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Development of an LC–MS method for determination of three active constituents of Shuang-huang-lian injection in rat plasma and its application to the drug interaction study of Shuang-huang-lian freeze-dried powder combined with levofloxacin injection

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ABSTRACT

A sensitive and specific high performance liquid chromatography coupled with mass spectrometric (LC–MS) method was developed and validated for the simultaneous determination of three main active constituents of Shuang-huang-lian injection with and without the combination use of levofloxacin injection in rat plasma. After addition of the internal standard rutin, plasma samples were protein precipitated with acetonitrile, the chromatographic separation was achieved on a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm), using a gradient mobile phase system of acetonitrile–water containing 0.05% formic acid. The analytes were detected without interference in the selected ion monitoring (SIM) mode with positive electrospray ionization. The linear range was 0.04–20 μg/mL for chlorogenic acid, 0.8–400 μg/mL for baicalin and 0.01–5.0 μg/mL for phillyrin, respectively. The accuracy (relative error, R.E.%) were between −2.7 and 3.4%, while the intra-day and inter-day precisions were less than 9.2 and 9.6% for the three analytes, respectively. This method was successfully applied to the drug interaction study of Shuang-huang-lian freeze-dried powder combined with levofloxacin injection after intravenous administration to rats. The results indicated that there were obvious differences in the pharmacokinetic behaviors after combination compared with only administration of Shuang-huang-lian injection.

1. Introduction

Shuang-huang-lian (SHL) injection, a modern formula prepared from three Traditional Chinese Medicines (TCM) including *Lonicera Japonica*, *Radix Scutellariae*, *Fructus Forsythiae*, mainly has anti-bacterial, anti-virus and anti-inflammation activities, which is put into clinic for curing the diseases including acute respiratory tract infection, bacterial infection, pneumonia, etc [1]. It contains various constituents, including phenylethanoid glycosides, lignans, flavonoids, etc. In clinical treatment, SHL injection co-administration with other drugs is common, but several of the combination may cause severe medicinal accidents without strict quality control and clinical compatibility experience [2]. Since it usually combines SHL injection with levofloxacin injection in clinic [3], however, both of the drugs had ever been reported caused severe adverse reactions [4–6], in consideration of pharmacokinetic drug–drug interaction, the safety of co-administrated SHL injection and levofloxacin injection is of great concern. Pharmacokinetic drug–drug interaction (DDI) is an unfavorable clinical event which is caused by abnormal increase or decrease of drug concentrations in the body as a consequence of co-administration of other drugs [7–9]. It reflects a major problem in drug development, even after approval, some drugs have to be withdrawn from the market due to DDI [10–12]. It is indispensable to collect sufficient information about the formula of injection and its compatible medicine, which makes the study of drug interaction more significant.

The qualitative and quantitative analyses of the ingredients in SHL for quality control have been studied using HPLC and LC–MS/MS methods [13–15]. Most of these methods were developed for assaying major index components such as chlorogenic acid, baicalin and forsythin. Besides, several literatures reported the *in vivo* pharmacokinetic study of chlorogenic acid, baicalin, and/or forsythin [16–19], although most of which made efforts to develop an effective method for determination of the main components and for pharmacokinetic study of SHL in rats [20,21], the methods applied to the combination of SHL injection with other drugs *in vivo* are still few.

Considering the research of the associated administration of SHL injection and levofloxacin injection has never been reported, this study is mainly focused on the interaction of two injections in pharmacokinetics to estimate the safety of the combination, which is trying to evaluate the changes in pharmacokinetic profiles of the

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three main components chlorogenic acid (CA), baicalin (BC), and phillyrin (PR) in SHL [22] injection with and without the combination use of levofloxacin injection.

2. Experimental

2.1. Materials, reagents and animals

SHL freeze-dried powders of injectable grade were obtained from the Second Chinese Medicine Factory, Harbin Pharm Group Co. Ltd. (Heilongjiang, China). Levofloxacin Hydrochloride and Sodium Chloride Injection were purchased from Guang Dong P.D. Pharmaceutical Co. Ltd. (Guangdong, China). The powders contained 8, 200 and 18 mg of chlorogenic acid, baicalin and phillyrin per gram, respectively, as determined by an HPLC assay. Chlorogenic acid (Fig. 1A), baicalin (Fig. 1B), phillyrin (Fig. 1C) and rutin (IS, Fig. 1D) were all purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China. Methanol and acetonitrile of HPLC-grade were both provided by Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (HPLC grade) was purchased from Concord Tech. Co. (Tianjin, China). Distilled water prepared with demineralized water was used throughout the study. All the other reagents were of analytical grade.

