

Simultaneous determination of baicalin, rhein and berberine in rat plasma by column-switching high-performance liquid chromatography

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Abstract

A simple HPLC method using column-switching and ultraviolet detection was developed for the simultaneous determination of baicalin (BA), rhein (RH) and berberine (BE) in rat plasma. Plasma samples were injected directly onto a Capcell Pak MF C₈ column (150 mm × 4.6 mm i.d.) to remove protein and to be pre-separated by an isocratic elution using 50 mmol/L phosphate sodium (pH 6.85)–acetonitrile (10:1, v/v). After the drug-containing fractions were transferred to a Kromasil C₁₈ column (150 mm × 4.6 mm i.d.) by a valve switching step, the valve position was switched back and the main separation was performed by an isocratic elution using triethylamine adjusted 20 mmol/L phosphoric acid (pH 6.78)–acetonitrile (4:1, v/v). The flow rate was always 1.0 mL/min. The calibration curve showed excellent linear relationship ($r \geq 0.9997$) over the concentration range of 0.4–7.9 µg/mL for baicalin, 0.2–7.8 µg/mL for rhein and 0.4–7.7 µg/mL for berberine in rat plasma. The intra- and inter-day assay precisions (R.S.D.) of three analytes were in the range of 0.34–4.3% and the accuracies were between 98.0% and 102.4%. Their recoveries were all greater than 95%. The method was successfully applied to the multi-constituents plasma concentration–time curve study after oral administration of a traditional Chinese medicine prescription *Xiexin-Tang* in rats.

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Keywords: HPLC; Plasma; Column-switching; Traditional Chinese medicine; Baicalin; Rhein; Berberine

1. Introduction

Chinese herbal medicine has attracted considerable attention all over the world. Hundreds of Traditional Chinese medicines (TCMs) are utilized to treat various diseases [1], which greatly promote the development of corresponding, effective and precise analytical methods. It is no doubt a remarkable progress that many studies have focused on simultaneous determination of multiple constituents in TCMs [2–5]. However, few reports about multi-constituents assay in the biological matrix have been documented [6].

Xiexin-Tang which consists of *Radix Scutellariae*, *Radix et Rhizoma Rhei* and *Rhizoma Coptidis* is one of the most classical traditional Chinese medicine prescriptions [7]. It is widely used for purging stomach-fire, to treat inflammation, haematemesis

induced by intense heat in the body, constipation, jaundice due to damp heat, and cholera, etc. [8].

Baicalin (BA), rhein (RH) and berberine (BE) are three representative bioactive constituents in the prescription. A method has been published earlier for the analysis of three components in traditional herbal medicine using capillary electrophoresis [4]. Many papers have studied their pharmacokinetics by HPLC with different sample pretreatment methods. The most routine approach is precipitation of plasma proteins using an organic reagent followed by centrifugation [9–13]. Furthermore, solid-phase extraction (SPE) [6,14] and microdialysis [15] are also common ways. However, these methods may not provide adequate selectivity, high recovery and may be difficult to be automated.

In the present paper, a column-switching system was applied to the simultaneous determination of plasma BA, RH and BE after oral administration of *Xiexin-Tang* decoction at a dose of 20 mL/kg in rats. Only 20 µL centrifuged plasma was demanded per assay. The primary separation was performed

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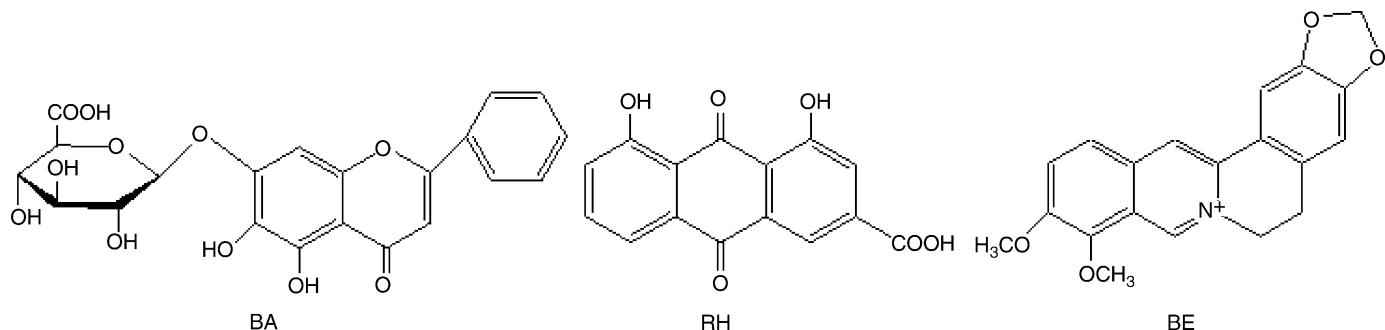


