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Rapid resolution liquid chromatography (RRLC) analysis and studies on the stability of Shuang-Huang-Lian preparations

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ABSTRACT

Shuang-Huang-Lian (SHL) is a traditional Chinese formula which comprises of three medicinal herbs: *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae*, and is commonly used to treat acute upper respiratory tract infection, acute bronchitis and light pneumonia. A simple, reliable and reproducible rapid resolution liquid chromatography (RRLC) method was developed for the quality control of SHL preparations, which baseline separates the major bioactive compounds within 6 min. The method uses a C18-HST column (2.5 μm, 100 mm × 3.0 mm) kept at 40 °C. The mobile phases consist of 0.1% phosphoric acid aqueous solution and acetonitrile. Flow rate is 1.0 ml/min and UV detection is performed at 327 nm from 0 to 4 min and 229 nm from 4 to 7 min. This method was further validated according to the ICH guidelines. Eight batches of commercial SHL preparations obtained from different pharmaceutical manufacturers as well as individual herbs were examined and their chromatographic profiles were compared. The stability test revealed that chlorogenic acid is stable only at acidic pH, and hence it is necessary to further evaluate and optimize the preparatory procedures and storage conditions for commercial SHL preparations.

1. Introduction

Shuang-Huang-Lian (SHL) is a traditional Chinese formula which comprises of three medicinal herbs: *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae*. SHL is often used to treat upper respiratory illness caused by virus or bacterial infection, such as tonsillitis, pharyngitis, pneumonia, acute enteritis, viral dysentery, etc. [1]. In April 2009, 1 month after the outbreak of influenza A H1N1 in China, Influenza A H1N1 Clinical Diagnostic and Treatment Program, published in 2009 by the Ministry of Health of the People's Republic of China shortlisted SHL oral solution as one of the recommended treatment against H1N1. Consequently, SHL preparations had received widespread attention and market demand increased dramatically in China.

According to Pharmacopoeia of People's Republic of China (PPRC), chlorogenic acid, baicalin, and forsythin are the marker compounds representing *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae*, respectively [2]. Chlorogenic acid, a monoester of caffeic acid with quinic acid, has received considerable attention for its wide distribution and potential biological effects [3]. A large number of studies revealed that chlorogenic acid has potential anti-inflammatory, analgesic, antipyretic [4], antimutagenic [5,6], and anticarcinogenic activities [7,8].

In 1985, Clifford [9] named chlorogenic acid, kryptochlorogenic acid and neochlorogenic acid as 5-O-, 4-O-, and 3-O-caffeoylquinic acid, respectively. These names were adopted by most of the researchers worldwide; however, according to International Union of Pure and Applied Chemistry (IUPAC), chlorogenic acid (CAS: 327-97-9), kryptochlorogenic acid (CAS: 905-99-7), neochlorogenic acid (CAS: 906-33-2) should be named as 3-O-, 4-O-, and 5-O-caffeoylquinic acid, respectively. These 3 isomers may have different pharmacological effects, and thus it is necessary to clarify the ambiguity in the nomenclature and measure each component separately. Chlorogenic acid was found to be not only the most abundant phenolic acid in various plant extracts but also the most active antioxidant constituent [10,11]. Although Huang & Yen [12] suggested that the antioxidant activities of 5-O-caffeoylquinic acid and 4-O-caffeoylquinic acid are almost the same as chlorogenic acid, no research has been conducted on the comparison of their anti-bacterial and anti-viral activities.

Abbreviations: SHL, Shuang-Huang-Lian; RRLC, Rapid resolution liquid chromatography; PPRC, Pharmacopoeia of People's Republic of China; ICH, The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; TCM, Traditional Chinese medicines; IUPAC, International Union of Pure and Applied Chemistry; RH, Relative humidity; LC, Liquid chromatography; MW, Multi-wavelength; LOD, Limit of detection; LOQ, Limit of quantification; DAD, Diode Array Detector; p*Ka*, Acid dissociation constant.