The SPF grade SD rats (male, 250 ± 5 g) were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The experimental rats were housed under the controlled conditions (22 ± 2 °C, relative humidity 50 ± 20%) with the natural light–dark cycle for 7 day before the experiment carried out. Before drug administration, they were fasted overnight and had free access to water. Animal study was carried out in accordance with the Guideline for Animal Experimentation of Shenyang Pharmaceutical University, and the protocol was approved by the Animal Ethics Committee of the institution.

2.2. Instruments and LC–MS conditions

The assay was performed on a Shimadzu (Japan) LC–MS 2010EV system equipped with an electrospray ionization (ESI) interface. The liquid chromatographic separation was achieved on a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm), which was preceded by a C_{18} guard column (4.0 mm × 2.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase was consisted of acetonitrile – 0.05% formic acid water at a flow rate of 0.8 mL/min (post-column split ratio 3:1). The initial mobile-phase composition was 22% mobile phase A (acetonitrile) – 78% mobile phase B (0.05% formic acid water). After sample injection, the initial mobile-phase composition was kept for 5.0 min, then changed linearly to 33% A in 1.0 min, and held constant for an additional 8.0 min, last, returned linearly to 22% A in 1.0 min for re-equilibration. The injection volume was 10 μL and the total analysis time was 15.0 min for each run. The column and autosampler tray temperature were maintained constant at 30 °C and 4 °C, respectively. The analytes and IS were all ionized by ESI source in positive ion mode under the following source conditions: nebulizing gas 1.5 L/min, CDL temperature 250 °C, heat block temperature 200 °C, detector voltage 1.75 kV, and the other parameters were fixed as the tuning file. Analysis was carried out in selected ion monitoring (SIM) for CA $[M+Na]^+$ m/z 377.10, BC $[M+H]^+$ m/z 447.05, PR $[M+Na]^+$ m/z 557.20, and IS $[M+Na]^+$ m/z 633.00, respectively. The data acquisition was performed by LC–MS Solution Version 3.0.

2.3. Standard solution and quality control samples

Stock solutions of CA, BC, and PR were prepared in methanol–water (50:50, *v/v*) at concentration of 2.0 mg/mL, 8.0 mg/mL and 0.5 mg/mL, respectively. IS stock solution was prepared in methanol at concentration of 0.5 mg/mL. A series of mixture standard working solutions with concentration 0.04–20 μg/mL for CA, 0.8–400 μg/mL for BC and 0.01–5.0 μg/mL for PR were obtained by diluting the mixture of the stock solution with methanol–water (50:50, *v/v*). The stock solution of IS was diluted to concentration of 10 μg/mL with methanol as working solution. All the solutions were stored at 4 °C.

Calibration standards of CA (0.04, 0.08, 0.4, 2.0, 4.0, 8.0, and 20 μg/mL), BC (0.8, 1.6, 8.0, 40, 80, 160, and 400 μg/mL) and PR (0.01, 0.02, 0.1, 0.5, 1.0, 2.0, and 5.0 μg/mL) were prepared by adding the residue obtained from evaporating 100 μL of the working solutions to 100 μL blank plasma. Three levels of quality control (QC) samples (0.08, 2.0, and 16 μg/mL for CA; 1.6, 40 and 320 μg/mL for BC; 0.02, 0.5, and 4.0 μg/mL for PR) in plasma were prepared separately in the same fashion.

2.4. Sample preparation

The 100 μL plasma sample spiked with 20 μL internal standard (10 μg/mL) and 50 μL hydrochloride (0.1 mol/L) was vortexed for 3 min after adding 400 μL acetonitrile as the precipitant. After the mixture being centrifuged at 4000 rpm for 5 min, the supernatant was transferred to another vial and evaporated to dryness at 35 °C under a slight stream of nitrogen. Then the residue was reconstituted with 100 μL of acetonitrile–water solution (22:78, *v/v*), 10 μL of which was used for LC–MS analysis.

