Simultaneous Determination of Forsythin, Baicalin and Chlorogenic Acid in Traditional Chinese Medicine Spray Disinfectant by HPLC

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Abstract [**Objectives**] To establish a high-performance liquid chromatography (HPLC) method for the simultaneous determination of forsythin, baicalin, and chlorogenic acid in a traditional Chinese medicine spray disinfectant. [**Methods**] The chromatographic separation was performed on a GL Sciences (19H0044724)-C_{18} column (4.6 mm × 250 mm, 5 μm) with a mobile phase of acetonitrile – 0.05% formic acid solution at a flow rate of 1 mL/min. The injection volume was 10 μL, detection wavelength was set at 280 nm, and column temperature was maintained at 25 ℃. [**Results**] The linear ranges of forsythin, baicalin, and chlorogenic acid were 10.5 – 52.5, 20.6 – 103, and 14.2 – 71 μg/mL, respectively, with good linear relationships between concentration and peak area (R^2 = 0.999 9). The relative standard deviations (*RSD*s) for precision and repeatability tests were all ≤1.0%. The average recoveries were 98.51%, 98.48%, and 97.71% for the three components, with *RSD*s of 0.96%, 0.97%, and 0.73%, respectively. [**Conclusions**] This method demonstrates strong specificity, high precision, excellent accuracy, and simplicity of operation, making it suitable for the simultaneous quantification of forsythin, baicalin, and chlorogenic acid in traditional Chinese medicine spray disinfectants. It provides a reliable basis for quality control and practical applications in animal breeding environments.

Key words Forsythin, Baicalin, Chlorogenic acid, high-performance liquid chromatography (HPLC), Traditional Chinese medicine spray disinfectant

1 Introduction

In recent years, the prevalence of infectious diseases such as avian influenza, African swine fever, brucellosis, East Coast fever, African animal trypanosomiasis, Newcastle disease, porcine reproductive and respiratory syndrome (PRRS), and foot-and-mouth disease has been increasing. Necessary disinfection of animal breeding environment can effectively prevent disease transmission. The traditional Chinese medicine spray disinfectant is prepared from nine herbal ingredients, including Forsythiae Fructus, Lonicerae Japonicae Flos, Scutellariae Radix, Magnoliae Officinalis Cortex, and Atractylodis Rhizoma. It demonstrates significant efficacy in purifying air, eliminating odors, activating orifices, disinfection, and sterilization. Additionally, it is naturally non-toxic, non-irritating, residue-free, and does not induce drug resistance, making it widely applicable for disinfection in animal breeding environments.

Forsythiae Fructus contains major components such as flavonoids, phenylethanes, lignans, and volatile compounds, among which forsythin, Forsythoside, and oleanolic acid are its primary active components responsible for pharmacological effects[1]. *In vitro* antimicrobial experiments on forsythin revealed its potent inhibitory activity against *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus agalactiae*. *In vivo* and *in vitro* studies on infections caused by *Pseudomonas aeruginosa* and *Escherichia coli* also demonstrated that forsythin exhibits therapeutic effects and reduces animal mortality[2]. Lonicerae Japonicae Flos primarily contains organic acids, flavonoids, volatile oils, and iridoid glycosides. The organic acids include chlorogenic acid, neochlorogenic acid, and cryptochlorogenic acid, which are among the main medicinal components of Lonicerae Japonicae Flos[3]. *In vitro* experiments confirmed that Lonicerae Japonicae Flos extract exhibits strong antibacterial activity against susceptible strains such as *Staphylococcus albus*, *S. aureus*, α-hemolytic streptococcus, β-hemolytic streptococcus, Gram-positive bacteria, and *Escherichia coli*[4]. Studies have verified that chlorogenic acid is the principal component responsible for its antibacterial effects[5-6]. Scutellariae Radix mainly contains baicalin, baicalein, wogonoside, and 5,7-dihydroxy-6-methoxyflavanone[7]. Baicalin is one of the key bioactive components of Scutellariae Radix, showing efficacy in alleviating lung symptoms in mice infected with *S. aureus*[8]. For drug-resistant bacteria, baicalin can act as an SOS inhibitor, significantly reducing the rifampicin resistance mutation rate in *S. aureus* induced by ciprofloxacin[9].