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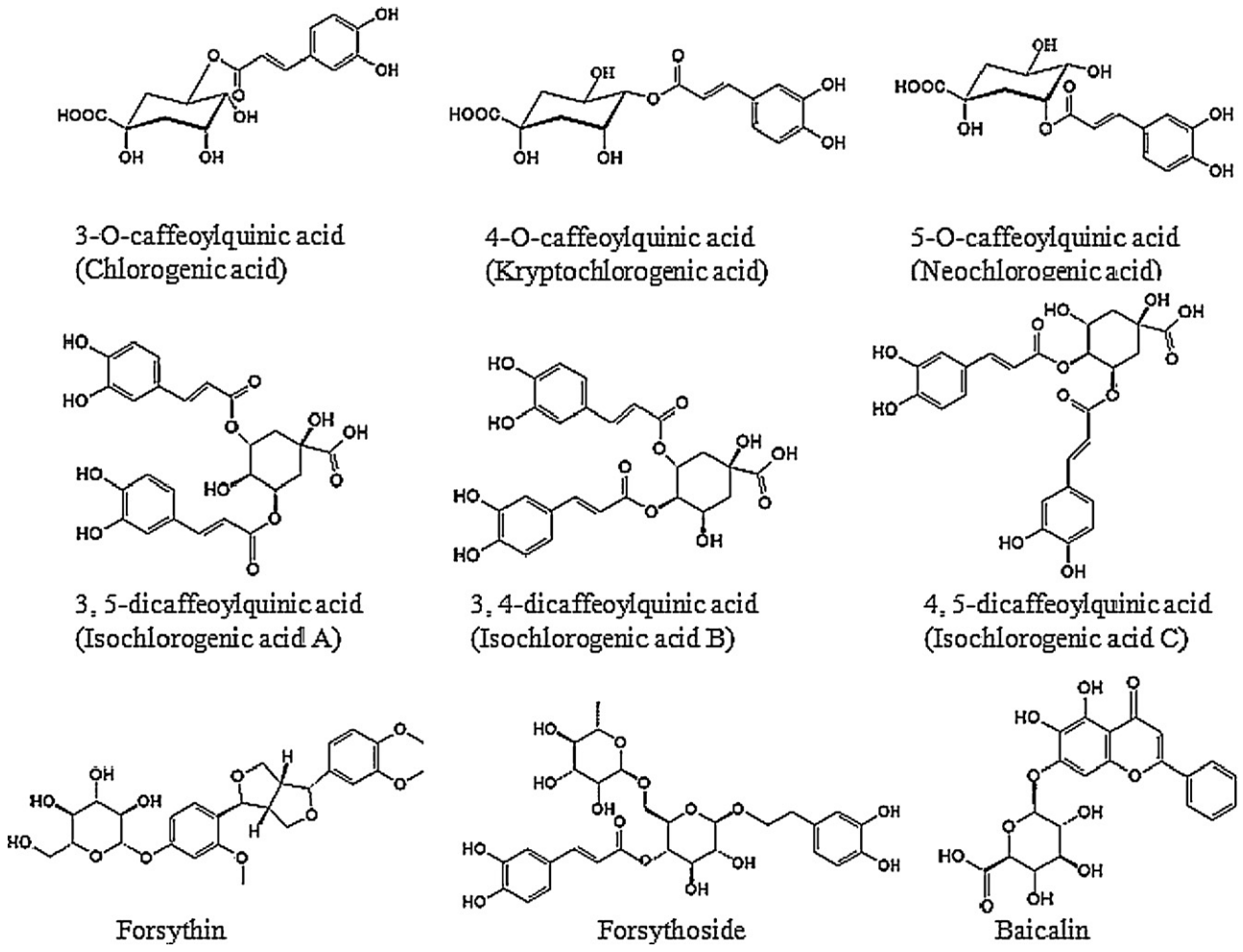

Fig. 1. Chemical structures of bioactive compounds: 3-O-caffeoylquinic acid (chlorogenic acid), 4-O-caffeoylquinic acid (kryptochlorogenic acid), 5-O-caffeoylquinic acid (neochlorogenic acid), isochlorogenic acid A, B, C, forsythin, forsythoside, and baicalin in SHL preparations.

Literature survey revealed that a variety of methods have been reported to analyze the active constituents in SHL preparations including capillary zone electrophoresis [13], liquid chromatography (LC) coupled with UV [1] or MS [14]. However, these methods either require longer analysis time or consume relatively large amounts of organic solvents used as mobile phase. RRLC method has become one of the most frequently applied approaches especially in the field of pharmaceutical analysis. It holds excellent peak shapes, enhanced reproducibility, high sensitivity, high-speed detection with reduced analysis cost, and is valuable for the quality control of herbal medicines [15]. To this purpose we have developed and validated an RRLC method, that allows simultaneous identification of more than 10 bioactive compounds and qualitatively determine the concentration of chlorogenic acid, forsythin, and baicalin in SHL preparations within 6 min. A stability study was also conducted to examine the effect of pH on the chemical composition profile of SHL preparations.

2. Materials and methods

2.1. Chemicals and materials

HPLC grade of acetonitrile (CH_3CN) and methanol (MeOH) were purchased from Fisher Scientific (Ottawa, Canada). Water was purified through a Nanopure Ultrapure Water System (Barnstead, USA) and was used for all the analyses. HPLC grade of phosphoric acid 85% (H_3PO_4) was purchased from EM Science (Darmstadt, Germany). External reference standard of chlorogenic acid (purity: 95.2%), baicalin (purity: 95.2%), and forsythin (purity: 98.9%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Forsythoside, 5-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, isochlorogenic acid A, B, and C reference standards were provided by Dr. Yu-Xin Zhou (Beijing, China). *Flos Lonicerae*, *Fructus Forsythiae* and *Radix Scutellariae* raw herbs were collected from Shandong, Hunan and Heilongjiang provinces (China). Eight batches of commercial SHL preparations were obtained from different pharmaceutical manufacturers in China (Fig. 1).

2.2. Sample preparation

Standard stock solution of chlorogenic acid, forsythin, and baicalin were prepared by dissolving a proper amount of compounds in 50% MeOH aqueous solution to a final concentration of 1 mg/ml and stored at 4 °C. Further calibration levels were prepared by diluting the above reference standard solution to 0.2, 0.4, 0.6 and 0.8 mg/ml.

Dried raw herbs of *Flos Lonicerae*, *Radix Scutellariae*, and *Fructus Forsythiae* were kept in the desiccator. About 5 g of herbal samples were ground into fine powder and 0.10 g were accurately weighed, then mixed with 10 ml 75% MeOH aqueous solution and sonicated at 37 °C for 15 min. After sonication, the volume was adjusted to 10 ml by 75% MeOH aqueous solution. Prior to injection, all liquid samples were filtered through a 0.20 μm syringe filter purchased

Table 1
Calibration curves, linear range, LOD, LOQ, and recovery for 3 reference compounds.

