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Simultaneous analysis of glycyrrhizin, paeoniflorin, quercetin, ferulic acid, liquiritin, formononetin, benzoic acid and isoliquiritigenin in the Chinese proprietary medicine Xiao Yao Wan by HPLC

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Abstract

A high performance liquid chromatography coupled with photodiode-array detection method was developed for simultaneous determination of glycyrrhizin, paeoniflorin, benzoic acid, quercetin, ferulic acid, formononetin, liquiritin and isoliquiritigenin in the Chinese proprietary medicine "Xiao Yao Wan" (XYW). The analysis was performed by reverse phase gradient elution, using an aqueous mobile phase (containing 0.1% phosphoric acid) modified by acetonitrile and detection made simultaneously at four wavelengths. The method was validated for accuracy, precision and limits of detection and quantification. Ten batches of XYW obtained from different pharmaceutical companies were analyzed and found to contain different amounts of the eight bioactive markers. This method could be used for quality assessment of this herbal medicine.

Keywords: Chinese proprietary medicine; Xiao Yao Wan; Quality evaluation; HPLC-DAD

1. Introduction

Traditional Chinese medicines (TCMs) often prescribed by Chinese physicians, extensively used to prevent and cure human diseases for thousands of years attract increasing attention in many fields up to the present time. These are usually composed of several herbs each with multiple constituents. As a result, the analysis of such a complex mixture presents a great challenge to the pharmaceutical analysts. Conventional research focuses mainly on determination of active components in herbs with all kinds of chromatography and related techniques (e.g. GC, HPLC, TLC and electrophoresis) [1–6]. In recent years, chemical fingerprinting has gained increasing attention to be used for the quality control [7–9].

Xiao Yao Wan (XYW), a well-known ancient Chinese herbal formula is included in Chinese Pharmacopoeia [10] for soothing the liver and strengthening the spleen, tonifying the blood and regulating menstruation. Today, it is used widely for irregular menstruation, distension pain in the chest and hypochondria, dizziness, blurry vision and loss of appetite.

The Chinese Pharmacopoeia records the formula of XYW as follows: 100 g of Radix Paeoniae Alba, 100 g of Radix Angelicae Sinensis, 100 g of Radix Bupleuri, 80 g of Radix et Rhizoma Glycyrrhizae Preparata Cum Melle, 100 g of Rhizoma Atractylodis Macrocephalae, 100 g of Poria, 20 g of Herba Menthae and 100 g of Rhizoma Zingiberis Recens.

Currently, there are a few analytical methods available for evaluating the quality of XYW [10–12], which are only able to determine one active ingredient, even though there are several biomarkers in XYW. The current system cannot control the quality of XYW to ensure its safety and efficacy in clinical applications effectively. Therefore, this study aims at developing a new HPLC method to quantify simultaneously eight bioactive markers, i.e. glycyrrhizin, paeoniflorin, benzoic acid, quercetin, ferulic acid, formononetin, liquiritin and isoliquiritigenin (Fig. 1) thus providing a validated method for the analysis of XYW suitable for the quality control of the products manufactured by the various pharmaceutical companies. Related studies found in early literatures showed that these compounds possess significant pharmacological actions,