In Traditional Chinese Medicine (TCM) theory, Forsythiae Fructus, Lonicerae Japonicae Flos, and Scutellariae Radix all possess the efficacy of clearing heat and detoxifying. Forsythiae Fructus is particularly effective in clearing heart fire and dispersing wind-heat in the upper energizer. Lonicerae Japonicae Flos demonstrates significant therapeutic effects against febrile diseases, heat-toxin-induced dysentery, and other heat-toxin disorders. Scutellariae Radix primarily clears lung heat and gallbladder heat, with functions of clearing heat, drying dampness, purging fire,

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and detoxifying. The combination of these three herbs exhibits synergistic effects and serves as the primary antimicrobial components of the traditional Chinese medicine spray disinfectant. According to the *Chinese Veterinary Pharmacopoeia* (2020 edition), the content determination indicators for these three herbs are forsythin, chlorogenic acid), and baicalin, respectively. This study aims to establish a high-performance liquid chromatography (HPLC) method for the simultaneous determination of these three components, with the goal of developing a simpler, more direct quantification approach to ensure the disinfection efficacy of the herbal disinfectant and enhance the controllability and stability of its quality.

2 Instruments and drugs

Agilent 1260 high performance liquid chromatograph (Agilent Technologies); electronic analytical balance (SECURA225D-1CN, precision 0.01 mg, Sartorius Group); PHB-4 portable pH meter (Shanghai INESA Analytical Instrument Co., Ltd).

Acetonitrile (chromatographic grade, Tianjin Bodi Chemical Co., Ltd.); formic acid (analytical grade, Tianjin Bodi Chemical Co., Ltd.); triethylamine (analytical grade, Yantai Sanhe Chemical Reagent Co., Ltd.); water was purified using the Sartorius Arium Comfort Ultrapure Water System; magnolol reference standard (99.8%, batch No.: 110729-201513), chlorogenic acid reference standard (99.8%, batch No.: 110735-201415), forsythin reference standard (99.8%, batch No.: 110821-201514), baicalin reference standard (batch No.: 110715-201720), atractylin reference standard (99.8%, batch No.: 111924-201605). All reference standards were purchased from Weifang Zhongke Medical Equipment Co., Ltd.; traditional Chinese medicine spray disinfectant (self-prepared).

3 Methods and results

3.1 Chromatographic conditions High performance liquid chromatograph (Agilent 1260, equipped with G7111A quaternary pump, G7129 A column incubator, M8413AA autosampler, G7114AUV ultraviolet detector and Lab Solution data processing workstation); electronic analytical balance (SECURA225D-1CN); PHB-4 portable pH meter.

Column: GL Sciences (19H0044724) C_{18} column (4.6 mm × 250 mm, 5 μm); mobile phase: acetonitrile (A) –0.05% formic acid solution (B), with gradient elution as follows: 0–10 min (15% A), 10–22 min (25% A), 22–30 min (40% A), 30–40 min (55% A), 40–50 min (70% A), 50–60 min (10% A); flow rate: 1 mL/min; detection wavelength: 280 nm; column temperature: 25 ℃; injection volume: 10 μL.

3.2 Preparation of solutions

3.2.1 Reference standard solutions. Accurately weighed baicalin reference standard was dissolved in methanol and diluted to a final concentration of 61.6 μg/mL[10]; accurately weighed chlorogenic acid reference standard was placed in a brown volumetric flask, dissolved in 50% methanol, and diluted to a concentration of 42.5 μg/mL, with storage below 10 ℃[11]; accurately weighed forsythin reference standard was dissolved in methanol and diluted to 31.5 μg/mL[12].

