

Study of Quality Standards of Xiao'er Qingre Enema Preparation in Hospitals

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Abstract: *Objective:* To establish the quality standards for the preparation of Xiao'er Qingre enema in hospitals. *Method:* Thin-layer chromatography (TLC) was used to identify Radix Isatidis and Glycyrrhizae in the prescription. The content of (R,S)-Goitrin was determined by high-performance liquid chromatography (HPLC). *Results:* In TLC identification, there was no interference between the negative controls of Radix Isatidis and glycyrrhizae, and the spots were well-separated. The HPLC results of (R,S)-Goitrin showed a good linear relationship between the peak area and concentration within the range of 0.476–95.2 $\mu\text{g}\cdot\text{mL}^{-1}$ ($r = 0.9999$). *Conclusion:* The TLC and HPLC methods established in this experiment are simple, reproducible, and specific, making them suitable for the quality control Xiao'er Qingre enema preparation in hospitals.

Keywords: Xiao'er Qingre enema; (R,S)-Goitrin; TLC; HPLC; Content determination

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1. Introduction

Hand foot and mouth disease (HFMD) is a common pediatric infectious disease mainly caused by enterovirus 71 and Coxsackie virus A16 infection^[1]. This disease is commonly found in children under 5 years old. It occurs year-round across all regions of China, with the incidence ranging from 37.01 to 205.06 per hundred thousand and the reported fatality rate ranging from 6.46 to 51.00 per hundred thousand in recent years^[2]. The main affected groups are children in nurseries and at home. HFMD can only be treated by isolating the child to avoid cross-infection, maintaining a light diet, ensuring good oral and skin care, fever control, and keeping the child calm. There are currently no specific anti-enterovirus drugs for this disease. While the early use of interferon α -2b spray or aerosol inhalation and intravenous ribavirin have shown some effectiveness in treating HFMD, there is lack of large-scale clinical trial studies, and the level of evidence supporting these treatments is currently low. In addition, ribavirin causes severe adverse reactions and reproductive toxicity. Common antiviral drugs, such as acyclovir, ganciclovir, and adenosine monophosphate, are ineffective towards HFMD^[3]. Therefore, there is an urgent need for effective antiviral drugs with fewer adverse reactions for HFMD treatment.

Xiao'er Qingre enema is composed of Radix Isatidis, wild honeysuckle, Glycyrrhiza, and other ingredients. Radix Isatidis and Wild honeysuckle clears heat and detoxify, while raw Glycyrrhiza clears heat and harmonizes the drugs. This combination aligns with the principles of Chinese medicine theory for HFMD, emphasizing the effects of relieving the exterior and interior conditions. HFMD patients are usually young children, thus it might be difficult for them to comply with the treatment or take their medicine. Enema can be administered through the rectum resulting in high adsorption and drug bioavailability. This is because it bypasses the first pass effect of the liver and digestion in the stomach and small intestine. Besides, this form of administration also avoids the potential irritation that oral medications may have on the stomach^[4]. However, there is currently no established quality standards for this form of administration. To ensure the safety, effectiveness, and controllability of clinical use, a quality standard for Xiao'er Qingre enema for children is established in this paper.

2. Instruments and drugs

JE1002 Electronic Balance (Shanghai Puchun Measuring Instrument Co., Ltd.), digital thermostatic water bath (Shanghai Pudong Physical Optics Instrument Factory, Model: HH-2), GL-200 Mini Camera-obscura UV System (Haimen Qilin Medical Instrument Factory), KQ-300E Ultrasonic Cleaning Machine (Kunshan Ultrasonic Instrument Co., LTD., with a power of 300W and frequency of 40KHz). Radix Isatidis control materials (batch No. 121177-201608), Glycyrrhiza control materials (batch No. 120904-201620), *Glycyrrhiza inflata* (121303-201704) were purchased from National Institutes for Food and Drug Control. Other reagents and the Xiao'er Qingre enema used were pure (batch No. 210101, 210102, 210103).

3. Methods

3.1 TLC identification of Radix Isatidis

20 mL of Xiao'er Qingre enema was extracted 3 times using ethyl acetate. The extract was steam dried and 1 mL methanol was added to dissolve the residue, which would be used for analysis. 0.5 g of Radix Isatidis was added into 20 mL of water and placed in a sonicator 30 minutes. The water layer was removed, and the solution would be the positive control. A negative control was also prepared. TLC was performed in accordance with the procedure in the Chinese Pharmacopoeia Appendix 0502 (2015 Edition). The positive and negative controls and the enema samples from the 3 extractions (5-10 µL each) were deposited on the TLC plate (silica plate). A mixture of petroleum ether and ethyl acetate (1:2) was used as the mobile phase. The TLC plate was dried and the dots were observed under UV light (254 nm).