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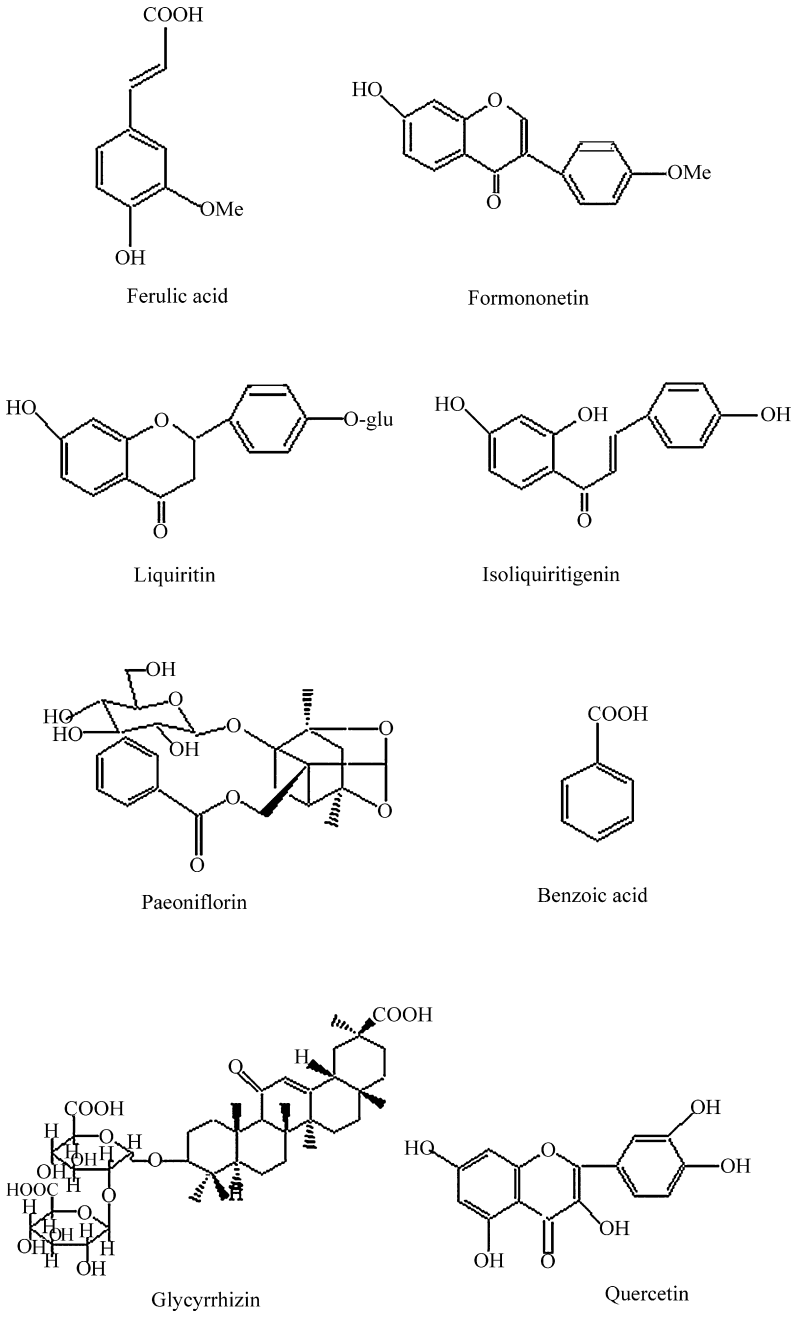

Fig. 1. Structures of bioactive markers.

especially immunomodulatory activity and antioxidant effect [13–20].

2. Experimental

2.1. Apparatus

The HPLC equipment used was Agilent HP-1100 system (Agilent, USA) including a HP-1100 quaternary pump, a degasser, a photodiode-array detector and HP ChemStation for LC 3D software. The column was a Hypersil C_{18} (250 mm × 4.6 mm i.d., 5 μm, Dalian Elite Analytical Instruments, China). SK3200LH ultrasonic cleaning instrument (Shanghai Kudos Ultrasonic Instrument Co., Shanghai, China).

2.2. Reagents and solution

Glycyrrhizin, paeoniflorin, benzoic acid, quercetin, ferulic acid and liquiritin were purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), isoliquiritigenin from Shanghai Touto Biotech Co. Ltd., and formononetin from Jianfeng Biotech Co. Ltd. (Tianjin, China). Ten batches of XYW samples were obtained from different pharmaceutical companies in China. They are abbreviated as Wx060202, Wx051022, Wx060504, Wx060323,

Wx051211, Ty051105, Jzt060110, Hw050310, Fc060108 and Tb040312. Radix Bupleuri, Radix Angelicae Sinensis, Radix Paeoniae Alba, Rhizoma Atractylodis Macrocephalae, Poria, Radix et Rhizoma Glycyrrhizae Preparata Cum Melle, Herba Menthae and Rhizoma Zingiberis Recens were purchased from a local drug store and identified by Professor Wenquan Wang. The voucher specimens were deposited in the School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, China.

Chromatographic grade acetonitrile was purchased from Fuchen Chemical Reagents Factory (Tianjin, China), and other chemicals were of analytical reagent grade. All aqueous solutions were prepared with deionized water.