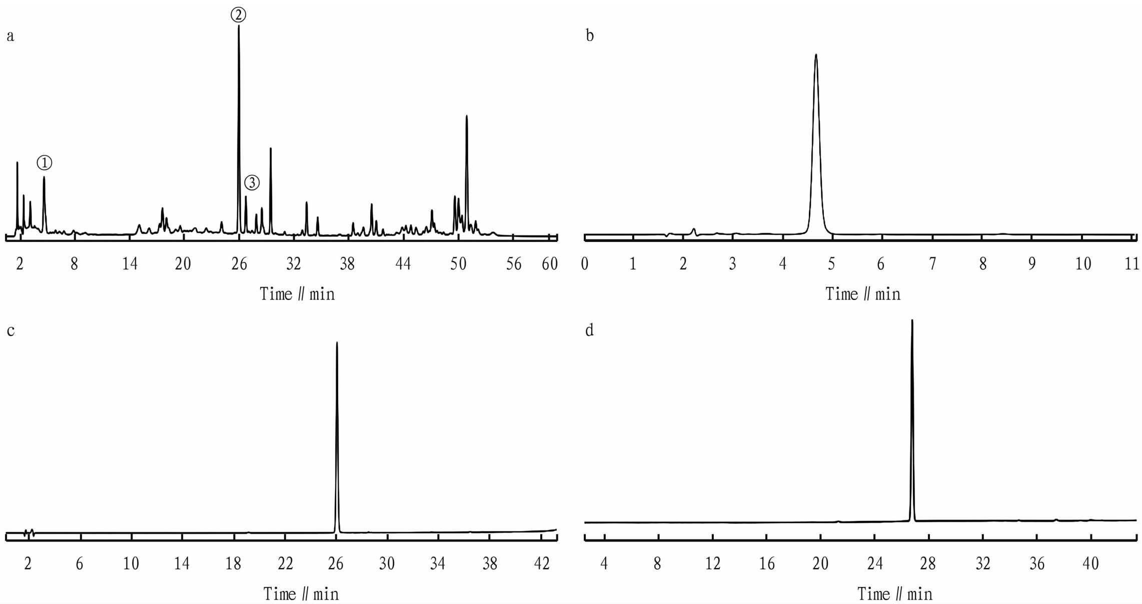
3.2.2 Test sample solution. 3 mL of the Traditional Chinese Medicine spray disinfectant was transferred to a 100 mL volumetric flask, dissolved in methanol, and diluted to the mark. The solution was filtered through a microporous membrane (0.45 μm), and the subsequent filtrate was collected for use.

3.3 Methodological investigation

3.3.1 System suitability and specificity test. An appropriate amount of the test sample solution, baicalin reference standard solution, chlorogenic acid reference standard solution, and forsythin reference standard solution were injected and analyzed under the chromatographic conditions specified in Section **3.1**. The chromatogram of the Traditional Chinese Medicine spray disinfectant test sample exhibited identical absorption peaks at retention times corresponding to those of baicalin, chlorogenic acid, and forsythin reference standards. The theoretical plate numbers calculated for the forsythin, baicalin, and chlorogenic acid peaks were all > 3 000, with resolutions >1.5, demonstrating satisfactory baseline separation (Fig. 1).