Fig. 1. Structures of baicalin (BA), rhein (RH) and berberine (BE).

on a polymer-coated restricted access material (MF C8) for deproteinization. The column-switching technique, allowing the direct and multiple injections of biological matrices without time-consuming extraction and arduous exploring of sample pre-treatment method, is an attractive means to achieve automatization of sample analysis. The recoveries of different concentration of three compounds all were stable and exceeded 95%.

Although the column-switching technique has been developed widely used for the fast analysis of a large variety of compounds in various biological matrices [16–20], the analysis objective realm is localized at Western medicines. Assaying complex TCM plasma samples in this way has not previously been reported.

2. Experimental

2.1. Chemicals and materials

Baicalin, rhein and berberine hydrochloride standard samples (Fig. 1.) were from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *Radix Scutellariae*, *Radix et Rhizoma Rhei* and *Rhizoma Coptidis* were purchased from the market and identified by Prof. Zhu Yeqing in School of Pharmacy, Fudan University (Shanghai, China). HPLC-grade acetonitrile was from TEDIA (Fairfield, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate were analytical grade from Xinhua Chemical Plant

(Shanghai, China). Phosphoric acid (A.R.) was purchased from Feida Co. Ltd. (Shanghai, China). Triethylamine (TEA, A.R.) was purchased from Lianchuang material Co. Ltd. (Suzhou, China). Pure distilled water was purchased from Shanghai Sparking dinking water Co. Ltd.

2.2. HPLC system

A column-switching system using two columns consisted of two Waters 510 pumps, a Waters 486 UV tunable absorbance detector, an on-line 0.22 μ m filter (Hanbon Sci.&Tech., Jiangsu, China), a 3994 injection valve with a 20 μ L ration loop and a 7000 switching valve (Rheodyne, USA) and a DC-1006 low-temperature bath (Hengping Instrument Co. Ltd. Shanghai, China); the configuration of which is shown in Fig. 2. The filter was purged daily. The chromatogram was recorded by HS2000 software (Empower Science and Techn. Co. Ltd., Hangzhou, China).

2.3. Chromatographic conditions

A Capcell Pak MF C₈ column (PC; 150 mm \times 4.6 mm i.d., Shiseido, Japan) was used for pre-separation of baicalin, rhein and berberine from plasma samples. The main separation was performed on a Kromasil C₁₈ column (AC; 150 mm \times 4.6 mm i.d., Eka Nobel, Sweden). The temperatures of both columns were maintained at 24 °C throughout the analytic process and

