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Quality Grade Evaluation of Niuhuang Qingwei Pills Based on UPLC and TCM Reference Drug—A Novel Principle of Analysis of Multiple Components in Ready-Made Chinese Herbal Medicine

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Abstract: Ready-made Chinese herbal medicine (RMCHM) is one of the most common types of synergistic herbal medicine used worldwide. It is based on composite herbal formulae (CHF), which makes quality control of this kind of traditional Chinese medicine (TCM) difficult, let alone distinguishing the good from the bad. Taking Niuhuang Qingwei Pills (NHQWP) as an example, this study reported the development of a novel principle of analysis of multiple components in RMCHM. Experimental procedures involved the selection of high-quality Chinese materia medica (CMM, individual medicinal plant parts used in the NHQWP) to prepare three batches of TCM reference drugs (TCMRD). Pure compounds of the active ingredients identified in the herbal formula including berberine hydrochloride, geniposide, forsythiaside A, 3,5-*O*-dicaffeoyl quinic acid, hesperidin, baicalin, glycyrrhizic acid, and chrysophanol in the three TCMRDs were analyzed as well as those in 49 batches of commercial products from 18 manufacturers by ultra-performance liquid chromatography (UPLC) method combined with wavelength switching. Using the TCMRD as the scientific ruler, quality grade specifications of NHQWP were proposed by comprehensive analysis of multiple components. Accordingly, 13, 28, and 8 batches of samples were primarily rated as first-grade, second-grade, and unqualified, respectively.

Keywords: ready-made Chinese herbal medicine (RMCHM); quality grade evaluation; traditional Chinese medicine reference drug (TCMRD); Niuhuang Qingwei Pills (NHQWP); ultra-performance liquid chromatography (UPLC); composite herbal formulae (CHF)

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1. Introduction

Ready-made Chinese herbal medicines (RMCHM) are frequently used in traditional Chinese medicine (TCM) treatment in China and some regions worldwide [1,2]. They are prescribed in the form of a combination of two or more processed-CMM known as decoction pieces based on well-used composite herbal formulae (CHF). Unlike pharmaceutical drugs, quality of RMCHM may differ due to the uneven quality of the initially used processed-CMM and the varied quality control ability of the manufacturers involved. Some herbal industries practice with undisclosed manufacturing procedures, such as providing lower amount of expensive CMM than prescribed, using low quality raw materials, regardless of possible prohibition. The mandatory standards are those specified in the pharmacopoeia

with the minimum requirements needed to be met. The authenticity of the finished products is not difficult to achieve due to the advances of chemical analysis, when assessed as described in the pharmacopeia procedures. However, it is not easy to distinguish the good from the bad finished products. Developing the quality grade evaluation system is a powerful means to solve the problem of ragged quality of TCM products. Currently, the study on quality grade has become a leading hotspot and new field for investigations on TCM herbal medications [3–7], which needs more innovative methods and more systemic and profound work to provide reasonable theory and a workable application.

A typical RMCHM is usually composed of several herbal decoction pieces containing numerous chemical components. Therefore, it is very difficult to effectively control the quality and to distinguish the good from the bad. Under this situation, the TCM reference drug (TCMRD) was proposed by the authors as the TCM standard formula for comparison [8], which shows broad application prospects in quality evaluation and control of RMCHM. TCMRD refers to the physical reference substance prepared with authenticated and standardized decoction pieces, and excipients. The reference drug was prepared in strict accordance with the statutory procedure and manufacturing process, and in compliance with good manufacturing practice (GMP). It is mainly used for the quality control of RMCHM to evaluate the authenticity of dosing (the correct raw material included) and the reliability of the dosing quantity (whether it is fully dosed according to the composite formula). The RMCHM is a synergistic herbal formula, and as such the different chemical ingredients included may affect each other during the preparation process and may change the chemical composition of final product. TCMRD is formulated according to the same herbal composition and prepared procedure accordingly, which can provide key information including the actual background and the transfer rates of constitutes from raw materials to preparations. The TCMRD can be applied as the measurement scale in assessment of the RMCHM products.