2.5. Method validation

The method was fully validated for selectivity, matrix effect, linearity, accuracy, precision, extraction recovery and stability.

Blank plasma samples from six rats were screened for selectivity. The matrix effect was evaluated by comparing the peak response of analytes (A) spiked in post-extracted blank plasma solutions with that of pure standard solution containing equivalent amounts of the compounds (B). The Ratio (A/B × 100)% was used to evaluate the matrix effect.

The linearity of the assay was assessed by analyzing the calibration curves (0.04–20 μg/mL for CA, 0.8–400 μg/mL for BC, and 0.01–5.0 μg/mL for PR) in plasma using least-squares linear regression of the peak area ratios of the analytes to the IS versus the nominal concentration of the calibration standard with a weighed factor ($1/C^2$). The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve with an acceptable accuracy within ±20% and the precision below 20%. The value of limit of detection (LOD) was calculated as the analyte concentration gave rise to peak whose height was 3 times the baseline noise.

QC samples at low, medium and high concentration were analyzed on three separate occasions with six replicates at each concentration per occasion to determine the accuracy and precision. Precision was defined as the relative standard deviation (R.S.D.%) while accuracy was defined as relative error (R.E.%). The recoveries of CA, BC and PR were determined at three QC levels with six replicates by comparing the peak areas from extracted samples with those in post-extracted blank plasma samples spiked with the analytes at the same concentration. The recovery of IS was determined in the same way at the concentration of 10 μg/mL.

Stability studies in plasma samples were also conducted at three QC levels in several different storage conditions: at room temperature for 12 h, at −80 °C for at least 7 day, after three freeze–thaw cycles, and 12 h after prepared at 4 °C.

2.6. Application of the method in pharmacokinetic study

The method was used to determine CA, BC, and PR in rat plasma after administering via tail vein of SHL injection (378 mg

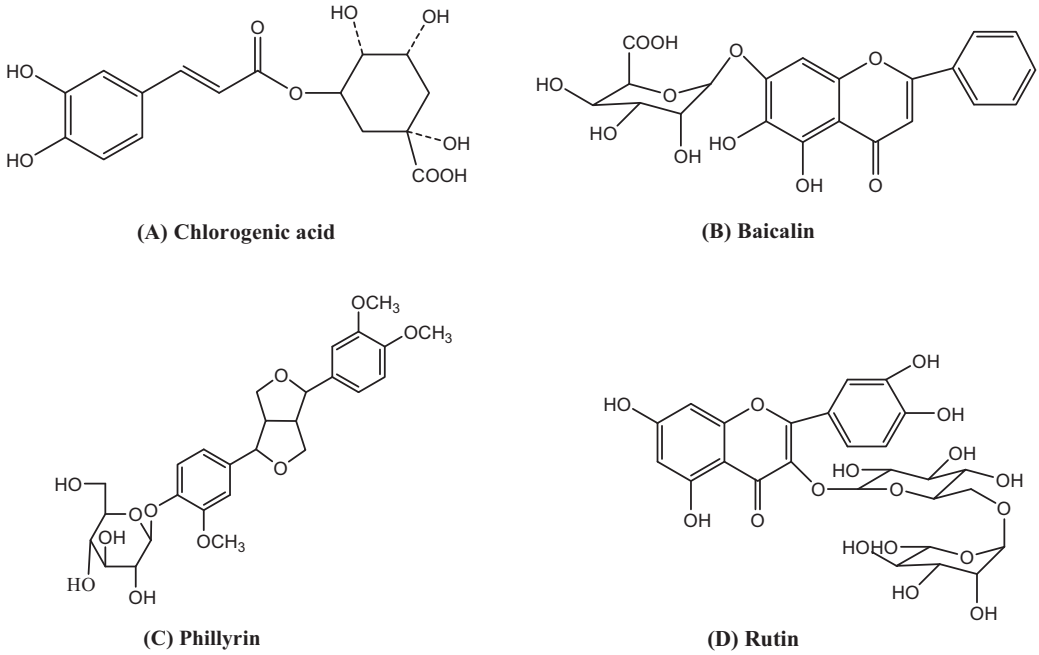

Fig. 1. Chemical structures of Chlorogenic acid (A), Baicalin (B), Phillyrin (C), and Rutin (D).

freeze-dried powder per kg weight), and the same dose of SHL injection which combined with levofloxacin hydrochloride and sodium chloride Injection (16 mg levofloxacin per kg weight), respectively. Animals were randomly divided into two groups, with six rats in each. Blood samples were collected from the suborbital vein before administration and at 5, 10, 15, 20, 30, 45, 60, 90, 120, 240, and 360 min after i.v. injection into heparinized polypropylene tubes, and then immediately centrifuged at 10,000 rpm for 5 min. Harvested plasma samples were stored at −80 °C until analysis.