Compounds	Chlorogenic acid	Baicalin	Forsythin
Regression equation	$y = 913.92x + 1.63$	$y = 362.90x + 0.63$	$y = 597.91x + 2.98$
r	0.9999	0.9998	0.9999
LOD (ng/ml) ($n = 3$)	4.38	10.09	9.90
RSD %	0.15	0.19	0.26
LOQ (ng/ml) ($n = 3$)	12.95	29.61	28.72
RSD %	0.18	0.27	0.32
Linear range (μg/ml)	50–500	50–500	50–500
Recovery ($n = 3$) %	100.4	98.6	99.5
RSD %	1.15	1.42	1.36

The y value is the peak response (peak height) under detection wavelength of analytes; the x value is the concentration of the analytes (μg/μl).

from VWR International (Mississauga, Canada). For commercial SHL preparations, a 1.0 ml liquid sample was diluted with purified water to 10 ml in a volumetric flask, and then filtered through a 0.20 μm syringe filter before RRLC analysis.

The preparation of SHL oral solution was recorded in Pharmacopoeia of People's Republic of China [2] as follows: 375 g of *Radix Scutellariae*, 375 g of *Flos Lonicerae*, and 750 g of *Fructus Forsythiae* were decocted, concentrated, extracted with ethanol, adjusted pH to 7 with HCl and NaOH, distilled to eliminate solvent and the residue were dissolved and diluted with water to 1000 ml. "LF" (*Flos Lonicerae* plus *Fructus Forsythiae*) liquid extract sample was prepared in laboratory according to the above method except no *Radix Scutellariae* raw herb was added and no pH adjustment was made. "LF" sample was kept at its original pH 3.5, which was verified by pH meter. SHL liquid extract sample at pH 7 was prepared exactly following the procedures stated in PPRC.

All samples for stability testing were prepared in triplicates and stored in Testequity 1000H series temp./humidity chamber (Moorpark, USA) under 40 °C, 75% relative humidity (RH). RRLC analyses were performed at time intervals of 0, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 40, 48, 56, 64, 72 days after preparation.

2.3. Apparatus and chromatographic conditions

RRLC analyses were performed on an Agilent 1200 SL HPLC equipped with a binary pump and a micro-vacuum degasser, a multi-wavelength (MW) detector, an auto-sampler, a thermostatted column compartment, and a Luna C18-HST column (2.5 μm, 100 mm × 3.0 mm) from Phenomenex (Torrance, CA, US). The mobile phase was selected as solvent A (H_2O + 0.1% H_3PO_4) and solvent B (CH_3CN) in the following gradients: 0–2 min, 12–20% B; 2–4 min, 20–26% B, 4–5 min, 26–35% B, 5–5.5 min, 35–100% B. Wavelength monitoring was performed at 0–4 min, 327 nm and 4–7 min, 229 nm. Column temp. was controlled at 40 °C and flow rate was set at 1.0 ml/min. Total analysis time was 6 min; the injection volume for all samples was set to 1 μl.

3. Results and discussion

3.1. Method development

Mobile phase composition and UV detection wavelength were taken into account while developing the RRLC method. Using MeOH as solvent B in mobile phase led to poor separation of the bioactive compounds and high backpressure. When replacing MeOH with CH_3CN, lower backpressure and better separation were achieved. The acid dissociation constant (p*K*a) of chlorogenic acid and baicalin are 3.59 [16] and 2.90 [17], respectively. It is a common practice to regulate the mobile phase pH at 1 to 2 units below the p*K*a of the peaks of interest, and thus H_2O + 0.1% H_3PO_4 at pH 2 was chosen as mobile phase A to improve the separation of bioactive compounds. By comparing the UV absorptions in mobile phase of reference compounds, it was found that chlorogenic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, isochlorogenic acid A, B, C, and forsythoside had characteristic absorption at 327 nm, while baicalin and forsythin had maximum absorption at 229 nm. Therefore, detection wavelength was selected as 327 nm (0–4 min) and 229 nm (4–7 min) to analyze all bioactive compounds in SHL preparations in a single run. A typical chromatogram of commercial SHL preparation OL-2 obtained under the optimized chromatographic conditions is shown in Fig. 4. It can be seen from the chromatogram that the method is capable of baseline separating the bioactive compounds in SHL preparations within 6 min.

3.2. Method validation

The method was validated according to ICH for validation of analytical procedures.

3.2.1. Calibration, sensitivity, linearity, and accuracy

Calibration curves were prepared by plotting the peak area against the corresponding concentrations. The regression lines are linear in the concentration range studied and the corresponding coefficients of correlation are shown in Table 1. Reference standard 5-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, forsythoside, isochlorogenic acid A, B, and C were only used for qualitative analysis. Peaks were assigned by spiking the samples with standard compounds, and comparing of the UV spectra and retention time. Good linear relationships ($r = 0.9999$ for chlorogenic acid and forsythin, 0.9998 for baicalin, respectively) are demonstrated over a range of 50–500 μg/ml. The mean regression equations for chlorogenic acid, forsythin, and baicalin are listed in Table 1. Fig. 2 shows the separation of mixed marker compounds 5-O-caffeoylquinic acid, chlorogenic acid, 4-O-caffeoylquinic acid, forsythoside, baicalin, and forsythin.