2.3. Preparation of sample solutions

Twelve pills of each batch of XYW were desiccated and ground into powder. 0.25 g of the powder sample accurately weighed was transferred into a 10 ml centrifuge tube and extracted by sonication three times (3× 30 min) at room temperature, each with 8 ml methanol. After centrifuged at 3000 rpm for 5 min, the supernatant was transferred into 25 ml volumetric flask and made up to volume with methanol. Before being injected into the HPLC system, all solutions were filtered through 0.45 μm membrane filters.

2.4. Preparation of drug positive control solutions and negative control solutions

According to the formula of XYW recorded by Chinese Pharmacopoeia, positive control solutions were prepared as follows: the eight dried herbal drugs were ground into powder. After accurately weighing according to the amount in the formula, they were separately prepared as the preparing method of sample solutions.

Negative control solutions were prepared as follows: except the aimed herbal drug, XYW negative sample was prepared with other herbal drugs as the formula. The solutions were prepared as described in Section 2.3.

2.5. Preparation of standard solutions

Stock solutions of the mixture of the reference compounds were prepared by dissolving accurately weighed portions of the standards in methanol, transferring them to 5 ml volumetric flasks, and then adding methanol to make up to volume. The stock solutions were further diluted to make different concentrations. The calibration curves were prepared with at least six appropriate concentrations.

2.6. Liquid chromatographic conditions

The HPLC linear gradient profile was as follows: water (containing 0.1% phosphoric acid), acetonitrile 97:3 v/v (0–3 min), 97:3 to 82:18 (3–30 min) and 50:50% (30–60 min) at a flow-rate of 0.8 ml/min. The volume injected was 20 μl. Then the column was re-equilibrated for 30 min, using water (containing 0.1% phosphoric acid), acetonitrile 97:3 before the next injection. The separation was carried out at 35 °C. Detection was made simultaneously at four different wavelengths, i.e. 254 nm, 230 nm, 280 nm and 320 nm, at the absorption maxima of the bioactive markers.

2.7. Validation of the HPLC method

2.7.1. Limits of detection and quantification

The dilute solution of the reference compounds was further diluted to a series of concentrations with methanol to assess the limits of detection (LOD) and quantification (LOQ). The LOD and LOQ were determined as signal-to-noise (S/N) ratio of 3 and 10, respectively.

2.7.2. Precision, reproducibility and accuracy

The intra- and inter-day precision was determined by analyzing calibration samples during a single day and on three consecutive days, respectively. To confirm the reproducibility, five different working solutions prepared from the Wx060202 were analyzed. The R.S.D. was taken as a measure of precision and reproducibility. Recovery test was used to evaluate the accuracy of this method. Accurate amounts of reference

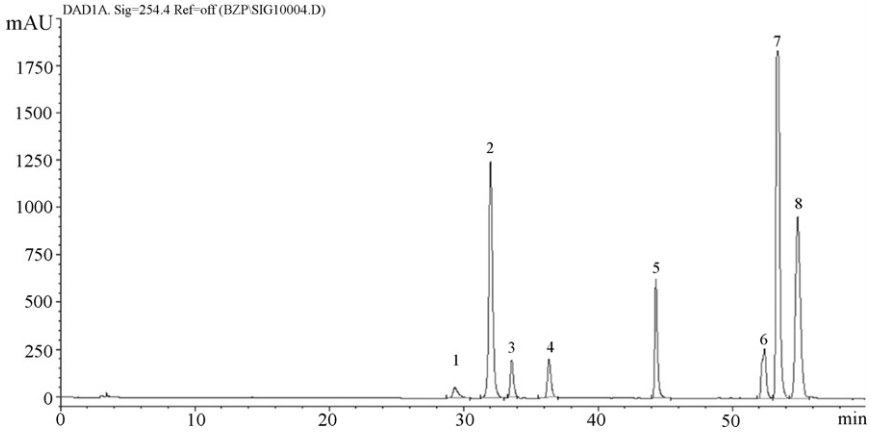

Fig. 2. Chromatogram of eight mixed bioactive markers at 254 nm: (1) paeoniflorin; (2) ferulic acid; (3) liquiritin; (4) benzoic acid; (5) quercetin; (6) formononetin; (7) isoliquiritigenin; (8) glycyrrhizin. Gradient program of acetonitrile: 3% (0 min)–3% (3 min)–18% (30 min)–50% (60 min).