Niuhuang Qingwei Pills (NHQWP) is a typical RMCHM used to treat stomach heat, mouth and tongue sores, swelling and painful gums, and sore throat; these are listed in the Chinese proprietary medicine (Zhong Cheng Yao) approved by the Ministry of Public Health [9]. It is a Chinese herbal formula consisting of 17 CMMs (See Table 1). NHQWP has been reported to promote gastrointestinal motility, decrease gastric acid volume and pepsin activity, and show a mild analgesia effect, which are consistent with its clinic indications [10]. It is administrated orally at a dosage of two pills per administration and two times a day. Studies focusing on qualitative and quantitative analysis of NHQWP using high-performance liquid chromatography (HPLC) [11–13], high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS-MS) [14], gas chromatography–tandem mass spectrometry (GC-MS-MS) [15], and near infrared spectroscopy (NIRS) [16] have been reported. The reference drug, TCMRD, prepared by the authors [17], was tested to detect if heavy metals and harmful elements [18], and ^{60}Co-γ irradiation [19], were present; the identification of whole-formula was carried out [20] by fingerprint analysis [21]. The respective assays of Gypsum Fibrosum [22], Borneolum Syntheticum [23], and Phellodendri Chinensis Cortex [24] in the formula were also carried out. However, no investigation has been conducted on simultaneous determination of multi-components for quality grade assessment of the commercial RMCHM.

In this study, a simultaneous analysis for comprehensive quality evaluation of seven CMMs (Gardeniae Fructus, Forsythiae Fructus, Chrysanthemi Flos, Aurantii Immaturus Fructus, Scutellariae Radix, Glycyrrhizae Radix et Rhizoma and Rhei Radix et Rhizoma) in the NHQWP using seven markers, berberine hydrochloride, geniposide, forsythiaside A, 3,5-*O*-dicaffeoyl quinic acid, hesperidin, baicalin, glycyrrhizic acid, and chrysophanol, was developed using ultra-performance liquid chromatography (UPLC) coupled with wavelength switching, which was also used to assay three batches of TCMRDs for NHQWP and 49 batches of commercial products from 18 manufacturers. Based on comprehensive analysis of the results, the quality grade specifications of NHQWP were proposed and the samples were rated accordingly to distinguish good and bad products. The integrated

strategy of multi-component analysis by UPLC and TCM Reference Drug to evaluate quality grade of NHQWP was demonstrated in Figure 1.

Table 1. Formulation information of Niuhuang Qingwei Pills.

No.	Ingredient	Ratio	No.	Ingredient	Ratio
1	Bovis Calculus Artifactus	2	10	Forsythiae Fructus	100
2	Scutellariae Radix	100	11	Gypsum Fibrosum	150
3	Rhei Radix et Rhizoma	100	12	Pharbitidis Semen	50
4	Glycyrrhizae Radix et Rhizoma	100	13	Gardeniae Fructus	100
5	Chrysanthemi Flos	150	14	Aurantii Immaturus Fructus	100
6	Platycodonis Radix	100	15	Scrophulariae Radix	100
7	Ophiopogonis Radix	50	16	Borneolum Syntheticum	25
8	Phellodendrl Chinensis Cortex	100	17	Sennae Folium	200
9	Menthae Haplocalycis Herba	50			