3.3.2 Linear relationship investigation. Accurately weighed baicalin reference standard was dissolved in methanol and diluted to the mark, then serially diluted to standard solutions with concentrations of 20.6, 41.2, 61.8, 82.4, and 103 μg/mL. Accurately weighed chlorogenic acid reference standard was dissolved in methanol, diluted to the mark, and further diluted to standard solutions with concentrations of 14.2, 28.3, 42.6, 56.8, and 71.0 μg/mL. Accurately weighed forsythin reference standard was dissolved in methanol, diluted to the mark, and serially diluted to standard solutions with concentrations of 14.2, 28.3, 42.6, 56.8, and 71.0 μg/mL. Each solution (10 μL) was injected into the HPLC system under the chromatographic conditions described in Section **3.1**. Peak areas were recorded, and calibration curves of peak area versus concentration were plotted. The regression equations were obtained as follows: Baicalin: $Y = 656.01X - 1.041$ ($R^2 = 0.999\,9$); chlorogenic acid: $Y = 227.31X - 2.743$ ($R^2 = 0.999\,9$); forsythin: $Y = 170.19X + 2.749$ ($R^2 = 0.999\,9$). Results demonstrated good linear relationships between peak area and concentration within the following ranges: baicalin: 20.6–103 μg/mL; chlorogenic acid: 14.2–71 μg/mL; forsythin: 10.5–52.5 μg/mL.

3.3.3 Precision test. Accurately pipetted 10 μL of each reference standard solution from Section **3.3** was injected six times repeatedly. The average peak areas obtained were 1 960.16 for baicalin, 680.27 for chlorogenic acid, and 515.46 for forsythin. The relative standard deviations (*RSD*) of the peak areas were calculated, and all *RSD* values were <2%, indicating excellent instrument precision (Table 1).



NOTE a. ① Chlorogenic acid, ② Baicalin, ③ Forsythin; b. Forsythin; c. Chlorogenic acid; d. Baicalin.

Fig. 1 High performance liquid chromatogram of test solution and reference solution

Table 1 Precision test results ($n = 6$)

Solution	Test times	Peak area	Mean of peak area	*RSD* // %
Baicalin	1	1 960.13	1 960.16	0.09
	2	1 962.57		
	3	1 958.35		
	4	1 961.27		
	5	1 963.48		
	6	1 961.93		
Chlorogenic acid	1	681.86	680.27	0.21
	2	682.41		
	3	680.27		
	4	678.34		
	5	679.58		
	6	679.17		
Forsythin	1	515.41	515.46	0.14
	2	514.76		
	3	515.27		
	4	516.87		
	5	514.64		
	6	515.82		

3.3.4 Repeatability test. Six aliquots of the Traditional Chinese Medicine spray disinfectant sample solution from the same batch were determined for content according to the method in Section **3.2.1**. The results are shown in Table 2, with all relative standard deviations (*RSD*s) < 2%, demonstrating satisfactory method repeatability.

3.3.5 Spiked recovery test. Accurately measured 3 mL of the previously determined traditional Chinese medicine spray disinfectant was transferred to a 100 mL volumetric flask in nine parallel replicates, which were divided into three groups: high-, medium-, and low-concentration. Each group received respective additions of 1.2, 1.0, and 0.8 mL containing baicalin (1.025 mg/mL), chlorogenic acid (0.364 5 mg/mL), and forsythin (0.166 mg/mL).

Table 2 Repeatability test results ($n = 6$)

Solution	Test times	Baicalin content // mg/mL	Average content mg/mL	*RSD* // %
Baicalin	1	1.025 7	1.024 6	0.12
	2	1.022 5		
	3	1.025 1		
	4	1.023 6		
	5	1.025 6		
	6	1.025 3		
Chlorogenic acid	1	0.364 7	0.364 1	0.12
	2	0.363 4		
	3	0.363 7		
	4	0.364 5		
	5	0.364 0		
	6	0.364 2		
Forsythin	1	0.166 4	0.165 9	0.24
	2	0.165 2		
	3	0.165 8		
	4	0.165 9		
	5	0.166 3		
	6	0.166 0		

The mixtures were subsequently dissolved in methanol and diluted to the mark. After filtration through a 0.45 μm microporous membrane, the solutions were analyzed under the chromatographic conditions specified in Section **3.1** to determine peak areas and calculate recovery rates. Results showed recovery rates of 98.48% for baicalin, 97.71% for chlorogenic acid, and 98.51% for forsythin, with all relative standard deviations (*RSD*) < 2%, demonstrating satisfactory recovery performance (Table 3).