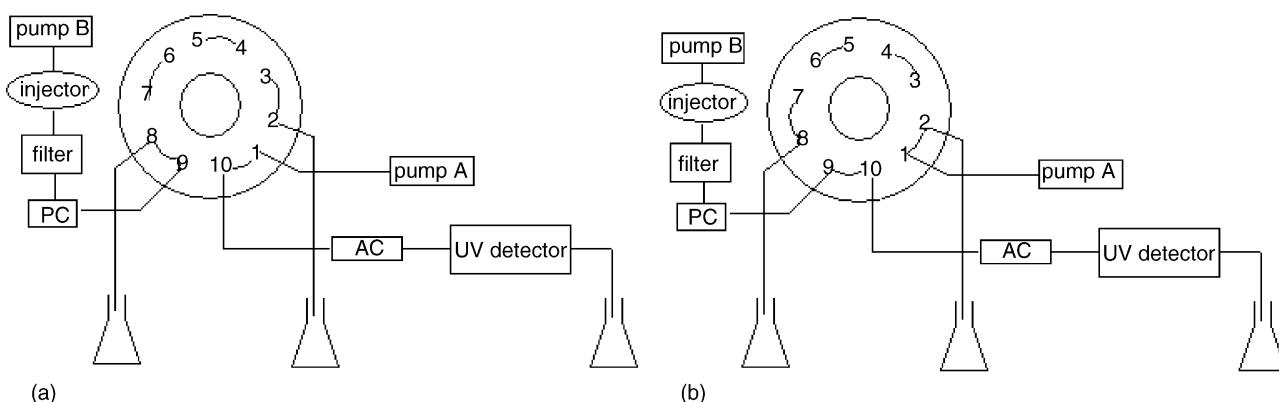


Fig. 2. Schematic diagram of the column-switching HPLC system: (a) valve position A and (b) valve position B. Surplus orifices 3–7 were sealed. PC: precolumn, AC: analytical column.

the eluate was monitored at 254 nm. The details for the analytical procedure were as follows.

Plasma samples were thawed at room temperature and centrifuged at $4800 \times g$ for 5 min. At the beginning of the analysis, the valve was placed in position A (Fig. 2). Twenty microlitres centrifuged plasma sample was injected onto the pre-column where plasma proteins and other interfering compounds were washed to waste using mobile phase B of 50 mmol/L phosphate sodium (pH 6.85)–acetonitrile (10:1, v/v) at a flow rate of 1.0 mL/min. In the meantime, the analytical column was equilibrated by mobile phase A of TEA adjusted 20 mmol/L phosphoric acid (pH 6.78)–acetonitrile (4:1, v/v). At 8 min, the valve was switched to position B. The plasma-free fractions were transferred from the pre-column to the analytical column still with mobile phase B at the same flow rate. At 26 min, the valve was switched back to position A and three target analytes were eluted by mobile phase A at a flow rate of 1.0 mL/min. The total run time per sample was about 60 min.

2.4. Standard solutions and spiked samples

Stock solutions of 79.36 $\mu\text{g}/\text{mL}$ baicalin, 77.92 $\mu\text{g}/\text{mL}$ rhein and 77.28 $\mu\text{g}/\text{mL}$ berberine were prepared by dissolved three standard samples in methanol, respectively, and then they were diluted with methanol for further concentration levels. These solutions were stored at -20°C . Spiked plasma samples were prepared by addition of the stock solutions to blank rat plasma giving final three concentration series. The concentration range of baicalin was 0.4–7.9 $\mu\text{g}/\text{mL}$, rhein 0.2–7.8 $\mu\text{g}/\text{mL}$ and berberine 0.4–7.7 $\mu\text{g}/\text{mL}$ ($n=6$).

2.5. Preparation of Xixin-Tang decoction

Three crude herbs *Radix Scutellariae*, *Radix et Rhizoma Rhei* and *Rhizoma Coptidis* (15.63, 15.63 and 31.25 g) were decocted twice in an earthen jug, each with 300 mL water, after they had been thoroughly soaked for 25 min. Strong fire was used until the water boiled, followed by mild fire for 30 min [21]. Finally the decoction was condensed to 200 mL. It had been determined that the dose of 20 mL/kg decoction was equivalent to 121.1 mg baicalin, 2.5 mg rhein and 27.6 mg berberine.

2.6. Method validation

Calibration curves for baicalin, rhein and berberine in plasma were generated by plotting the peak area versus the concentrations in the plasma. The limit of detection (LOD) was determined as the concentration of drug giving a signal-to-noise ratio about 5:1. The limit of quantification (LOQ) was obtained as $\text{S}/\text{N} \geq 10$. Three concentrations (high, medium and low) of baicalin, rhein and berberine-spiked plasma standard samples were quantified to evaluate the recovery, precision [denoted as the relative standard deviation (R.S.D.) of replicate assay] and accuracy. Two concentrations of plasma standard samples were stored at -20°C for 0, 6, 12 and 18 days. Stability was exhibited by the curve of determined concentrations to different storage time.