Table 1
Regression data, LODs and LOQs for the eight bioactive markers in HPLC

Analyte	Calibration curve	r^2	Linear range (μg/ml)	LOD (ng)	LOQ (ng)
Glycyrrhizin	$Y = 6.4066X - 0.6488$	0.9992	0.080–0.800	5.6	15.1
Paeoniflorin	$Y = 32.226X - 101.71$	0.9996	0.060–0.600	2.9	7.2
Quercetin	$Y = 105.752X + 4.496$	0.9996	0.010–0.060	1.7	5.3
Liquiritin	$Y = 38.938X + 90.982$	0.9994	0.100–0.800	2.8	7.6
Ferulic acid	$Y = 82.068X - 3.754$	0.9995	0.010–0.100	1.4	6.1
Isoliquiritigenin	$Y = 91.582X - 9.913$	0.9986	0.010–0.080	1.8	5.8
Formononetin	$Y = 292.76X + 16.59$	0.9983	0.004–0.020	1.2	2.9
Benzoic acid	$Y = 160.986X + 43.283$	0.9991	0.020–0.180	2.3	6.4

Table 2
Precision and reproducibility of the eight bioactive markers

Analyte	Precision				Reproducibility ($n = 5$)	
	Intra-day ($n = 3$)		Inter-day ($n = 3$)		Mean (mg/g)	R.S.D. (%)
	Mean (mg/g)	R.S.D. (%)	Mean (mg/g)	R.S.D. (%)		
Glycyrrhizin	11.481	1.90	11.592	1.00	11.472	1.50
Paeoniflorin	3.191	1.40	3.230	0.20	3.254	1.71
Quercetin	0.812	2.12	0.819	1.62	0.803	1.85
Liquiritin	2.118	0.87	2.121	0.48	2.126	0.92
Ferulic acid	0.025	1.35	0.026	1.19	0.026	2.03
Isoliquiritigenin	0.089	1.72	0.091	1.03	0.092	2.54
Formononetin	0.008	1.21	0.008	1.34	0.008	0.61
Benzoic acid	0.583	1.34	0.591	0.96	0.574	2.67

compounds were added to 1# sample, and then extracted and analyzed as described in Sections 2.3 and 2.6. The average recoveries were estimated by the formula: recovery (%) = (amount found − original amount) / amount added × 100%, and R.S.D. (%) = (S.D./mean) × 100%.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

HPLC parameters were optimized by investigating the influence of the mobile phase and detection wavelength on resolution and sensitivity. The initial separation of the XYW extracts was carried out on a Hypersil C_{18} column using mixtures of acetonitrile and water as the mobile phase. Good separation was not achieved although a gradient elution method was employed. Considering the presence of acidic ingredients in the herbal extraction, a small amount of H_3PO_4 was added to the mobile phase in order to suppress the ionization of these compounds. The optimum mobile phase was achieved with an aqueous phase (containing 0.1% phosphoric acid) with different amounts of acetonitrile in the gradient elution mode. The use of a buffer system for sodium dihydrogen phosphate was also attempted but did not give better separation.

In order to analyze multiple components on the HPLC chromatogram, the spectra of all peaks in the chromatogram of XYW were investigated with photodiode-array detection. 254 nm (glycyrrhizin, quercetin and formononetin), 230 nm (paeoniflorin and benzoic acid), 320 nm (ferulic acid and isoliquiritigenin), 280 nm (liquiritin) were selected as detection wavelength for determination (Fig. 2).

Table 3
Recovery of the eight bioactive markers

Analyte	Added (mg)	Recorded mean (mg)	Recovery mean (%)	R.S.D. (%) ($n = 5$)
Glycyrrhizin	0.893	0.909	101.8	2.08
Paeoniflorin	0.542	0.535	98.7	1.05
Quercetin	0.182	0.186	102.3	2.37
Liquiritin	0.368	0.371	100.8	0.98
Ferulic acid	0.025	0.024	95.3	2.81
Isoliquiritigenin	0.031	0.030	97.2	1.73
Formononetin	0.017	0.018	103.9	4.04
Benzoic acid	0.071	0.069	96.8	1.78

Table 4
The mean contents of bioactive markers in XYW samples ($n = 3$)