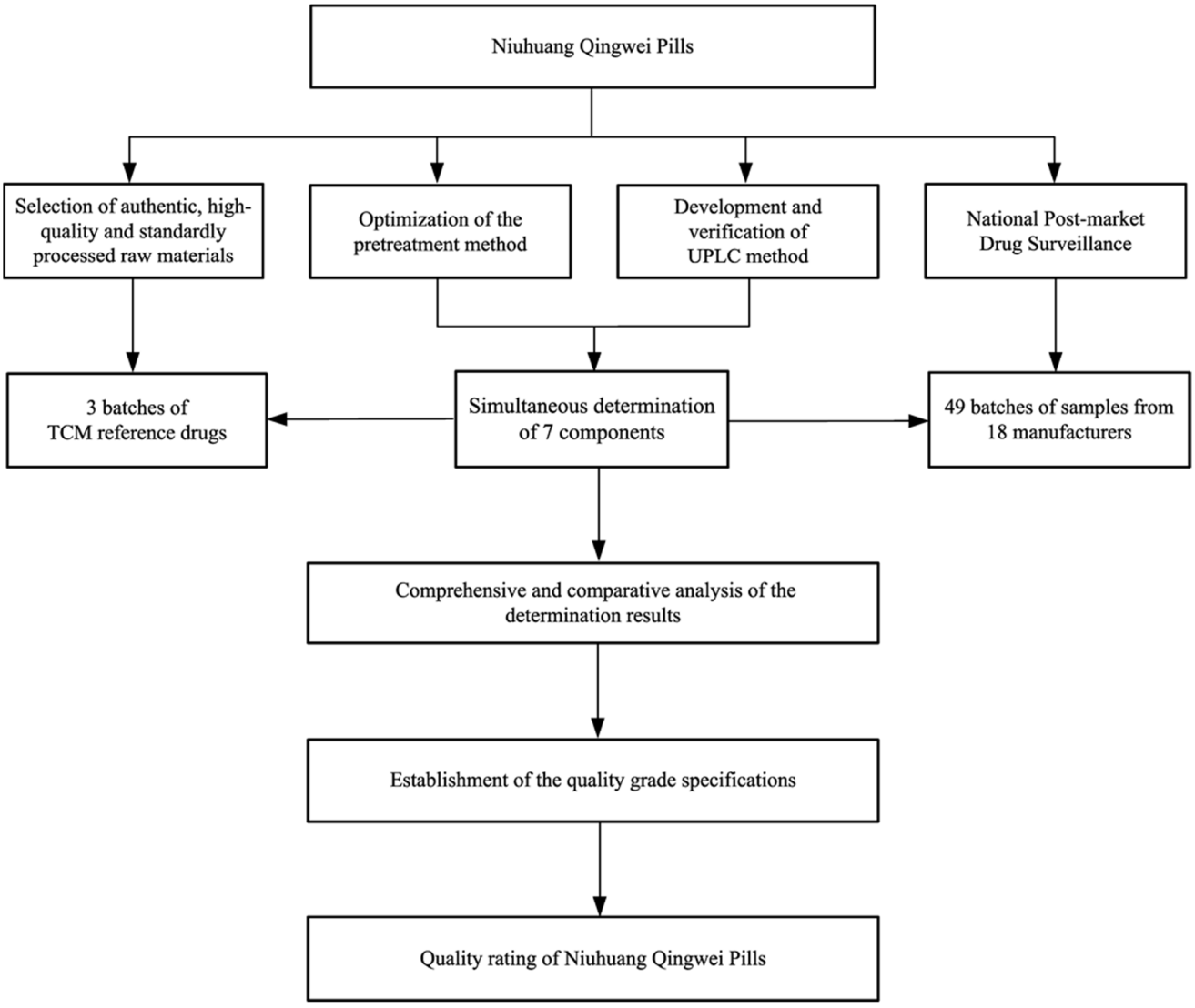

Figure 1. Flow diagram illustrating the overall procedures for quality grade evaluation of Niuhuang Qingwei Pills.

2. Materials and Methods

2.1. Materials

Forty-nine batches of Niuhuang Qingwei Pills (NHQWP) from 18 manufacturers were collected via National Post-market Drug Surveillance. All the samples were produced according to the statutory procedure and manufacturing process [9] and were authorized by their corresponding manufacturers. Respectively, 3 batches of authentic, high-quality and standardly processed CMMs including Bovis Calculus Artifactus, Scutellariae Radix, Rhei Radix et Rhizoma, Glycyrrhizae Radix et Rhizoma, Chrysanthemi Flos, Platycodonis Radix, Ophiopogonis Radix, Phellodendrl Chinensis Cortex, Menthae Haplocalycis Herba, Forsythiae Fructus, Gypsum Fibrosum, Pharbitidis Semen, Gardeniae Fructus, Aurantii Immaturus Fructus, Scrophulariae Radix, Borneolum Syntheticum, and Sennae Folium were bought from 3 different suppliers of CMMs,(Beijing Tongrentang Co., Ltd., Beijing, China, Sichuan Neautus Traditional Chinese Medicine Co., Ltd., Chengdu, China and Sinopharm Group Beijing HuaMiao Pharmaceutical Co., Ltd., Beijing, China). The origin of each CMM was authenticated by Associate Professor Shuai Kang using macroscopy and microscopy according to the Chinese Pharmacopoeia, edition 2020 (ChP 2020). The results of chemical analysis performed by the authors indicated that all the raw materials met their own national drug standards, including that of ChP 2020. For future reference, the specimens were deposited at the Traditional Chinese Medicine Herbarium, National Institutes of Food and Drug Control (Beijing, China). Refined honey, the excipient of the big honeyed pills, was kindly offered by Beijing Tongrentang Co., Ltd. (Beijing, China). Then, 3 batches of TCM reference drugs (TCMRDs) for NHQWP were prepared using the above-mentioned raw materials and excipient in strict accordance with the statutory procedure and manufacturing process, and in compliance with good manufacturing practice (GMP). The primary procedure was as follows [9]: Scutellariae Radix, Rhei Radix et Rhizoma, Glycyrrhizae Radix et Rhizoma, Chrysanthemi Flos, Platycodonis Radix, Ophiopogonis Radix, Phellodendrl Chinensis Cortex, Menthae Haplocalycis Herba, Forsythiae Fructus, Gypsum Fibrosum, Pharbitidis Semen, Gardeniae Fructus, Aurantii Immaturus Fructus, Scrophulariae Radix, and Sennae Folium were pulverized to fine powder. Bovis Calculus Artifactus and Borneolum Syntheticum were triturated with the abovementioned powder. Subsequently, all the raw materials were sifted and mixed well. Big honeyed pills were created by adding 100 g of refined honey to each 100 g of the mixed powder. For specificity validation, negative control without Gardeniae Fructus, Forsythiae Fructus, Chrysanthemi Flos, Aurantii Immaturus Fructus, Scutellariae Radix, Glycyrrhizae Radix et Rhizoma, and Rhei Radix et Rhizoma was prepared by the same method with appropriate amounts of the rest of the herbal materials and the refined honey according to the formulation of NHQWP.