3.4 Content determination of traditional Chinese medicine spray disinfectant Three batches of Traditional Chinese Medicine disinfectant samples were taken. Test sample solutions were prepared according to the method in Section **3.2**, and analyzed under

Table 3 Results of Spiked recovery test

Components	Spike volume mg	Original content mg	Measured value mg	Recovery rate %	Mean value %	*RSD* %
Baicalin	0.820 0	1.025 1	1.842 9	99.73	98.48	0.98
	0.820 0	1.025 1	1.822 2	97.21		
	0.820 0	1.025 1	1.836 7	98.97		
	1.025 0	1.025 1	2.041 8	99.19		
	1.025 0	1.025 1	2.021 6	97.22		
	1.025 0	1.025 1	2.035 3	98.56		
	1.230 0	1.025 1	2.249 9	99.58		
	1.230 0	1.025 1	2.226 7	97.69		
	1.230 0	1.025 1	2.232 6	98.17		
Chlorogenic acid	0.291 8	0.364 8	0.648 9	97.37	97.71	0.74
	0.291 8	0.364 8	0.650 9	98.05		
	0.291 8	0.364 8	0.648 0	97.04		
	0.364 8	0.364 8	0.719 0	97.09		
	0.364 8	0.364 8	0.726 4	99.12		
	0.364 8	0.364 8	0.721 0	97.64		
	0.437 7	0.364 8	0.796 0	98.51		
	0.437 7	0.364 8	0.789 5	97.02		
	0.437 7	0.364 8	0.791 8	97.55		
Forsythin	0.132 6	0.165 7	0.298 5	100.18	98.51	1.09
	0.132 6	0.165 7	0.295 8	98.13		
	0.132 6	0.165 7	0.296 0	98.25		
	0.165 7	0.165 7	0.326 9	97.29		
	0.165 7	0.165 7	0.330 2	99.28		
	0.165 7	0.165 7	0.328 0	97.95		
	0.198 8	0.165 7	0.360 9	98.21		
	0.198 8	0.165 7	0.364 5	100.02		
	0.198 8	0.165 7	0.359 2	97.31		

the chromatographic conditions specified in Section **3.1** to obtain peak areas[14]. The content was then calculated based on the peak areas (Table 4).

Table 4 Results of Content determination of traditional Chinese medicine spray disinfectant (mg/mL, $n = 4$)

Batch No.	Baicalin content	Chlorogenic acid content	Forsythin content
20220412	1.025 1	0.364 8	0.165 7
20220413	1.024 8	0.366 4	0.166 8
20220414	1.025 4	0.365 5	0.165 0

3 Discussion

The *Chinese Veterinary Pharmacopoeia* (2020 edition) specifies high-performance liquid chromatography (HPLC) for content determination of Forsythiae Fructus, Scutellariae Radix, and Lonicerae Japonicae Flos, with control indices set as forsythin, baicalin, and chlorogenic acid, respectively. The chromatographic conditions for forsythin are acetonitrile-water (25 : 75) with detection at 277 nm; for baicalin, methanol-water-phosphoric acid (47 : 53 : 0.2) at 280 nm; and for chlorogenic acid, acetonitrile-0.4% phosphoric acid solution (13 : 87) at 327 nm. Given the close detection wavelengths of forsythin (277 nm) and baicalin (280 nm), a unified wavelength of 280 nm was selected. As both forsythin and chlorogenic acid utilize acetonitrile-water systems, and baicalin/chlorogenic acid mobile phases incorporate phosphoric acid, initial attempts with isocratic elution (acetonitrile-0.4% phosphoric acid solution) yielded unsatisfactory results. Through iterative optimization, the final chromatographic conditions were established as gradient elution with acetonitrile-0.05% formic acid solution.

