1 ± 509	1.2	9.1
Hyodeoxycholic acid	120	125.9 ± 6.5	4.9	5.2
	1800	1918.1 ± 51.3	6.6	2.7
	4000	3896.0 ± 281	−2.6	7.2

Table 4
Pharmacokinetic parameters of geniposide, baicalin, cholic acid and hyodeoxycholic acid following the 4 mL/kg Qingkailing injection to rats via ip (equivalent to 0.68, 17.28, 7.88, and 3.44 mg/kg of geniposide, baicalin, cholic acid and hyodeoxycholic acid, respectively)

Parameter	Unit	Geniposide	Baicalin	Cholic acid	Hyodeoxycholic acid
$t_{1/2\alpha}$	h	0.386	0.436	0.235	0.121
Vd	L/kg	0.001	0.019	0.014	0.002
CL	L/h/kg	0.001	0.0002	0.001	0.001
AUC_{0-4}	mg/L h	638	16000	5942	3143
$AUC_{0-\infty}$	mg/L h	658	80800	11872	3344
MRT_{0-4}	h	386	0.813	0.903	0.125
$MRT_{0-\infty}$	h	0.010	1.42	0.681	0.185

3.3. Extraction recovery

The extraction recoveries were determined at three concentration levels and the results are shown in Table 2. For cholic acid and hyodeoxycholic acid, the background response in blank serum was subtracted.

3.4. Accuracy and precision

The data from QC samples were calculated to estimate the accuracy, intra-day and inter-day precision of the method. The results are presented in Tables 2 and 3.

3.5. Stability

The CV (%) of geniposide, baicalin, cholic acid and hyodeoxycholic acid in 24 h at room temperature was 2.2, 3.5, 3.0, and 2.9, respectively. The result showed good stability of these four components.

3.6. Application

The present method was applied to the pharmacokinetic investigations of geniposide, baicalin, cholic acid and hyodeoxycholic acid following the 4 mL/kg Qingkailing injection to rats. The concentrations for cholic acid and hyodeoxycholic acid found in serum after administration of Qingkailing were much higher than the values found in blank serum samples, and the blank serum was subtracted from the real sample for quantification.

Pharmacokinetic parameters were estimated by 3p97 software package (version 1.1, Chinese Pharmacological Association) and the results are listed in Table 4, and the concentration–time profiles are shown in Fig. 3.

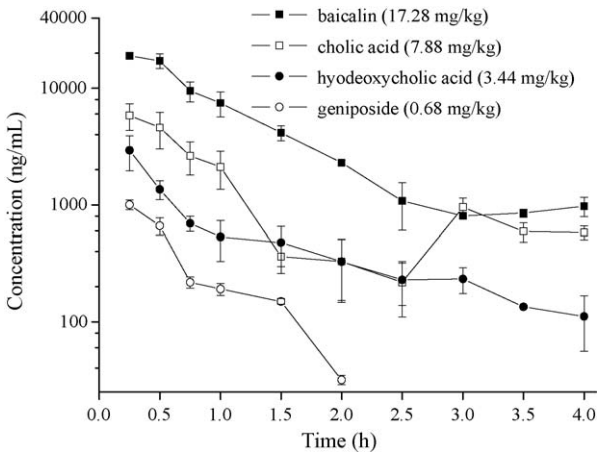

Fig. 3. Mean serum concentration–time profiles ($n = 7$) of geniposide, baicalin, cholic acid and hyodeoxycholic acid following the 4 mL/kg Qingkailing injection to rat (via ip) (after 3.0 h, the concentration of geniposide was lower than the LOQ).

Following the administration of the Qingkailing injection, the concentrations of baikalin, cholic acid and hyodeoxycholic acid were all fitted to a two-compartment model. The concentrations of geniposide declined rapidly and were fitted to a linear one-compartment model. Previous investigation on pharmacokinetics for baicalin in Qingkailing injection [7] also reported the same result.

4. Conclusion

In conclusion, this paper described a simple, rapid, and specific LC–MS–MS method for simultaneous determination of geniposide, baicalin, cholic acid and hyodeoxycholic acid in serum. Only 50 μL of serum was required for the sample preparation procedures. The method has been successfully applied to pharmacokinetic studies of these four compounds in Qingkailing injection in rats.

Acknowledgements

This work was financially supported by the Chinese National Key Technologies R&D program (no. 2004BA721A13), National Natural Science Foundation of China (30300469) and Tsinghua Basic Research Foundation (JCqn2005038).

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