Contents (mg/g)	Wx 060202	Wx 051022	Wx 060504	Wx 060323	Wx 051211	Ty 051105	Jzt 060110	Hw 050310	Fc 060108	Tb 040312
Glycyrrhizin	11.471	10.342	10.343	10.870	11.075	10.177	12.564	4.401	12.282	8.468
Paeoniflorin	3.254	3.693	2.905	3.060	3.745	4.397	3.109	2.943	3.716	3.047
Quercetin	0.801	0.623	0.987	1.110	0.972	1.725	0.694	0.779	0.376	0.351
Liquiritin	2.126	1.873	1.521	1.864	2.070	1.671	1.271	0.486	1.470	1.258
Ferulic acid	0.026	0.032	0.020	0.026	0.028	0.133	0.101	0.056	0.093	0.082
Isoliquiritigenin	0.092	0.125	0.129	0.100	0.086	0.119	0.064	0.093	0.039	0.066
Formononetin	0.008	0.008	0.008	0.008	0.007	0.008	0.018	0.007	0.009	0.013
Benzoic acid	0.572	0.206	0.793	0.620	0.304	0.155	0.238	0.186	0.682	0.615

3.2. Determination of the bioactive markers in XYW

3.2.1. Method validation for quantitative determination of the bioactive markers

The biomarkers in XYW were identified by comparing the retention times and the UV spectra with those of the reference standards. Furthermore, the HPLC profiles of the different positive control solutions and negative control solutions (solutions without an individual herb in the preparation) indicated that there were no interferences for the biomarkers.

For determination of the bioactive markers, a calibration curve for each marker was constructed and tested for linearity. Their regression equations were calculated in the form of $Y = AX + B$, where X and Y (μg/ml) were peak area and compound concentration injected, respectively. The results and the LOD and LOQ for each compound are shown in Table 1. The results of precision, reproducibility and accuracy test are shown in Tables 2 and 3. The retention times of glycyrrhizin, paeoniflorin, quercetin, benzoic acid, ferulic acid, liquiritin, formononetin and isoliquiritigenin were 55.45 ± 0.31, 29.24 ± 0.11, 44.48 ± 0.20, 36.94 ± 0.13, 32.10 ± 0.10, 33.63 ± 0.18, 52.37 ± 0.32 and 53.48 ± 0.38 min, R.S.D. < 0.8% ($n = 6$).

3.2.2. Sample analysis

This method was applied to the simultaneous determination of the eight bioactive markers in 10 batches of XYW preparations. The assay results are listed in Table 4.

According to the Chinese Pharmacopoeia, the content of paeoniflorin in XYW is required to be more than 2.5 mg/g. This criterion is met in all samples. However, there was a wide variation in the content of the eight bioactive markers in XYW products from different companies (Table 4). Compared with other samples, the content of glycyrrhizin in Hw050310 and Tb040312 samples was considerably lower. Even in the five batches produced by the same company the contents of the eight bioactive markers were also different appreciably. It is therefore, questionable whether all the herbal products will give rise to the same efficacy. Currently, there are no limits imposed on glycyrrhizin, benzoic acid, quercetin, ferulic acid, formononetin, liquiritin and isoliquiritigenin or other active ingredients in Chinese Pharmacopoeia. However, this should be implemented in the future to ensure the quality and safety of the XYW preparations.

Although the formula of 10 batches of XYW is the same, the process of preparation is different. The preparing process of sample Hw050310 is as follows: according to the formula in