2.2. Chemicals and Reagents

Geniposide (110749-201718), forsythiaside A (111810-201707), 3,5-*O*-dicaffeoyl quinic acid 111782-201807), hesperidin (110721-201818), baicalin (110715-201821), and ammonium glycyrrhizinate (110731-201720) were from National Institutes for Food and Drug Control. Chrysophanol (RS01391020) was obtained from Shanghai Standard Technology Co., Ltd (Shanghai, China). They were all used as reference standards. HPLC grade methanol, acetonitrile, and formic acid were purchased from Merck (Darmstadt, Germany). De-ionized water was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Preparation of Standard Solutions

Stock solutions of reference standards for each marker components were prepared at a concentration of 1 mg/mL with 70% (v/v) methanol. Then, appropriate volumes of the stock solutions were measure accurately and diluted with 70% (v/v) methanol to produce a series of mixed working standard solutions at five concentration levels (seen Table 2).

Table 2. Concentration levels of working standard solutions for multi-component determination of Niuhuang Qingwei Pills.

Analyte	Working Standard				
	1	2	3	4	5
Geniposide	0.02	0.04	0.06	0.08	0.1
Forsythiaside A	0.02	0.04	0.06	0.08	0.1
3,5-*O*-dicaffeoyl quinic acid	0.01	0.02	0.04	0.06	0.08
Hesperidin	0.008	0.02	0.04	0.25	0.5
Baicalin	0.06	0.12	0.18	0.24	0.3
Glycyrrhizic acid [1]	0.01	0.02	0.03	0.04	0.05
Chrysophanol	0.001	0.002	0.004	0.006	0.008

[1] The weight of glycyrrhizic acid = the weight of ammonium glycyrrhizinate/1.0207.

2.4. Preparation of Solutions of Sample, Reference Drugs, Negative Control, and Chinese Materia Medicas

NHQWP samples and NHQWP reference drugs obtained under the weight variation test were cut into pieces. One g of each piece was accurately weighed and transferred into a conical flask, into which 50 mL of methanol was accurately added. The conical flask and the whole mixture was weighed, then was heated under reflux at 65 °C for 4 h. The content was then allowed to cool to ambient temperature. The flask was weighed again and the lost weight was replenished with methanol. The mixture then filtered through a 0.22 μm filter and the filtrate was subjected for further analysis. Then negative control solution, containing no active ingredients for specificity validation, was prepared in the same manner as the sample solution. To determine the chemical markers' transfer rates from raw materials to the finished products of reference drugs, respectively 3 batches of Gardeniae Fructus, Forsythiae Fructu, Chrysanthemi Flos, Aurantii Immaturus Fructus, Scutellariae Radix, Glycyrrhizae Radix et Rhizoma, and Rhei Radix et Rhizoma were pulverized to fine powder and processed with the same method mentioned above to prepare the solutions of herbal materials.

2.5. Instrument and Operating Conditions

The analysis of all herbal sample extracts and filtrates was performed using a Waters ACQUITY UPLC™ system (Waters Co., Milford, MA, USA) equipped with a binary solvent manager, a sample manager, a column compartment, and a photo diode array (PDA) detector. The software Empower was used for data acquisition. The separation was performed on a Waters Acquity UPLC™ BEH C_{18} column (100 mm × 2.1 mm, 1.8 μm) using a linear gradient elution of 0.5% formic acid in acetonitrile (A) and 0.5% (*v*/*v*) formic acid in water (B): 0–10 min, 5%–15% (*v*/*v*) A; 10–20 min, 15% (*v*/*v*) A; 20–25 min, 15%–20% (*v*/*v*) A; 25–30 min, 20% (*v*/*v*) A; 30–35 min, 20%–25% (*v*/*v*) A; 35–50 min, 25%–80% (*v*/*v*) A, at a flow rate of 0.2 mL/min. The column temperature was set at 40 °C and the injection volume was 2 μL. Multi-wavelength scanning was applied and the detection wavelengths were set at 238 nm for geniposide, 330 nm for forsythiaside A, 327 nm for 3,5-*O*-dicaffeoyl quinic acid, 284 nm for hesperidin, 276 nm for baicalin, 251 nm for glycyrrhizic acid, and 258 nm for chrysophanol, respectively. Three injections were performed for each solution.