The limit of detection (LOD) was determined by calculation of the signal-to-noise ratio of 3:1. The LOD for chlorogenic acid, forsythin, and baicalin is 4.38, 9.90, 10.09 ng/ml, respectively ($n = 3$, RSD = 0.15%, 0.26%, 0.19%). The limit of quantification (LOQ) was determined by the calculation of signal-to-noise ratio of 10:1. The LOQ for chlorogenic acid, forsythin, and baicalin is 12.95, 28.72 and 29.61 ng/ml, respectively ($n = 3$, RSD = 0.18%, 0.32%, 0.27%).

The accuracy of the analytical method was evaluated using the recovery test. The recovery tests were performed by spiking a known quantity of the 3 reference standard to 1 ml of commercial SHL preparation OL-2. The fortified sample was then diluted and analyzed in triplicates as described in Section 2.2. For each sample, 3 concentration levels (approximately equivalent to 0.8, 1.0 and 1.2 times of the concentration of the sample) were tested. The results of the recovery tests were obtained by comparing the results from original samples and fortified samples. The mean recoveries are from 98.6% to 100.4% with RSD less than 1.42% for 3 reference compounds.

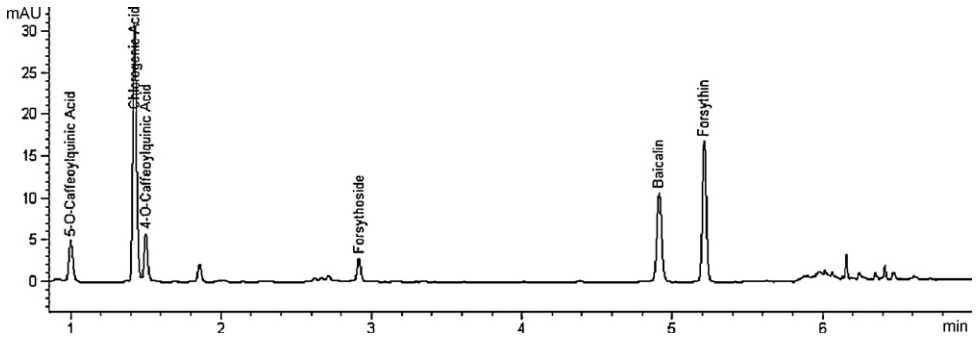

Fig. 2. Chromatogram of reference compounds mixture: 5-O-caffeoylquinic acid, chlorogenic acid, 4-O-caffeoylquinic acid, forsythoside, baicalin, and forsythin using the proposed method (Column: Phenomenex Luna C18-HST (2.5 μm, 100 mm × 3.0 mm), kept at 40 °C, mobile phases: A – H_2O + 0.1% H_3PO_4 and B – CH_3CN in the following gradients: 0–2 min, 12–20% B; 2–4 min, 20–26% B, 4–5 min, 26–35% B, 5–5.5 min, 35–100% B, flow rate: 1.0 ml/min, injection volume: 1 μl, UV detection: 0–4 min at 327 nm, 4–7 min at 229 nm).

As shown in Figs. 2 and 3 chlorogenic acid isomers gave 3 well-separated peaks. The 5-O-caffeoylquinic acid eluted first at about 1.01 min, followed by chlorogenic acid at 1.38 min and then 4-O-caffeoylquinic acid with a retention time of 1.44 min.

3.2.2. System suitability test and precision

A system suitability test was performed. Table 2 summarizes the retention time, peak area, the number of theoretical plates, tailing factor and resolution. The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by testing a single commercial SHL sample OL-2, at the same concentration and during the same day. The intermediate precision was studied by analyzing and comparing the assays on each of the 3 successive days. Retention time RSD within a single day ranged from 0.04% to 0.29% and peak area RSD ranged from 1.49% to 1.88%. Retention time RSD on triplicate injections on 3 successive days ranged from 0.06% to 0.32% and peak area RSD ranged from 1.03% to 1.92%. The results show that the variance for both retention time and peak area for triplicate injections of the same sample analyzed on 3 successive days tended to be higher than the variance for a single day.

3.2.3. Specificity and selectivity

The specificity of the RRLC method was demonstrated by the completed separation of 5-O-caffeoylquinic acid, chlorogenic acid, 4-O-caffeoylquinic acid, forsythoside, isochlorogenic acid A, B, C, baicalin, and forsythin in commercial SHL sample OL-2 (Fig. 4). No interference or overlapping was observed from the degraded products. Moreover, the purity of these peaks was further confirmed by analyzing the same sample by a Diode Array Detector (DAD).

3.2.4. Ruggedness

The ruggedness test was performed by two analysts testing 3 batches of commercial SHL preparations (OL-1, OL-2, and OL-3). Each analyst prepared samples in duplicates and used separate mobile phase solution. The RSD ($n = 4$) of the chlorogenic acid, baicalin, and forsythin concentrations in all of the tested samples was less than 1.5%, indicating acceptable ruggedness.

3.2.5. Robustness

The influence of the different chromatographic parameters upon the separation on sample solution of commercial SHL preparation OL-2 was evaluated by systematically varying the following factors: pH of the mobile phase A at 1.8, 2.0 and 2.2, flow rate at 0.95, 1.0 and 1.05 ml/min, and column temp. at 38, 40 and 42 °C. Only one factor was changed while the others were kept constant. The effects of the parameters on the resolution of reference compound peaks were evaluated and summarized in Table 3. All critical separations were achieved with the indicated minimum baseline resolution. Furthermore, satisfactory separation and symmetric peaks were achieved using any of the above conditions, indicating the robustness of this method in the tested range.