2.7. Sample collection

Male Sprague–Dawley rats (249 ± 9 g) were purchased from the Center of Experimental Animals, Fudan University, China, and fasted overnight (at least 14 h) prior to the experiment of oral administration of Sanhuang Xiexin-Tang decoction with a dose of 20 mL/kg. Blood samples (0.3–0.5 mL) were collected into heparinized tubes from the tail at 0, 0.25, 0.5, 1.0, 1.5, 2, 3, 5, 8, 12 and 24 h post dose. Following centrifugation (6000 rpm, 10 min), the plasma samples were harvested and stored at -20°C until analysis.

3. Results and discussion

3.1. Chromatography

The Capcell Pak MF column is a mixed-functional restricted access material (RAM). It can elute biological matrix components in the void volume and retain small molecules of constituents [16]. To minimize protein denaturation and to obtain good recovery, a neutral eluent is appropriate for this type of columns. A MF Ph-1 column (20 mm \times 4 mm i.d.) was once tried to use in the system. Although the short column was efficient to deproteinize, it was less efficient in pre-separation of so many compounds in the complex TCM sample so that a great number of peaks stacked in the final chromatogram. Therefore a long MF C₈ column (150 mm \times 4.6 mm i.d.) was chosen.

The results of conditioning experiments to determine the switching time zone are shown in Fig. 3, which was obtained by connecting the end of MFC₈ column directly to the UV detector. The retention times (RTs) of baicalin, rhein and berberine were around 8.7, 16.7 and 19.9 min, respectively. Before 8 min, not only nearly all plasma proteins but also some more hydrophilic compounds than baicalin were eluted to waste. Since berberine is a basic constituent, the tailing of its peak was dramatically severe. Injecting about 15 $\mu\text{g}/\text{mL}$ berberine plasma sample, the peak tailed to 26 min. In order to insure berberine to be completely transferred to the analytical column, the final switching

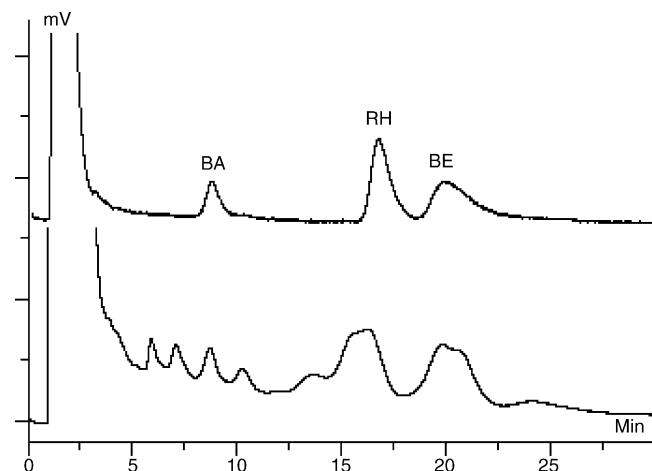


Fig. 3. MF C₈ column chromatogram of standard samples (baicalin: 7 min; rhein: 16.7 min; berberine: 19.9 min) (upper) and plasma obtained 0.25 h after a 20 mL/kg oral dose of Xixin-Tang (lower).

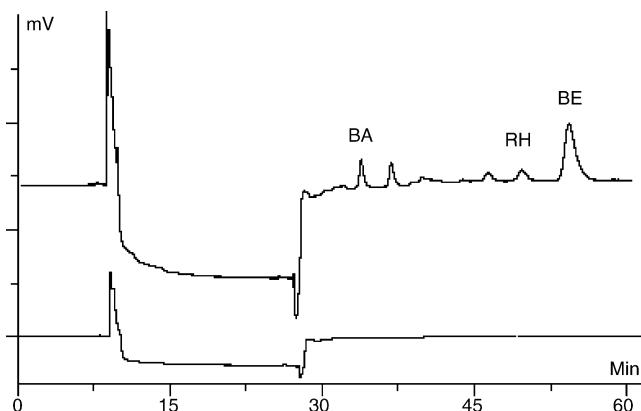


Fig. 4. Representative chromatogram of plasma obtained 2 h after a 20 mL/kg oral dose (upper) and blank plasma (lower).

time zone was set as 8–26 min. However, their RTs could be slightly changed along with the use of the pre-column and the change of the temperature. So keep the operation temperature constant and determine periodically the actual RTs before experiment were very important. The time zone could be slightly adjusted according to the RT change.