2.6. Method Validation

The method was validated for specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy (recovery), and precision as per the recommendations laid down by International Conference on Harmonization (ICH) guideline [25]. The specificity of the method was assessed by comparing the UPLC chromatograms obtained from NHQWP and the negative control. The linearity was determined by the establishment of the calibration curves at seven concentration levels. The obtained peak areas and the concentrations of all markers were subjected to least-squares regression to calculate the calibration equation and correlation coefficient (*r*). LOD and LOQ were separately determined

at the signal-to-noise ratio (S/N) values of 3 and 10, respectively. To confirm the precision, the variations of the mixed standard solutions were determined. For the intra-day test, the solution were examined for six replicates within a single day, while for the inter-day test, the solutions were analyzed for 3 consecutive days. For the repeatability test, six test solutions were prepared from the same sample and analyzed. Accuracy was determined using a recovery test by the standard addition method. Corresponding amounts of each reference standards were added to 0.5 g of the sample pieces. The mixture was extracted and analyzed by the proposed procedure. Six replicates were performed.

3. Results and Discussion

3.1. Development of the UPLC Method

To achieve simultaneous determination of seven analytes with better resolution and shorter duration, the UPLC chromatographic conditions were optimized. Ultraviolet (UV) spectra of the chemical markers in solutions of reference standards and samples were scanned. The maximum absorption for the seven markers for analyses (geniposide, forsythiaside A, 3,5-*O*-dicaffeoyl quinic acid, hesperidin, baicalin, glycyrrhizic acid, and chrysophanol) were set at 238 nm, 330 nm, 327 nm, 284 nm, 276 nm, 251 nm, and 258 nm respectively, based on which the multi-wavelength switching was programmed. A Waters Acquity UPLC™ BEH C18 column, the most used reversed phase C_{18} column with wide adaptability, was found to have satisfactory separation and peak capacity. The concentration of formic acid in the mobile phase was optimized. A series of mobile phases including acetonitrile–water, acetonitrile-0.2% (v/v) formic acid in water, acetonitrile-0.5% (v/v) formic acid in water, and 0.5% (v/v) formic acid in acetonitrile-0.5% (v/v) formic acid in water were examined. The results indicated that 0.5% (v/v) formic acid in acetonitrile-0.5% (v/v) formic acid in water could achieve better resolution than other systems, especially for the peak of baicalin. Additionally, different gradient profiles were applied to improve the separation of NHQWP by varying the ratio of the mobile phase during the elution process and the optimum gradient was finally picked out through numerous empirical attempts. The flow rate is also crucial for achieving optimal separation. A decrease in the flow rate achieved better resolution, but longer analysis duration at the same time. The best resolution with a lower retention time was obtained at the flow rate of 0.2 mL/min. The column temperature is another important parameter to be controlled. An increase of the column temperature improved the resolution, until an inflection point appeared at 45 °C. Based on the results, 40 °C was selected as the optimal column temperature value. Representative chromatograms of each of the seven reference standards and a typical sample under different wavelengths are shown in Figure 1. The analytes were identified by comparing their retention time and UV spectra with those of each reference standards. Besides, spiked sample with reference standards showed no additional peaks, which further confirmed the identities of the peaks.

3.2. Optimization of the Pretreatment Method

In order to improve the sensitivity and reliability of the method, concentrations of the analytes were used as criteria to optimize the pretreatment parameters including the extracting solvent, the extracting method, the extracting time, and the volume of the extracting solvent. Different ratios of methanol in the range of 50%–100% (v/v) were added to water as the extracting solutions. The results indicated that the extraction efficiencies of hesperidin, baicalin, and chrysophanol increased with increasing amount of methanol while those of geniposide, forsythiaside A, 3,5-*O*-dicaffeoyl quinic acid, and glycyrrhizic acid varied slightly in 50% (v/v) methanol, 75% (v/v) methanol, and 100% (v/v) methanol. Therefore, methanol was selected as the extracting solvent for the further experiment. To investigate the effect of the extracting method on the extraction efficiency, the powdered NHQWP was ultrasonicated (300 W, 40 kHz) then heated under reflux at 65 °C for 1 h, respectively. It was demonstrated that the extraction efficiencies of seven analytes were much higher when heated under reflux. The refluxing time was optimized in the range of 1–6 h.