3.3. RRLC analysis of raw herbs in SHL preparations—Flos Lonicerae, Fructus Forsythiae, and Radix Scutellariae

To investigate the correlation between the chromatogram of SHL preparations and raw herbs, *Flos Lonicerae, Fructus Forsythiae*, and *Radix Scutellariae* herb were analyzed by the developed method and their overlapped RRLC chromatogram is shown in Fig. 3. Chlorogenic acid is the absolute predominant compound accounted for 97.8% of total peak area, 5-O-caffeoylquinic acid only exists in tiny amount (2.2% total peak area) and no 4-O-caffeoylquinic acid was

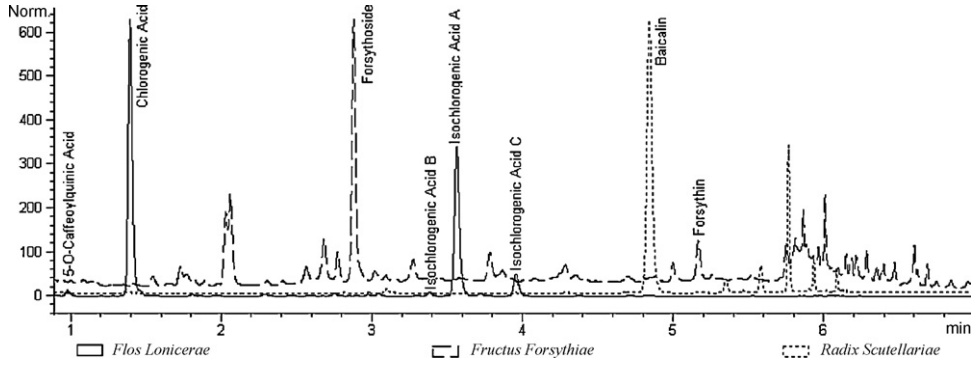

Fig. 3. RRLC chromatogram of raw medicinal herbs in SHL preparations: *Flos Lonicerae*, *Fructus Forsythiae*, and *Radix Scutellariae*. The chromatographic conditions are the same as for Fig. 2.

Table 2
System suitability, precision, number of theoretical plates, tailing factor and resolution.

	Retention time (min)	Peak area	# of theoretical plates	Tailing factor	Resolution[a]
Chlorogenic acid					
Intra-day					
Mean (n = 3)	1.386	95.31	12,354	1.05	1.60
Range	1.382–1.390	92.51–97.42	12,195–12,125	1.04–1.06	1.59–1.61
RSD (%)	0.23%	1.88%	3.65%	0.72%	0.61%
Inter-day					
Mean (n = 9)	1.388	95.72	12,377	1.05	1.60
Range	1.382–1.394	92.51–98.59	11,817–13,340	1.04–1.07	1.58–1.63
RSD (%)	0.31%	1.92%	4.27%	1.11%	1.26%
Baicalin					
Intra-day					
Mean (n = 3)	4.868	537.94	82,413	1.05	5.15
Range	4.865–4.870	531.37–547.21	79,610–84,468	1.04–1.06	5.09–5.19
RSD (%)	0.04%	1.81%	2.37%	0.60%	0.69%
Inter-day					
Mean (n = 9)	4.868	537.10	84,467	1.05	5.16
Range	4.864–4.871	530.28–547.21	79,610–86,903	1.04–1.06	5.08–5.20
RSD (%)	0.06%	1.03%	2.45%	0.60%	0.81%
Forsythin					
Intra-day					
Mean (n = 3)	5.158	28.15	120,994	1.05	3.53
Range	5.156–5.160	27.92–28.88	119,081–124,051	1.03–1.06	3.52–3.55
RSD (%)	0.29%	1.49%	1.56%	1.12%	0.50%
Inter-day					
Mean (n = 9)	5.158	27.96	121,052	1.05	3.53
Range	5.155–5.160	27.56–28.88	119,081–124,051	1.03–1.07	3.51–3.55
RSD (%)	0.32%	1.21%	1.42%	1.35%	0.62%

[a] Resolution is calculated using the most adjacent peak.

Table 3
Summary of resolution values for method robustness testing.

Method parameter	Chlorogenic acid	Baicalin	Forsythin
Column temp. (°C)			
38	1.62	6.46	4.69
40	1.60	5.16	3.53
42	1.37	5.08	3.12
pH of mobile phase A			
1.8	1.57	5.18	3.46
2.0	1.60	5.16	3.53
2.2	1.43	4.80	3.62
Flow rate (ml/min)			
0.95	1.28	3.19	3.48
1.00	1.60	5.16	3.53
1.05	1.38	3.21	3.21

detected. Similarly, isochlorogenic acid A is the predominant compound, isochlorogenic acid B and C only exist in small amount. Chlorogenic acid is the naturally occurring predominant form not only in *Flos Lonicerae* but also in other plants such as potato tuber [18] and prunes (*Prunus domestica* L.) [19], whilst other chlorogenic acid isomers only exist in small amount.

Table 4
The contents of 3 marker compounds: chlorogenic acid, baicalin, and forsythin in 8 commercial SHL preparations (n = 3).