Final chromatograms obtained from the main analytical column are shown in Fig. 4. Attributed to the pre-separation of MF C₈ column, the chromatograms were quite clean. The peaks of three analytes (RT: baicalin 34.2 min, rhein 50.0 min and berberine 54.7 min) were all baseline separation. The extra appearing peaks were other Chinese medicine ingredients in the decoction which had not been identified at present because of the lack of standard reference substances and the limitation of the existing analysis method.

Table 3
Recovery of BA, RH and BE in plasma (*n*=6)

Compounds	Concentration (μg/mL)	Recovery (%)
BA	7.936	96.06
	3.174	97.77
	0.7936	95.71
RH	7.792	96.13
	3.117	96.49
	0.4052	97.60
BE	7.728	96.75
	3.091	97.19
	0.7728	95.26

3.2. Method validation

The calibration curves of peak areas versus the concentrations of baicalin, rhein and berberine in plasma were all linear. The regression equations, correlation coefficients and linearity ranges are shown in Table 1. The LOD of baicalin was lower than 39.7 ng/mL, rhein 2.2 ng/mL and berberine 193.2 ng/mL using 20 μL plasma. Their corresponding LOQs were 0.08, 0.004 and 0.4 μg/mL, respectively.

The intra- and inter-day precision and accuracy of the assay are summarized in Table 2. The R.S.D. of three analytes were in the range of 0.34–4.3% and the accuracies were between 98.0% and 102.4%. The recovery was evaluated as the ratio of standard plasma peak areas divided by those of methanol containing the corresponding concentration. Recovery greater than 95% meant that the analytes were almost completely separated from plasma proteins through the pre-column (Table 3). Xixin-Tang plasma sample was stable at -20 °C for at least 12 days (Fig. 5).

Table 1
Standard work curves of three compounds

Compound	Regression equation	Concentration range (μg/mL)	Slope R.S.D. (%)	Intercept R.S.D. (%)	Correlation coefficient
Baicalin	$Y=27,007X + 373.76$	0.3968–7.936	0.6	194.1	0.9999
Rhein	$Y=98,238X + 4,624.2$	0.1558–7.792	0.5	45.5	0.9999
Berberine	$Y=61,614X - 11,722$	0.3864–7.728 ^a	1.1	25.9	0.9997

^a Berberine was always calculated as berberine hydrochloride.

Table 2
Reproducibility and accuracy for determination of BA, RH and BE in plasma (*n*=6)

Compounds	Theoretical concentration (μg/mL)	Intra-day			Inter-day		
		Determined concentration (mean ± S.D.) (μg/mL)	R.S.D. (%)	Accuracy (%)	Determined concentration (mean ± S.D.) (μg/mL)	R.S.D. (%)	Accuracy (%)
Baicalin (BA)	7.936	8.015	0.34	101.00	7.902	3.53	99.57
	3.174	3.127	2.11	98.52	3.126	3.39	98.49
	0.7936	0.8051	3.11	101.45	0.7926	2.25	99.87
Rhein (RH)	7.792	7.742	0.62	99.36	7.612	2.51	97.70
	3.117	3.066	2.08	98.35	3.152	4.25	101.14
	0.4052	0.4021	2.56	99.24	0.4035	3.53	99.58
Berberine (BE)	7.728	7.847	2.35	101.54	7.724	2.10	99.95
	3.091	3.029	1.87	97.99	3.032	1.91	98.09
	0.7728	0.7784	1.02	100.72	0.7912	2.81	102.38

Accuracy (%) = (determined concentration)/(theoretical concentration) × 100%.