When the time exceeded 4 h, the contents of all markers reached the dynamic equilibrium. Hence, an extraction time of 4 h was considered sufficient. Figure 2 summarized the UPLC finger prints of the seven key markers of the extracts from the NHQWP samples after going through all the analytical procedures.

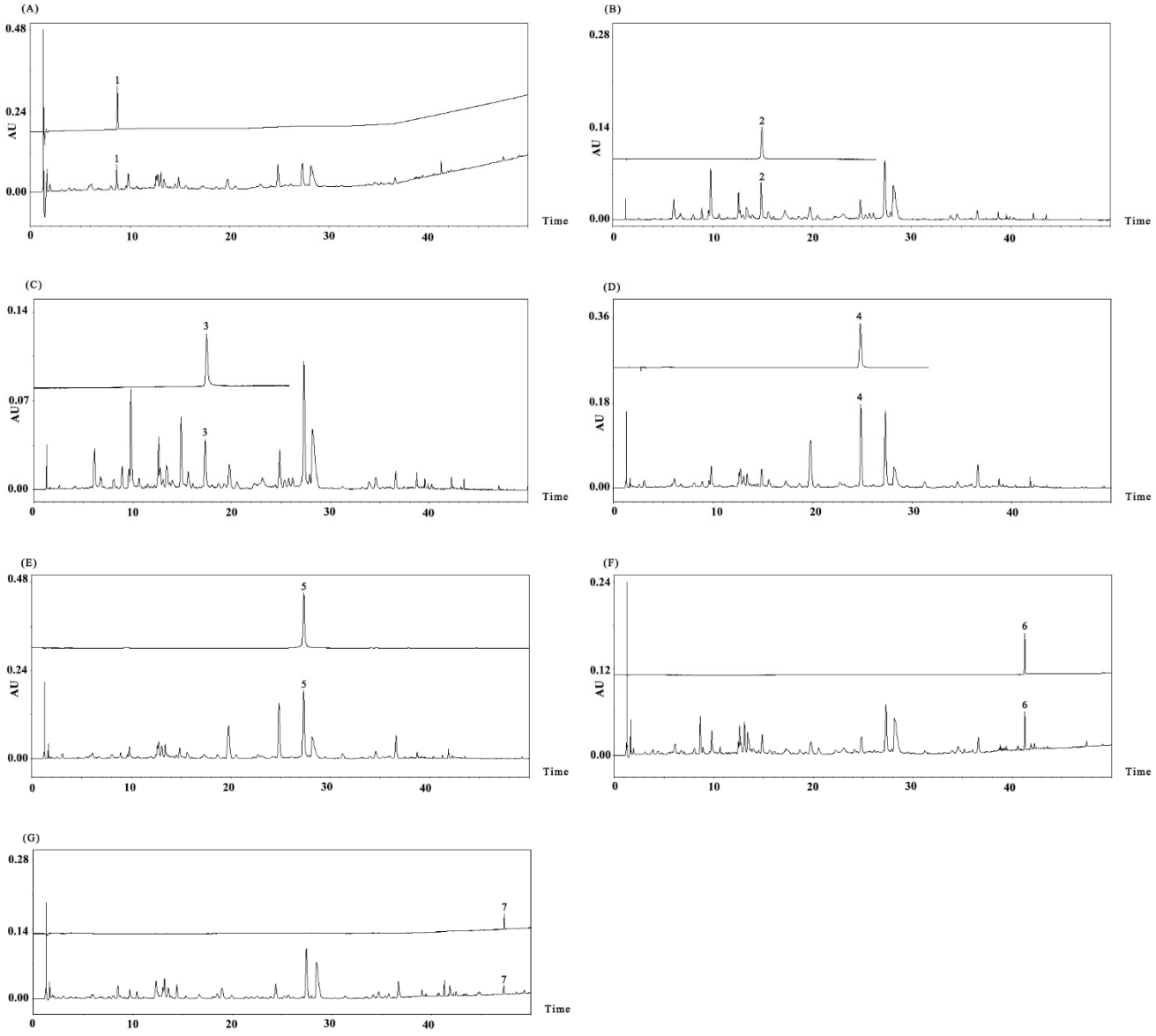

Figure 2. Chromatograms of the reference solutions of geniposide (1), forsythiaside A (2), 3,5-*O*-dicaffeoyl quinic acid (3), hesperidin (4), baicalin (5), glycyrrhizic acid (6), and chrysophanol (7) and the sample solution of NHQWP (s) at 238 nm (**A**), 330 nm (**B**), 327 nm (**C**), 284 nm (**D**), 276 nm (**E**), 251 nm (**F**), and 258 nm (**G**), respectively.