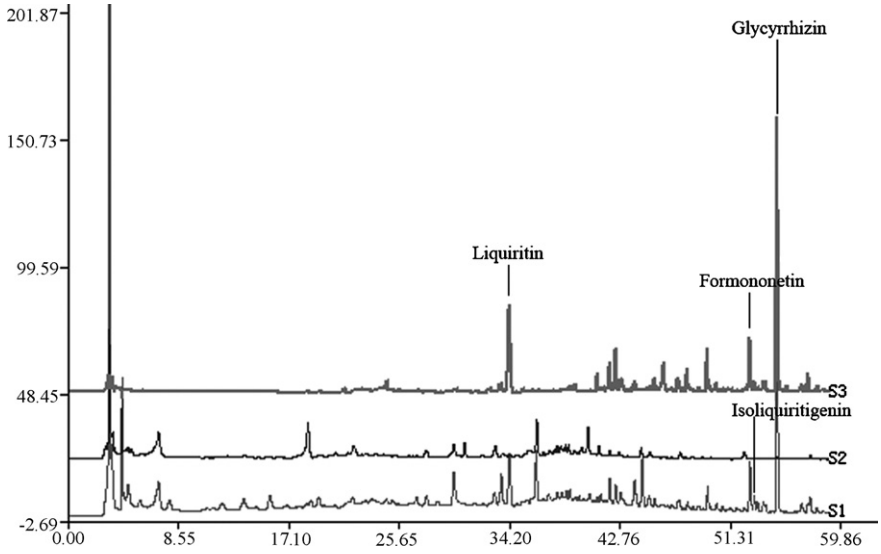

Fig. 3. Chromatogram of XYW (S1), negative control solutions (S2) and positive control solutions of Radix et Rhizoma Glycyrrhizae Preparata Cum Melle (S3) at 254 nm. Gradient program of acetonitrile: 3% (0 min)–3% (3 min)–18% (30 min)–50% (60 min).

Chinese Pharmacopoeia, all the herbs were ground and blended except Rhizoma Zingider Recens, and the prepared powders were mixed thoroughly with the water extract of Rhizoma Zingideris Recens to produce the pills. The preparing process of the other samples is different: Radix Bupleuri, Rhizoma Zingiberis Recens, Herba Menthae and 50 g of Radix Angelicae Sinensis were extracted to obtain volatile oil 1. The residue, Rhizoma Atractylodis Macrocephalae and Poria were extracted with water by boiling to obtain extract 2. Twenty grams of Radix et Rhizoma Glycyrrhizae Preparata Cum Melle, Radix Paeoniae Alba and 50 g of Radix Angelicae Sinensis were ground and blended to obtain powder 3. Sixty grams of Radix et Rhizoma Glycyrrhizae Preparata Cum Melle was extracted with water to obtain extract 4. Finally, the volatile oil 1, the extracts 2, 4 and the powder 3 were mixed to produce the pills. The specifications of this XYW pill indicated that eight pills are equivalent to 3 g of herbal drugs. Compared with the first preparing process, using the second method the content of some biomarkers in XYW was improved.

In addition to the process of preparation different growth environment of the medical plant can also influence the content of the bioactive markers in the finished product. In order to ensure the consistency of the crude herbs, many companies have adopted the good agricultural practice (GAP) for traditional Chinese medicine.

3.2.3. Adscription of the bioactive markers

In order to identify the origins of these bioactive markers from each herb, a comparative study was carried out with and without certain kind(s) of herb(s) by using drug positive control solutions and negative control solutions (Section 2.4).

For example, as shown in Fig. 3, the peaks of liquiritin, glycyrrhizin, formononetin and isoliquiritigenin were attributed to Radix et Rhizoma Glycyrrhizae Preparata Cum Melle; and not observed in the negative control solutions, indicating that these components were contributed by the herb Radix et Rhizoma Glycyrrhizae Preparata Cum Melle. Furthermore, it was also shown that there was no interference from the concomitant herbs in the course of the determination of these bioactive markers. Accordingly, paeoniflorin and benzoic acid originate from Radix Paeoniae Alba, ferulic acid from Radix Angelicae Sinensis and quercetin from Radix Bupleuri.

Polysaccharides, glycolipids, oleoresin and essential oil which possess weak UV absorption characters, are the main bioactive constituents of Poria [21], Rhizoma Atractylodis Macrocephalae [22], Herba Menthae and Rhizoma Zingiberis Recens [23] resulting in difficulties in their detection by HPLC-UV. GC–MS [22] and LC–MS [24] were successfully used for the qualification of these herbs.

4. Conclusion

A HPLC method was developed for simultaneous determination of glycyrrhizin, paeoniflorin, benzoic acid, quercetin, ferulic acid, formononetin, liquiritin and isoliquiritigenin to evaluate the quality of XYW. The method has been validated and can be used with reasonable confidence, for both identification and quantification of the eight components found in XYW. Ten batches of samples from different pharmaceutical companies were analyzed. The amount of different bioactive markers varied considerably indicating differences in the quality of these products. This raised the question of whether the various XYWs would exhibit equivalent efficacy. The proposed HPLC method can be used to identify and assess the XYW preparations and applied for quality control of this TCM.

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