The pharmacokinetic parameters of CA, BC, and PR were calculated by the non-compartmental analysis of plasma concentration vs. time data using the DAS 2.1 software package (Chinese Pharmacological Society). The comparison of pharmacokinetic parameters between administration of the single and the combination was possessed by SPSS 16.0 (Statistical Package for the Social Science).

3. Results and discussion

3.1. LC–MS optimization

Under the electrospray ionization condition chosen, CA, BC, PR and IS all exhibited higher sensitivity in the positive mode than in the negative mode, and the most abundant ions is $[M+H]^+$ for BC (*m/z* 447.05), while for CA, PR and IS, the circumstance is more complicated. There are $[M+H]^+$ ions, $[M+Na]^+$ ions and $[M+K]^+$ ions, and the $[M+Na]^+$ ions for CA (*m/z* 377.10), PR (*m/z* 557.20) and IS (*m/z* 633.00) was the most sensitive. In addition, the response of $[M+Na]^+$ was more stable and showed better linearity than $[M+H]^+$ for CA, PR and IS in SIM mode. So the quantitative analysis was carried out in SIM mode at $[M+H]^+$ *m/z* 447.05 for BC, $[M+Na]^+$ *m/z* 337.10, 557.20 and 633.00 for CA, PR and IS, respectively.

The mobile phase played a critical role in achieving good chromatographic behavior (including peak symmetry and short analysis time) and appropriate ionization. With addition of formic acid to the mobile phase, the peak symmetry of CA, BC and PR was both improved greatly, so the concentration of formic acid in mobile phase was optimized from 0.005% to 0.2%, and referring to the results, acetonitrile–water containing 0.05% formic acid was adopted with a gradient mobile phase system for sufficient ionization response, good peak symmetry and proper retention time for the analytes and IS.

3.2. Sample preparation

Due to the strong polarity and hydrophilicity of this three compounds of glycoside, the liquid–liquid extraction performed unsatisfactorily with low recoveries less than 40% and poor sensitivity, testing several extractants such as ethyl acetate, diethyl ether, mix solvents consist of isopropanol–ethyl acetate with different ratios, etc. Comparatively, protein precipitation is a simple and fast method and easy to control the operating conditions to get a satisfactory repeatability and recovery. This research investigated several precipitants like methanol, acetonitrile, acetone and trifluoroacetic acid, etc. It is found that the precipitant composed of acetonitrile got the maximum recovery with less interference of the endogenous substances. Finally acetonitrile was chosen for the precipitant and the matrix effect satisfied the criteria.

3.3. Method validation

3.3.1. Selectivity

No endogenous interference was observed at retention time of CA (5.9 min), BC (12.2 min), PR (12.9 min) and IS (9.1 min) because of the high selectivity of SIM mode. Typical chromatograms of blank plasma, blank plasma spiked with CA, BC, PR and IS, plasma sample after i.v. administration of SHL injection combined with levofloxacin injection are shown in Fig. 2.

3.3.2. Linearity and LLOQ

The calibration curves were linear over the concentration range of 0.04–20 μg/mL for CA, 0.8–400 μg/mL for BC and 0.01–5.0 μg/mL for PR, with the correlation coefficient above 0.99 for all these three constituents. The representative linear regression equations for CA, BC and PR were $Y = 4.242 \times 10^{-1}\ X - 2.850 \times 10^{-2}$, $Y = 8.04 \times 10^{-1}\ X - 2.05 \times 10^{-2}$, $Y = 2.759\ X - 1.440 \times 10^{-2}$, respectively. The LLOQ