SHL sample #:	Chlorogenic acid (mg/ml)	Baicalin (mg/ml)	Forsythin (mg/ml)
OL-1	0.98 ± 0.001	11.75 ± 0.01	0.48 ± 0.002
OL-2	1.01 ± 0.001	12.84 ± 0.02	0.46 ± 0.001
OL-3	1.03 ± 0.002	13.23 ± 0.01	0.41 ± 0.002
OL-4	1.06 ± 0.002	15.21 ± 0.01	0.45 ± 0.001
OL-5	0.79 ± 0.002	11.96 ± 0.01	0.35 ± 0.001
OL-6	0.74 ± 0.001	12.27 ± 0.02	0.30 ± 0.001
COL-1	0.98 ± 0.001	28.7 ± 0.03	0.89 ± 0.003
AS-1	1.23 ± 0.002	16.75 ± 0.02	0.47 ± 0.001

3.4. RRLC analysis of commercial SHL preparations

Eight batches of commercial SHL preparations collected from different manufacturers were tested by the described RRLC method. These samples include one oral spray sample, one concentrated SHL oral solution sample and six regular strength oral solution samples. The analysis results are summarized in Table 4.

PPRC 2005 edition established the quality control standards for SHL oral solution as follows: the chlorogenic acid, forsythin, and baicalin concentration should not be less than 0.6, 0.3 and

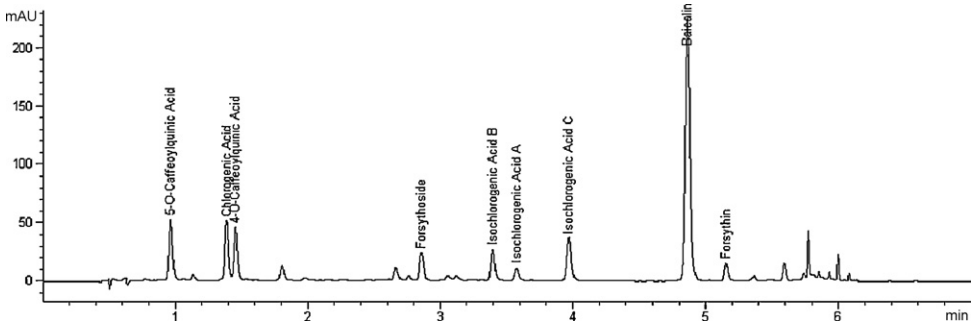

Fig. 4. Typical chromatogram of a commercial SHL oral solution (Sample #: OL-2). The chromatographic conditions are the same as for Fig. 2.

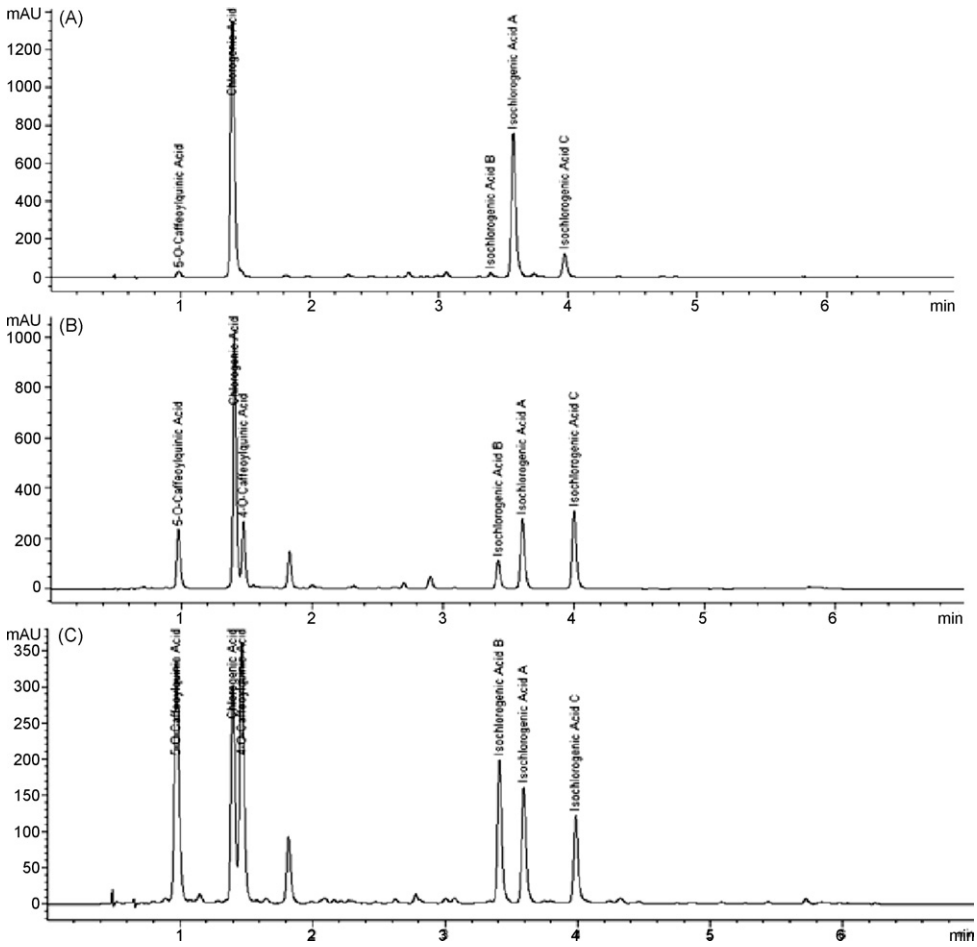

Fig. 5. RRLC chromatograms of *Flos Lonicerae* herbal liquid extract (A) pH 3.5, (B) pH 7.0, (C) pH 11, after 48 h storage at room temp. The chromatographic conditions are the same as for Fig. 2.