3.3. Verification of the UPLC Method

Specificity of the analytical method was confirmed by comparing the UPLC chromatograms obtained from solutions of the NHQWP sample and the negative control without Gardeniae Fructus, Forsythiae Fructus, Chrysanthemi Flos, Aurantii Immaturus Fructus, Scutellariae Radix, Glycyrrhizae Radix et Rhizoma, and Rhei Radix et Rhizoma. The seven analytes were well separated without interference (See Figure 2), indicating that the selectivity of the method was acceptable for further quantification. Linearity of the method was validated by plotting the peak area (y) versus the concentrations of the injected reference standards (x). Good linear relations were observed for all the analytes, with correlation coefficients (r^2) > 0.995. The LODs ($S/N \geq 3$) and LOQs ($S/N \geq 10$) of the

seven analytes ranged from 0.18 to 0.38 μg/mL and from 0.61 to 1.31 μg/mL, respectively. The results of linearity, LOD, and LOQ are summarized in Table 3. To confirm the precision, repeatability, and accuracy of the method, the obtained values of tests of intra-day and inter-day precisions, repeatability, and recovery were performed and are presented in Table 4. The relative standard deviations (RSDs) were below 3.0%, and the average recoveries for all the analytes were in the range of 101.1% to 103.8%. Therefore, this UPLC method is sensitive, precise, and accurate for quantification of multiple components in NHQWP.

Table 3. Linear range, regression equation, coefficient of determination (r^2), LOD, and LOQ for UPLC analysis of seven analytes.

Analyte	Linear Range (mg/mL)	Regression Equation $y = ax + b$	r^2	LOD (μg/mL)	LOQ (μg/mL)
Geniposide	0.04~1.07	$y = 8.46 \times 10^6 x - 2.65 \times 10^5$	1.0000	0.25	0.85
Forsythiaside A	0.04~1.10	$y = 1.09 \times 10^7 x - 3.65 \times 10^5$	1.0000	0.36	1.25
3,5-*O*-dicaffeoyl quinic acid	0.02~0.49	$y = 2.20 \times 10^7 x - 3.30 \times 10^5$	1.0000	0.38	1.31
Hesperidin	0.01~0.52	$y = 1.03 \times 10^7 x - 4.58 \times 10^4$	0.9998	0.27	0.92
Baicalin	0.09~0.87	$y = 1.82 \times 10^7 x - 1.20 \times 10^6$	1.0000	0.35	1.19
Glycyrrhizic acid	0.02~0.55	$y = 4.92 \times 10^6 x - 8.69 \times 10^4$	1.0000	0.18	0.61
Chrysophanol	0.002~0.06	$y = 1.82 \times 10^4 x - 3.68 \times 10^4$	0.9999	0.19	0.62

Table 4. Precisions, repeatability, and recovery of seven analytes.

Analyte	Precisions		Repeatability	Recovery	
	Intraday RSD (%, n = 6)	Interday RSD (%, n = 3)	RSD (%, n = 6)	Mean (%)	RSD (%, n = 6)
Geniposide	1.1	2.1	2.4	103.8	2.2
Forsythiaside A	1.6	1.6	2.8	103.5	1.7
3,5-*O*-dicaffeoyl quinic acid	1.9	1.5	2.1	102.5	1.0
Hesperidin	1.4	1.3	2.9	102.8	2.4
Baicalin	1.2	2.3	2.6	101.1	2.4
Glycyrrhizic acid	1.1	1.6	1.0	103.1	1.9
Chrysophanol	1.8	2.9	2.8	102.7	1.5