3.2. TLC identification of Glycyrrhiza

10 mL of Xiao'er Qingre enema was extracted 3 times using water-saturated n-butanol. The extract was washed with 30 mL of water-saturated n-butanol for 2 times. The water layer was discarded and the residue was dissolved using 1 mL methanol. The positive control was prepared by first adding 1 g of glycyrrhiza into 30 mL of water-saturated n-butanol and placing the mixture in a sonicator for 20 minutes. The solution was filtered, the filtrate was evaporated, and 1 mL of methanol was added to the residue to dissolve it. A negative control was also prepared. TLC was performed in accordance with the procedure in the Chinese Pharmacopoeia Appendix 0502 (2015 Edition). 10 µL of the extract, 10 µL of the negative control, and 2 µL of the positive control were deposited onto the TLC plate. A mixture of dichloromethane, methanol, and water (40:10:1) was used as the mobile phase. The plates were dried, sprayed with 10% ethanol sulfate solution, and then heated at 105 °C until the spots were visible.

3.3 Determination of (R,S)-Goitrin by HPLC

3.3.1. Instrument and parameter settings

The column used was Hypersil Gold C18 (4.6 mm × 250 mm, 5 μm), with acetonitrile-water (5:95) as the mobile phase and the flow rate set to 1 ml·min⁻¹. The detection wavelength was 241 nm and the column temperature was 30°C. The number of theoretical plates was determined according to the (R,S)-Goitrin chromatographic peak, which was not less than 5000. The amount of sample used for each analysis was 10 μL.

3.3.2. Preparation of solutions

3.3.2.1. Preparation of stock solution

476 μg·mL⁻¹ (R,S)-Goitrin solution was prepared by dissolving suitable amount of (R,S)-Goitrin in methanol.

3.3.2.2. Preparation of reference solution

1 mL of the stock solution was added into a 50 mL volume flask and topped up with 30 % methanol to prepare 9.52 μg·mL⁻¹ (R,S)-Goitrin solution

3.3.2.3 Preparation of sample recovery solution

3 mL of stock solution was added into a 10 mL volumetric flask and diluted with methanol to the graduation point. The final concentration of the solution was (R,S)-Goitrin 142.8 μg·mL⁻¹.

3.3.2.4. Preparation of test solution

Xiao'er Qingre enema was extracted with ethyl acetate 3 times. The extract was dried through evaporation and the residue was dissolved in 5 mL methanol transferred to a 25 mL volumetric flask and diluted with 30 % methanol. The solution was filtered through 0.45 μm filter membrane and the filtrate was taken as the test solution.

3.3.3. Tests

3.3.3.1. Specificity test

Samples of the remaining ingredients of the enema other than Radix Isatidis were prepared. The negative control was prepared according to the method in Section 3.3.2.4. The samples were then analyzed using HPLC.

3.3.3.2. Precision test

The (R, S)-Goitrin reference solution (47.6 μg·ml⁻¹) was injected repeatedly for 6 times.

3.3.3.3. Linear relationship investigation

1 mL of stock solution was added into a 5 mL, 10 mL, 25 mL, 50 mL, 100 mL, and a 1000 mL volumetric flask, respectively and diluted with 30 % methanol to the graduation point. The solutions described above were injected in a 10 μL volume. The concentration of the reference substance (expressed in μg·ml⁻¹) was used as the *x*-axis variable, and the corresponding peak area values were plotted on the *y*-axis to conduct linear regression analysis. The derived regression equation is $y = 1.324x - 0.5957$, where *y* represents the peak area and *x* represents the concentration of the reference substance. The high correlation coefficient ($r = 0.9999$) indicates a strong linear relationship, and the analysis was based on a sample size of 6.

3.3.3.4. Stability test

The same test solution was injected with 10 μL at 0, 2, 4, 8, 12, and 24h, respectively, and the peak area was determined.

3.3.3.5. Repeatability test

10 bottles of Xiao'er Qingre enema were mixed together and six 10 mL samples were taken from the mixture. Xiao'er Qingre enema was extracted with ethyl acetate 3 times. The extract was dried through evaporation and the residue was dissolved in 5 mL methanol transferred to a 25 mL volumetric flask and diluted with 30% methanol. The solution was filtered through 0.45 μm filter membrane and the filtrate was taken as the test solution. The (R, S)-Goitrin content in 10 μL of the extract was determined.

3.3.3.6. Sample recovery test

Six 5 mL samples of (R, S)-Goitrin were prepared and placed in a separator funnel for further processing. A precise measurement of 1 mL of the sample recovery solution was taken, resulting in a total of six portions, each placed in an evaporation dish. Methanol was then evaporated from each portion. Subsequently, each evaporation dish was washed with 20 mL of ethyl acetate, and the resulting solutions were transferred to the corresponding spare sample-containing separator funnel for subsequent steps in the procedure.