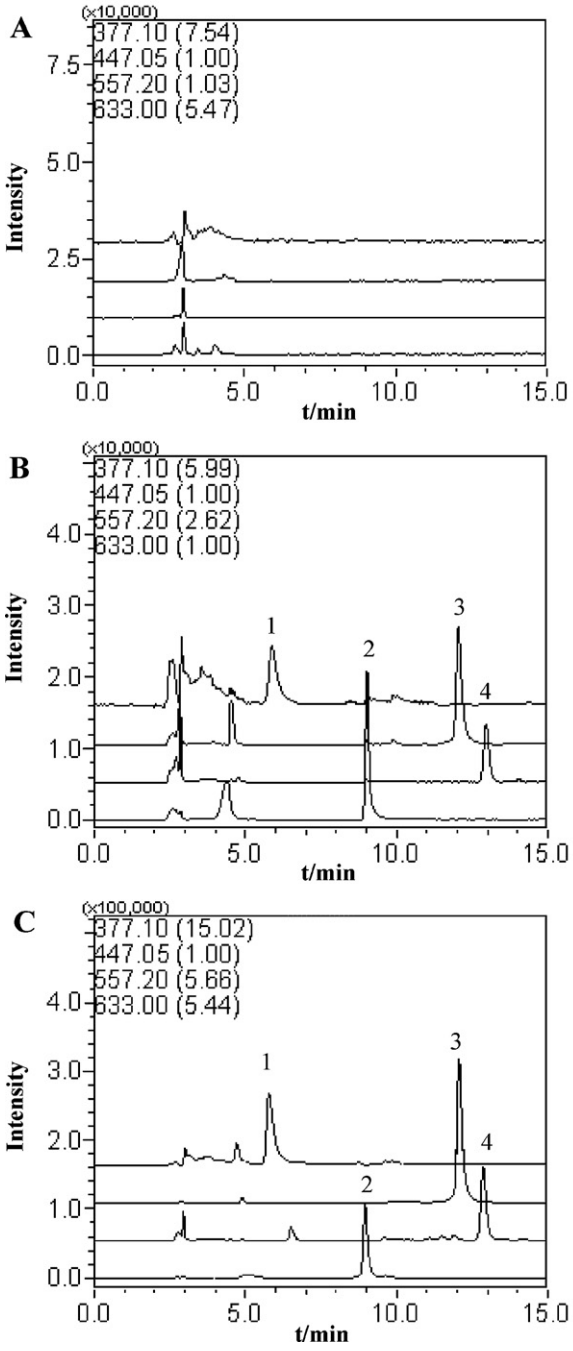

Fig. 2. Representative chromatograms obtained from LC–MS analysis of CA, BC, PR and IS in rat plasma: (A) blank rat plasma; (B) blank plasma spiked with 0.04, 0.8, 0.01 and 10 μg/mL for CA, BC, PR and IS, respectively; and (C) rat plasma sample collected at 30 min after intravenous injection of SHL at a dose of 378 mg/kg. Peak: (1) CA at m/z 377.10, (2) IS at m/z 447.05, (3) BC at m/z 557.20, and (4) PR at m/z 633.00.

were 0.04, 0.8 and 0.01 μg/mL for CA, BC and PR, whilst the LOD with an S/N ratio of >3 were 4.0, 10 and 1.0 ng/mL for CA, BC and PR, respectively.

3.3.3. Precision and accuracy

The intra-day precision, inter-day precision and accuracy of CA, BC and PR are summarized in Table 1. All the results of the tested samples were within the acceptable criteria of ±15%.

Table 1
Precision and accuracy of CA, BC and PR in rat plasma ($n = 6$).

	Nominal concentration (μg/mL)	Precision (RSD%)		Accuracy (RE%)
		Intra-day	Inter-day	
CA	0.08	6.8	7.2	−0.5
	2.0	7.1	7.5	0.3
	16	7.0	7.1	−2.7
BC	1.6	6.0	6.4	−1.5
	40	6.9	7.1	0.5
	320	3.4	3.2	−2.0
PR	0.02	9.2	9.6	−0.1
	0.5	7.2	7.1	3.4
	4.0	5.9	6.2	−0.9

3.3.4. Recovery and matrix effect

The results represented in Table 2 show that extraction recoveries of CA, BC, PR and IS ranged from 71.3 to 85.6%. And the matrix effects of these four analytes were between 86.9 and 94.6%, indicating that no significant ion enhancement/suppression effect of biological matrix was observed for the analytes. There was no relevant difference in extraction recovery or matrix effect at different concentration levels.