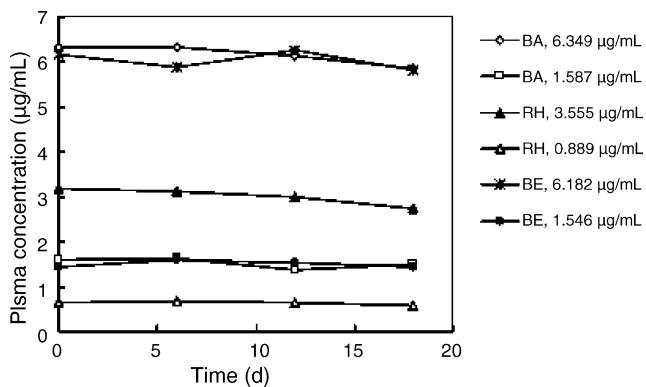
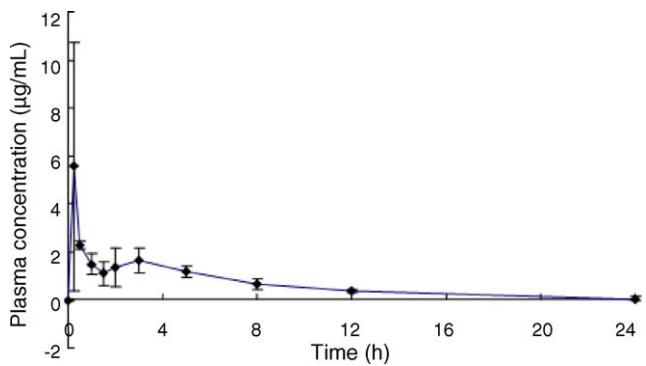
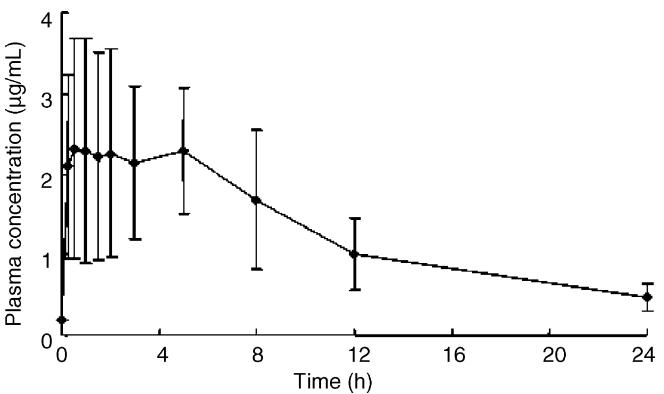


Fig. 5. Stabilities of BA, RH and BE in plasma.

Fig. 6. Mean plasma concentrations of baicalin after oral administration of Xixin-Tang decoction at a dose of 20 mL/kg in rats ($n=5$).

3.3. Plasma concentration–time curves of baicalin, rhein and berberine in rats

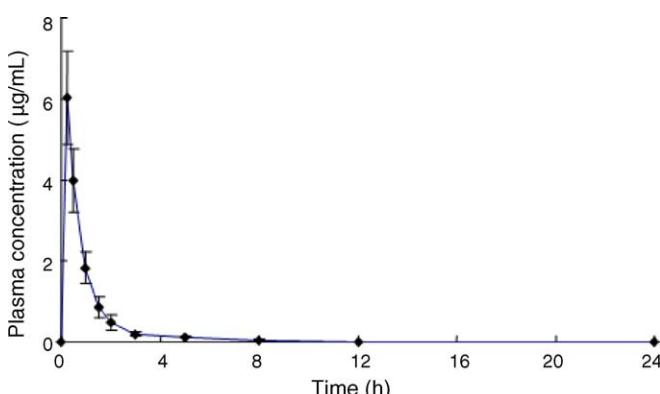
The developed method here was applied to simultaneous determination of concentrations of baicalin, rhein and berberine in plasma after oral administration of Xixin-Tang decoction at a dose of 20 mL/kg in rats. Mean plasma concentration–time curves of three analytes are shown in Figs. 6–8. There were two extrema in the baicalin plasma concentration–time curve. The C_{\max} (mean value) was 5.57 µg/mL appearing at 15 min, and the other extremum (1.63 µg/mL) appeared at 3 h. The two extremum times both was ahead of the data reported by Akao

Fig. 8. Mean plasma concentrations of berberine after oral administration of Xixin-Tang decoction at a dose of 20 mL/kg in rats ($n=5$).