8 mg/ml, respectively. The marker compounds contents in these 8 batches of commerical SHL all exceed the minimum standards of PPRC. Comparing the chromatogram of SHL preparation (Fig. 4) with the chromatograms of raw herbs (Fig. 3), most of the peaks detected in SHL preparations except 4-O-caffeoylquinic acid could be found in raw herbs. However, the chromatographic profile of the *Flos Lonicerae* component in commercial SHL preparation is significantly different from the raw herb. Chlorogenic acid is the predominant compound in *Flos Lonicerae* raw herb; in contrast, 5-O-caffeoylquinic acid, chlorogenic acid, and 4-O-caffeoylquinic acid were all detected in comparable amounts in commercial SHL preparations, suggesting that the chemical composition of the *Flos Lonicerae* component has changed during commercial SHL preparations manufacturing process.

Our study also revealed that the proportion of each chlorogenic acid isomer is relatively constant in the 8 commercial SHL preparations. Based on peak area, the average percentage of 5-O-caffeoylquinic acid, chlorogenic acid, and 4-O-caffeoylquinic acid in total chlorogenic acid isomers are 36.37%, 34.68%, and 28.95%, respectively ($n = 3$, RSD = 3.03%, 5.72%, 3.96%). In other words, the chemical composition profiles of commercial SHL preparations are stable.

3.5. Effect of pH on the concentration of individual chlorogenic acid isomer

Griffiths and Bain [18] reported that chemical isomerization is the major mechanism for the increase of the relative amount of 5-O-caffeoylquinic acid and 4-O-caffeoylquinic acid in potato tubers and at higher pH values isomerization may be important. Therefore, we hypothesized that pH plays a crucial role in the isomerization of chlorogenic acid isomers. To study the effect of pH on the concentration of chlorogenic acid isomers, 3 samples of *Flos Lonicerae* herbal liquid extract at pH 3.5, 7.0 and 11.0 were prepared and analyzed by the described method and their RRLC chromatograms are shown in Fig. 5.

In sample A (pH 3.5) chlorogenic acid is the predominant isomer, while 5-O-caffeoylquinic acid only exists in tiny amount and 4-O-caffeoylquinic acid could not be detected at all. This shows a typical chromatographic profile of *Flos Lonicerae* herb at its physiological pH. In sample B (pH 7) isomerization of 5-O-caffeoylquinic acid and 4-O-caffeoylquinic acid from chlorogenic acid was observed after 48 h of room temp. storage. In sample C (pH 11) the amount of chlorogenic acid decreased dramatically accompanied by the increasing of 5-O-caffeoylquinic acid and 4-O-caffeoylquinic acid isomers.

The above observations suggest that chlorogenic acid is very sensitive to pH change. In acidic conditions it is the predominant isomer in *Flos Lonicerae* (Fig. 5A). At neutral or basic conditions, the isomerization of chlorogenic acid to 5-O-caffeoylquinic acid and 4-O-caffeoylquinic acid was observed within 48 h. Friedman and Jurgens [20] demonstrated that chlorogenic acid is stable to acidic pH, to heat, but not to high pH and the pH and time dependent spectral transformations are not reversible. Similar trends were also observed in isochlorogenic acid isomers. Isochlorogenic acid A is the predominant form in *Flos Lonicerae* herb; however, at neutral or basic pH its content drops rapidly, meanwhile isochlorogenic acid B and C increase to similar level of isochlorogenic acid A.

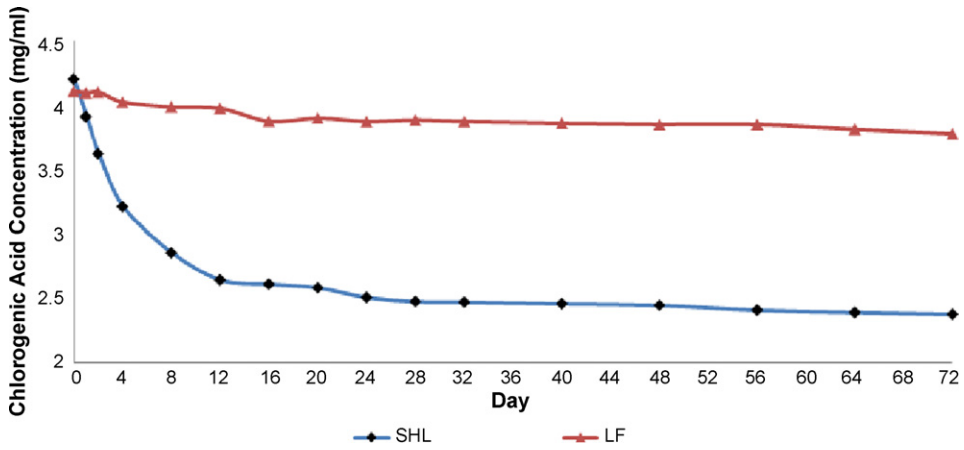

Fig. 6. The trend of the chlorogenic acid concentration change in "LF" (*Flos Lonicerae* plus *Fructus Forsythiae*) and SHL liquid extract samples at pH 7. The chromatographic conditions are the same as for Fig. 2.