3.3.5. Stability

The stability study showed that CA, BC, PR and IS in plasma were stable within three freeze–thaw cycles, and showed no significant degradation of analytes for 12 h at ambient temperature and for 7 day at −80 °C. The analytes in post-prepared solution were also stable at ambient temperature for 12 h. It was reported that the chlorogenic acid was unstable under the circumstance of light [23]. So, all the procedures were carried out without the exposure to the strong light (Table 3).

3.4. Pharmacokinetic study

The developed method has been successfully used for the pharmacokinetic study of CA, BC and PR in rat plasma after intravenous administration of SHL injection. The mean concentration–time curve of these three constituents with and without co-administration of levofloxacin injection are presented in Fig. 3 and the corresponding pharmacokinetic parameters are shown in Table 4. There was no significant difference for PR between groups which indicated co-administration of levofloxacin injection had no substantial effect on the plasma concentration of PR, whereas the MRT of BC, half-life of CA and BC were changed significantly as the result of co-administration of levofloxacin injection ($p < 0.01$). We can see the concentration of CA and BC in rat plasma of combinational samples are obvious higher than that

Table 2
Recovery and matrix effect of CA, BC, PR and IS ($n = 6$).

	Concentration (μg/mL)	Recovery (%)		Matrix Effect (%)	
		Mean ± SD	RSD	Mean ± SD	RSD
CA	0.08	73.5 ± 3.2	4.4	86.9 ± 5.5	6.3
	2.0	75.8 ± 3.3	4.4	88.2 ± 2.9	3.4
	16	71.3 ± 1.8	2.5	88.6 ± 2.3	2.6
BC	1.6	80.6 ± 4.7	5.9	87.0 ± 1.6	1.8
	40	83.6 ± 1.0	1.2	89.5 ± 3.9	4.4
	320	80.9 ± 6.1	7.5	94.6 ± 2.8	3.1
PR	0.02	81.4 ± 7.5	5.8	88.5 ± 3.9	4.1
	0.5	81.8 ± 5.5	9.3	90.8 ± 2.4	2.7
	4.0	85.6 ± 2.7	6.8	92.5 ± 5.9	6.5
IS	5.0	78.5 ± 2.6	3.1	92.7 ± 2.1	2.3

Table 3
Stability of CA, BC, and PR in rat plasma ($n = 3$).

	Concentration spiked (μg/mL)	Post-prepared solution RSD (%)	Three freeze–thaw cycles, RSD (%)	12 h at ambient temperature RSD (%)	7 day at −80 °C RSD (%)
CA	0.08	2.2	5.8	2.1	7.0
	2.0	7.4	10.7	5.5	10.5
	16	6.8	3.2	1.6	4.5
BC	1.6	3.0	8.2	4.2	10.1
	40	1.9	1.1	3.2	1.2
	320	1.1	1.1	0.8	1.0
PR	0.02	5.6	6.4	4.7	3.2
	0.5	3.5	1.1	3.3	2.7
	4.0	2.4	5.5	2.7	3.7

Table 4
Pharmacokinetic parameters of CA, BC and PR in rat plasma after intravenous administration of SHL injection with and without co-administration of levofloxacin injection ($n = 6$).

Parameters	CA		BC		PR	
	Single (mean ± SD)	Combination (mean ± SD)	Single (mean ± SD)	Combination (mean ± SD)	Single (mean ± SD)	Combination (mean ± SD)
CL_z (mL/min kg)	8.7 ± 3.3	8.9 ± 2.5	13.0 ± 6.0	10.0 ± 3.7	105.2 ± 40.9	108.0 ± 26.9
$t1/2_z$ (min)	18.2 ± 1.1	20.4 ± 2.4*	14.4 ± 1.5	16.1 ± 2.6**	22.7 ± 3.7	22.7 ± 3.8
V_z (L/kg)	0.230 ± 0.079	0.253 ± 0.069	0.259 ± 0.105	0.298 ± 0.098	3.43 ± 1.49	3.56 ± 1.18
AUC_{0-t} (mg/L min)	384.3 ± 147.3	366.2 ± 120.5	7191 ± 3647	6665 ± 3256	73.1 ± 31.3	65.0 ± 15.7
$AUC_{0-\infty}$ (mg/L min)	388.6 ± 149.5	372.3 ± 123.4	7219 ± 3678	6712 ± 3303	74.7 ± 32.4	66.5 ± 16.2
MRT_{0-t} (min)	24.5 ± 3.6	26.7 ± 2.8	18.3 ± 3.8	20.3 ± 4.4*	22.9 ± 4.1	23.7 ± 3.7
$MRT_{0-\infty}$ (min)	25.8 ± 4.0	27.9 ± 4.6	18.6 ± 4.1	21.1 ± 5.0*	25.5 ± 5.5	26.6 ± 5.1