This study developed an HPLC method for simultaneous quantification of three bioactive components (baicalin, chlorogenic acid, and forsythin) in traditional Chinese medicine spray disinfectants. The method demonstrates high accuracy and repeatability, providing a scientific basis for product quality control and supporting industrial development of herbal air disinfectants for veterinary applications in pet care.

References

[1] JING FT, LI F, ZHANG TY, *et al.* Recent research progress on chemical components and biological activities of *Forsythia suspensa*[J]. Journal of Chinese Medicinal Materials, 2023, 46(1): 242–251. (in Chinese).
[2] DUAN LJ, HU LQ, ZHANG Q, *et al.* Effect of different forsythia preparations on influenza virus *nucleoprotein* gene expression after transfection[J]. China Journal of Chinese Medicine, 2015, 30(1): 71–73. (in Chinese).
[3] XIAO L, BAO ZX, YU Y, *et al.* Chemical components from *Lonicerae japonicae* flos and their antibacterial and anti-inflammation activity *in vitro* [J]. Central South Pharmacy, 2022, 20(8): 1773–1780. (in Chinese).
[4] PENG WX, HAN NN, DAI Q, *et al.* Research progress of bacteriostatic effect of Shuanghuanglian preparation andits compositions on *Escherichia coli* and *Staphylococcus aureus in vitro*[J]. Chinese Journal of Veterinary Drug, 2023, 57(7): 62–67. (in Chinese).
[5] LI L, SUN J. Comparative on content of chlorogenic acid and bacteriostasis of extracts from different parts of polyploid and diploid Honeysuckle [J]. Food Science and Technology, 2012, 37(8): 225–227, 231. (in Chinese).
[6] WANG HJ, WU GJ, LI HR, *et al.* Extraction of chlorogenic acid from flos lonicerae and its antibacterial activity[J]. Journal of Beijing Agricultural College, 2003, 18(4): 262–265. (in Chinese).
[7] JIANG YT, HE C, REN TT, *et al.* Research progress on chemical components and pharmacological effects of *Scutellaria baicalensiss*[J]. Science & Technology in Chemical Industry, 2022, 30(6): 93–100. (in Chinese).
[8] XIE YM. Mechanism of action of baicalin on BT cell injury induced by *Mannheimia haemolytica* and pneumonia in mice[D]. Daqing: Heilongjiang Bayi Agricultural University, 2023. (in Chinese).
[9] LU QQ, SUN L. Research progress on new dosage forms of baicalein preparations[J]. Journal of Shenyang Pharmaceutical University, 2023, 40(9): 1253–1264. (in Chinese).
[10] LI XY, LI ZJ, XU HH, *et al.* Study on TLC identification and quantitative determination of Tonglin tablets[J]. China Pharmaceuticals, 2011, 20(18): 28–29. (in Chinese).
[11] XUAN TF. Study on quality standard of Yinhuang granules[J]. China Pharmaceuticals, 2006(5): 52–53. (in Chinese).
[12] YU W. Quality analysis of Shuanghuanglian oral liquid circulating in Chongqing[J]. China Pharmaceuticals, 2021, 30(19): 61–64. (in Chinese).
[13] YUSUPU KDN, BAI MN, TAN R, *et al.* Quality standard study for Yuningxin soft capsule[J]. China Pharmaceuticals, 2015, 24(24): 162–163. (in Chinese).
[14] LIU CQ, KE YC, HE BY, *et al.* Simultaneous determination of forsythin and baicalin in chere chaihu oral liquid by HPLC[J]. Traditional Chinese Drug Research & Clinical Pharmacology, 2011, 22(1): 105–107. (in Chinese).
[15] CHEN L, LI M, HUANG JQ, *et al.* Simultaneous determination of metronidazole and bilirubin in galculus bovis and metronidazole capsules by HPLC[J]. China Pharmaceuticals, 2023, 32(22): 95–98. (in Chinese).