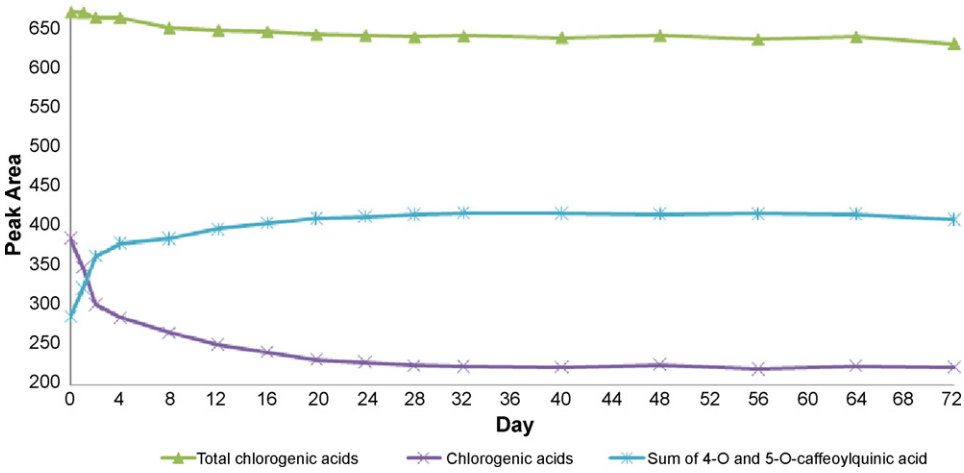

Fig. 7. The relationship of peak area (chlorogenic acid, the sum of 4-O and 5-O-caffeoylquinic acid and total of 3 chlorogenic acid isomers) and storage time of SHL liquid extract sample at pH 7. The chromatographic conditions are the same as for Fig. 2.

3.6. Stability studies of SHL preparations

As discussed in Section 3.3, chlorogenic acid and isochlorogenic acid A are very sensitive to pH. At pH 7 isomerization of chlorogenic acid and isochlorogenic acid A was observed within 48 h. To further investigate the stability of SHL preparations at different pH, 1000 ml "LF" (*Flos Lonicerae* plus *Fructus Forsythiae)* liquid extract sample at pH 3.5 and 1000 ml SHL liquid extract sample at pH 7 were prepared as described in Section 2.2.

For the "LF" liquid extract sample at pH 3.5, no significant change in chromatogram was observed after 72 days stability testing, implying that chlorogenic acid is stable in acidic conditions. In contrast, the chromatogram of SHL sample at pH 7 after 72 days stability testing is significantly different from the overnight sample. The content of chlorogenic acid decreased, in the meantime the contents of 4-O and 5-O-caffeoylquinic acid increased gradually. No significant change in the contents of forsythoside, forsythin, and baicalin was observed in 72 days, suggesting that the stability of these compounds is independent of pH. Fig. 6 demonstrates the trend of chlorogenic acid concentration change during stability test. The "LF" sample was found stable for at least 72 days in accelerated storage conditions since no significant change in chlorogenic acid concentration was observed. For SHL sample at pH 7, the chlorogenic acid concentration decreased 32% from 4.2 mg/ml to 2.85 mg/ml within 8 days. And hence in order to preserve the chemical profile integrity of *Flos Lonicerae*, it was advised to prepare and store SHL preparations in acidic conditions.

Fig. 7 was plotted to illustrate the change over time with respect to the peak area of chlorogenic acid, the sum of 4-O and 5-O-caffeoylquinic acid, and the total of 3 chlorogenic acid isomers. Although the peak area of chlorogenic acid decreased and the sum of 4-O and 5-O-caffeoylquinic acid increased over time, the total peak area of 3 chlorogenic acid isomers only slightly decreased. Taken together, the results strongly suggest that the conversion of chlorogenic acid to 4-O and 5-O-caffeoylquinic acid is attributed to chemical isomerization.

4. Conclusion

The method developed allows an efficient determination of the bioactive compounds i.e., chlorogenic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, forsythin, forsythoside, isochlorogenic acid A, B, C and baicalin in SHL and "LF" preparations. Baseline separation of the studied analytes was achieved within 6 min. The method was shown to be rugged, transferable and it satisfied the requirements of linearity, precision and selectivity to quantify the bioactive compounds in finished products.

Chlorogenic acid and isochlorogenic acid A are the predominant compounds in *Flos Lonicerae* herb. The stability studies revealed that these two compounds are stable in acidic conditions, while at

neutral and basic pH, isomerization of chlorogenic acid to 4-O and 5-O-caffeoylquinic acid, as well as the isomerization of isochlorogenic acid A to B and C occurred rapidly. This finding explains the inconsistency in the chromatogram of commercial SHL preparations and individual herbal components. Forsythoside, forsythin, and baicalin are stable in both acidic and basic condition, and no significant concentration change was observed in stability testing. Based on the observation in this study, it is suggested to further evaluate and optimize PPRC's preparatory procedures and storage conditions for commercial SHL preparations.

This RRLC method is efficient not only in the raw herbs quality control screening but also in the analysis and evaluation of commercial SHL preparation, as well as other formulated products containing one or more of *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae* herbal component such as "Jin-Hua-Qing", "Yin-Qiao-Jie-Du-Wan", and "Lian-Hua-Qing-Wen".

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