3.3.4. Sample determination

3 batches of Xiao'er Qingre enema sample solution were prepared according to Section 3.3.2.4., and the sample was injected into the HPLC column.

4. Results

4.1. TLC identification of Radix Isatidis

All samples of the enema showed a spot with the same R_f value as the positive control. There was no interference in the negative control. The results are shown in Figure 1.

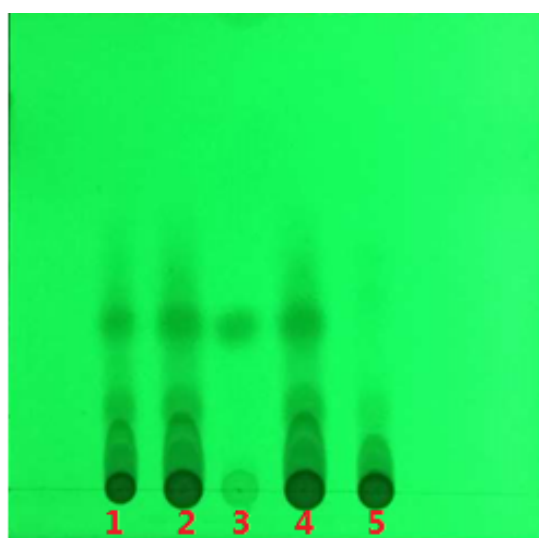


Figure 1. TLC results for Radix Isatidis. 1, 2, 4 are the enema samples, 3 is the positive control, 5 is the negative control

4.2 TLC identification of Glycyrrhiza

There were 1~2 spots of the same color in the chromatographic position corresponding to the control material. Negative solution has no interference. The results are shown in Figure 2.

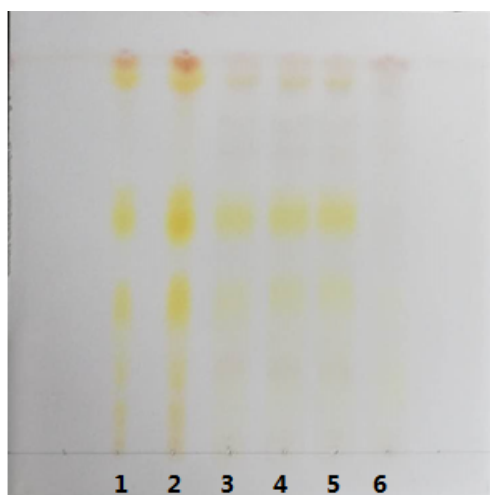


Figure 2. TLC results for Glycyrrhiza: (1) Glycyrrhiza inflata, (2) Glycyrrhiza, (3)-(5) enema samples, (6) negative control

4.3. Determination of (R,S)-Goitrin by HPLC

4.3.1. Specificity

There was no interference in the negative control, indicating that the method had good specificity. The results are shown in **Figure 3**.