et al. (2.4 and 9 h) [11]. The rhein plasma concentration monotonically decreased with time and the C_{\max} value (6.03 µg/mL) appearing at the beginning. After 12 h, the signal of rhein was lower than its detection limit. Lee et al. [12] showed C_{\max} was reached between 1 and 2 h when humans were used as experiment subjects. Berberine was eliminated very slowly in the rat body. The C_{\max} (mean value) was 2.31 µg/mL (0.5 h) and the high concentration level lasted until 5 h. The high standard deviation was due to the huge individual difference existing among the rats. In large numbers of repeated experiments, the plasma berberine concentration value of different rats fluctuated intensely along with repeated concentrations of other two components, the berberine even was under LOD in some rats. The reason is still studied. The reported T_{\max} was 0.63 h [13] or around 3 h [22] in rabbits and 2.37 h in humans [13]. The appearance of the concentration platform was obviously different with those reported previously. The cooperation effect of multitudinous compounds in prescription might be one of the significant reasons. C_{\max} of three compounds all appeared very early. This should be relative to the administrated drug which was a complex prescription besides animal species difference. It also explained why the prescription was a first-aid medicine for haematemesis in ancient China.

3.4. Method comparison with existing reports

There are several reports on assaying baicalin, rhein and berberine in blood. He et al. [9] measured baicalin in rat plasma with electrochemistry detection. The intra- and inter-day recoveries were $98.2 \pm 0.88\%$ and $95.9 \pm 0.63\%$ in a 0.5–5 µg/mL range. Li et al. [10] reported pharmacokinetic study of baicalin in rabbits. The intra- and inter-day precisions (R.S.D.) were 3.59–5.14% and 3.57–6.06% in a 15.16–58.94 µg/mL range. The recoveries were 87.26–94.77% for the same samples. Li et al. [13] reported an assay method for berberine in rabbit and human serum with intra- and inter-day precisions (R.S.D.) of 3.49–5.11% and 4.45–5.47% in the 0.02–0.64 µg/mL range. The average recovery was $99.85 \pm 3.20\%$ and the LOD was 0.02 µg/mL. The reports cited above contained sample extraction step using organic solvents. Zhang et al. [14] showed that the intra- and inter-day precisions were 1.18–1.77% and

Fig. 7. Mean plasma concentrations of rhein after oral administration of Xixin-Tang decoction at a dose of 20 mL/kg in rats ($n=5$).

1.94–5.92% for 0.10–10.0 µg/mL for baicalin. Corresponding accuracies were –3.6 to 5.0% and –1.7 to 2.0, respectively, which were calculated as relative errors. The LOQ was 0.05 µg/mL and the recoveries were 79.3–91.0%. Solid phase extraction using the Oasis® HLB cartridge was employed for sample preparation. Tsai and Tsai [15] showed a microdialysis process to obtain berberine plasma samples. The intra- and inter-assay precisions (R.S.D.) were 1.3–5.5% and 0.04–10.4% for 0.05–50.00 µg/mL. Their accuracies were 0.6–10.0% and –4.0 to 4.0%, respectively. The average recovery in blood (0.5, 1 and 5 mg/mL) was $33.56 \pm 2.5\%$. All the reported method mentioned above had to go through a time-consuming and arduous pretreatment step.

Considering the results above, the submitted method can be used as a simpler procedure with similar precision and accuracy but with higher and more stable recovery. Sample demand (20 µL) is very little. It makes possible that multiple constituents of TCM in biological fluid can be simultaneously determined by automatization.

4. Conclusion

A direct injection column-switching HPLC method was developed for the simultaneous determination of baicalin, rhein and berberine in rat plasma. The sample pretreatment was minimal and only 20 µL of plasma was need for the assay. Because of the pre-separation in the longer MF C₈ column, the main separation on the analytical column was simplified. A comparatively clean chromatogram was obtained though a complex TCM plasma sample was injected. The method showed excellent precision, accuracy, stability and high recovery. The suitability of the method was proved in the plasma concentration-time curve study of baicalin, rhein and berberine after oral administration of Xie Xin Tang decoction in rats.

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