* $p < 0.05$.
** $p < 0.01$.

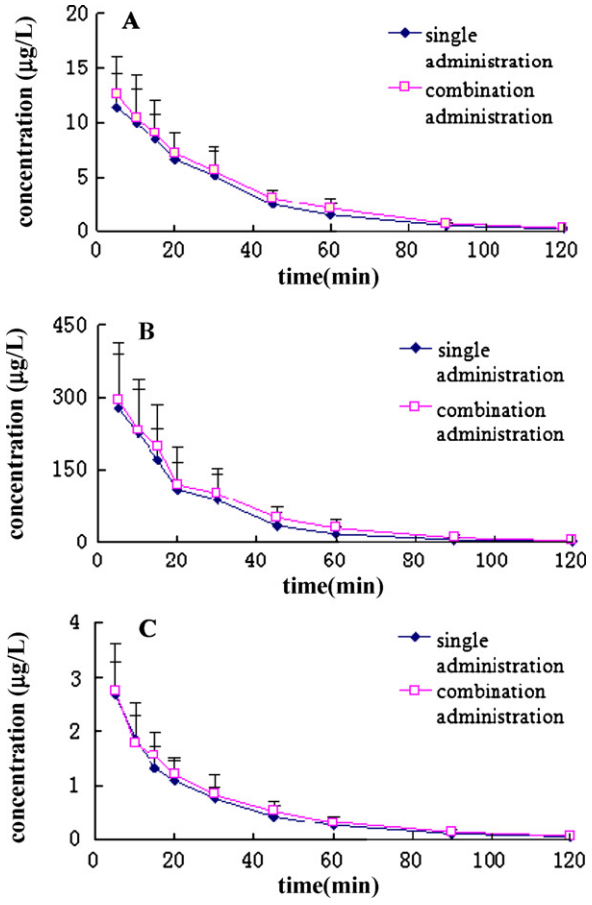

Fig. 3. Mean concentration–time curves of CA (A), BC (B), and PR (C) in rat plasma after intravenous administration of SHL injection with and without co-administration of levofloxacin injection ($n = 6$).

of single administration samples in Fig. 3, indicates that there were differences of CA and BC in metabolism between intravenous administration of SHL and co-administration of levofloxacin injection.

CA and BC exist in many TCM injections, and have extensive pharmacological effects, but both of them may be the main components in SHL injection which can cause anaphylactic reaction. Small molecular compounds of CA and BC are hapten, which may be combined with the plasma protein to become antigen when directly access the blood circulation, thus leads to the adverse reaction [24,25]. Levofloxacin, an optically active isomer of ofloxacin, is a fluoroquinolone with a broad spectrum of antibacterial activity [26]. Fluoroquinolones exert their bactericidal effect by inhibiting the DNA gyrase, which introduces negative superhelical twists into bacterial DNA, and thus is essential for replication and transcription [27]. The variation observed in the result maybe due to competitive combination with plasma protein.

As is reported, CA and BC are the allergens which may cause the occurrence of allergic reaction after administration [28]. Consequently, the prolonged exposure time of CA and BC, the rise of MRT of BC may increase the chance for adverse reaction. In the present study, we confirmed the potential of danger for co-administration of SHL with levofloxacin injection.

4. Conclusion

A specific, simple, and efficient LC–MS method has been developed and validated for the simultaneous determination of CA, BC and PR in rat plasma. The method has been successfully applied to the pharmacokinetics study of SHL injection powder. The pharmacokinetic results are useful for evaluating the clinical efficacy and safety of the combinatorial administration of SHL and levofloxacin injection. Such findings suggested that the special caution should be taken when SHL injection have been used with the combination of levofloxacin injection in therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.04.036.

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