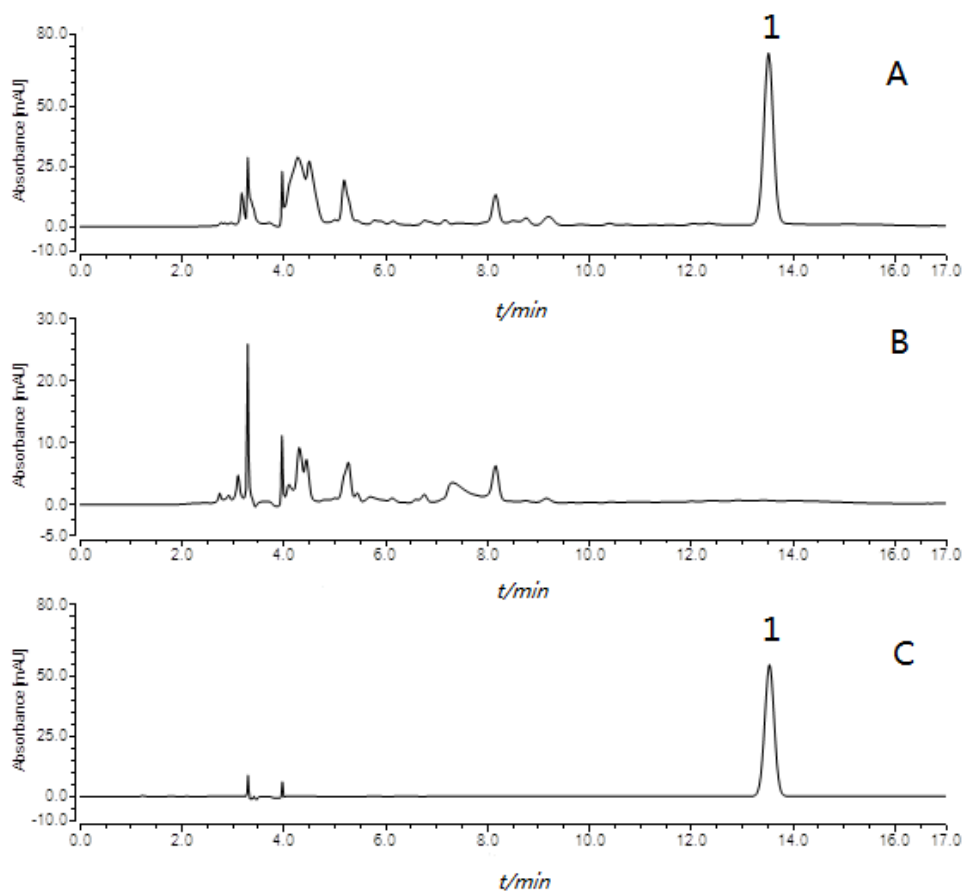


Figure 3. HPLC results: (A) Xiao'er Qingre enema sample, (B) negative control, (C) pure (R,S)-Goitrin

4.3.2. Precision

The average peak area was 61.63 and the relative standard deviation (RSD) was 0.03 %, indicating that the instrument was precise.

4.3.3. Linearity

(R, S)-Goitrin demonstrated good linearity within the range of 0.476–95.2 $\mu\text{g}\cdot\text{mL}^{-1}$.

4.3.4. Stability

The average peak area of (R,S)-Goitrin was 15.513 and, with an RSD of 0.59%, indicating that the test solution was stable within 24 h.

4.3.5. Repeatability

The average content of (R,S)-Goitrin was 29.55 $\mu\text{g}\cdot\text{mL}^{-1}$, with an RSD of 1.27 %, indicating good repeatability.

4.3.6. Sample recovery

The average recovery rate was calculated, and the results are shown in **Table 1**.

Table 1. Recovery test on (R,S)-Goitrin

Sample volume (mL)	Amount of sample (μg)	Amount added (μg)	Measured quantity (μg)	Recovery (%)	Average recovery (%)	RSD (%)
5	147.750	142.800	288.949	98.88		
5	147.750	142.800	285.420	96.41		
5	147.750	142.800	282.812	94.58	95.38	2.27
5	147.750	142.800	284.655	95.87		
5	147.750	142.800	280.263	92.80		
5	147.750	142.800	281.635	93.76		

4.3.7. Sample determination

The (R,S)-Goitrin content in 3 batches of Xiao'er Qingre enema was determined, and the results are shown in **Table 2**.

Table 2. Content determination of three batches Xiao'er Qingre enema

Batch number	(R,S)-Goitrin content ($\mu\text{g}\cdot\text{mL}^{-1}$)
210101	29.55
210102	34.35
210103	27.48

5. Discussion

5.1. TLC identification investigation

In this experiment, TLC identification was carried out on seven herbs of the whole prescription. However, only the negative controls of Radix Isatidis and Radix Glycyrrhizae had no interference, and the negative controls of the other herbs had interference. Therefore, only Radix Isatidis and Radix Glycyrrhizae were chosen as the

indicators for quality control of Xiao'er Qingre enema.

5.2. Selection of mobile phase

In the experiment, methanol-water^[5], methanol-0.02% phosphoric acid water^[6], acetonitrile-water, acetonitrile-0.02% phosphoric acid water, acetonitrile-0.1% formic acid were used as mobile phases. The results showed that acetonitrile-water (5:95) had the best separation effect, so acetonitrile-water was selected as the mobile phase.

5.3. Content determination index selection

Xiao'er Qingre enema contains Radix Isatidis, which has anti-inflammatory, antiviral and antipyretic effects^[7]. The active ingredient in Radix Isatidis that has antiviral properties is (R,S)-Goitrin^[8,9]. (R, S)-Goitrin can exert both indirect and direct antiviral effects by regulating the immune function of the body^[10]. *In vitro* studies have demonstrated the antiviral activity of (R, S)-Goitrin^[11], which operates not by directly killing the virus but by inhibiting its proliferation and adsorption^[12]. Consequently, in this study, the content of (R, S)-Goitrin served as the evaluation index for Xiao'er Qingre enema.

5.4. Selection of preparation method of test solution

In the experiment, analysis was performed using various sample sizes (5 mL, 10 mL, 20 mL), but the sample size of 10 mL showed the most moderate peaks. Additionally, the polar impurities were effectively eliminated through ethyl acetate extracted, which led to a reduction in background interference. The experimentation on the extraction times using ethyl acetate indicated that three rounds of extraction yielded the optimal results.

6. Conclusion

The TLC and HPLC methods established in this experiment are simple, reproducible, and specific, making them suitable for the quality control for Xiao'er Qingre enema preparation in hospitals.

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Disclosure statement

The authors declare no conflict of interest.

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