3.4. Simultaneous Determination of 7 Components in the Samples from Manufacturers

The assay markers for Gardeniae Fructus, Forsythiae Fructus, Chrysanthemi Flos, Aurantii Immaturus Fructus, Scutellariae Radix, Glycyrrhizae Radix et Rhizoma, and Rhei Radix et Rhizoma Geniposide used in ChP 2020 were geniposide, forsythiaside A, 3,5-*O*-dicaffeoyl quinic acid, synephrine, baicalin, glycyrrhizic acid, and chrysophanol, respectively. Among them, synephrine is a strongly polar alkaloid, which cannot be reserved on the C_{18} column by elution of routine mobile phase. However, the ion-pair reagent containing potassium dihydrogen phosphate and sodium dodecyl benzene sulfonate is not suitable for multi-component determination with wavelength switching. So hesperidin, another index ingredient in Aurantii Immaturus Fructus was chosen as the marker for assay of the RMCHM. According to the literature [26–32], geniposide, forsythiaside A, 3,5-*O*-dicaffeoyl quinic acid, hesperidin, baicalin, glycyrrhizic acid, and chrysophanol were active compounds of their corresponding CMMs. In all, 49 batches of Niuhuang Qingwei Pills (NHQWP) from 18 pharmaceutical manufacturers were quantified. As shown in Table 5, there were differences among the contents of the seven components in different samples. It was well-recognized that CMMs were mostly from cultivated herbs, whose contents were inevitably influenced by uncontrollable natural factors. So, the quality of TCM could be reflected by the contents of the markers but might not necessary in a proportional relation However, obvious lower contents always indicated poor quality or insufficient amount of raw materials. The contents of geniposide and baicalin varied gently with RSDs below 10% while those of forsythiaside A, 3,5-*O*-dicaffeoyl quinic acid, and glycyrrhizic acid changed within a wider range with RSDs around 20%. Violent fluctuations were observed

for contents of hesperidin and chrysophanol with RSDs of 57% and 48%, respectively. The variations indicated uneven quality of the raw materials and that would result in the differences of internal quality and pharmaceutical effects of the finished products.

Table 5. Contents (mg/g) of 7 components in 49 batches of Niuhuang Qingwei Pills from 18 pharmaceutical manufacturers (n = 2).

No.	Manufacturer	Geniposide	Forsythiaside A	3,5-*O*-dicaffeoyl Quinic Acid	Hesperidin	Baicalin	Glycyrrhizic Acid	Chrysophanol
1	A	1.66	1.36	0.62	8.95	3.10	0.62	0.13
2	A	1.51	1.54	0.54	8.16	3.23	0.64	0.08
3	A	1.70	1.41	0.51	7.55	3.09	0.65	0.10
4	B	1.76	1.85	0.74	8.63	3.39	0.71	0.40
5	B	1.80	1.86	0.75	8.68	3.31	0.69	0.39
6	C	1.67	1.20	0.73	10.18	3.40	0.72	0.13
7	C	1.67	1.79	0.74	10.64	3.46	0.72	0.10
8	C	1.62	1.91	0.75	10.49	3.42	0.72	0.12
9	D	1.58	1.33	0.53	1.77	3.01	0.73	0.28
10	D	1.62	1.39	0.51	1.21	3.13	0.97	0.30
11	D	1.71	1.70	0.56	1.16	2.84	0.77	0.21
12	E	1.60	1.40	0.90	9.08	3.71	0.69	0.22
13	E	1.76	1.07	0.70	2.12	3.27	0.75	0.18
14	F	1.66	1.45	0.71	0.93	3.31	0.69	0.25
15	F	1.63	1.19	0.48	1.33	3.12	0.65	0.10
16	F	1.78	2.06	0.61	1.79	3.18	0.70	0.19
17	G	1.75	1.56	0.73	1.13	3.48	0.82	0.16
18	G	1.81	1.76	0.65	0.99	3.47	0.61	0.10
19	G	1.69	1.82	0.65	1.05	3.50	0.61	0.10
20	H	1.58	1.81	0.59	8.67	3.05	0.70	0.24
21	H	1.77	1.66	0.54	8.78	3.17	0.74	0.20
22	H	1.78	1.75	0.57	8.71	3.16	0.76	0.19
23	I	0.93	0.95	0.47	6.15	2.64	1.57	0.22
24	J	1.38	0.97	0.51	5.63	2.87	0.52	0.10
25	J	1.47	1.12	0.51	9.26	2.95	0.58	0.09
26	J	1.74	1.12	0.51	9.67	3.02	0.78	0.10
27	K	1.56	1.27	0.65	7.23	3.27	0.63	0.10
28	K	1.59	1.55	0.66	7.49	3.25	0.62	0.12
29	K	1.58	1.54	0.65	7.44	3.30	0.63	0.11
30	L	1.42	1.68	0.71	6.06	3.29	0.62	0.08
31	L	1.43	1.58	0.66	6.36	3.04	0.64	0.10
32	L	1.47	1.58	0.65	6.14	3.38	0.62	0.09
33	M	1.55	1.18	0.76	10.15	2.92	0.70	0.16
34	M	1.38	1.13	0.70	9.48	3.06	0.68	0.13
35	M	1.40	1.20	0.58	9.45	3.03	0.60	0.12
36	N	1.46	1.48	0.71	7.11	2.23	0.69	0.21
37	N	1.47	1.46	0.54	8.98	2.84	0.60	0.22
38	N	1.44	1.57	0.54	8.13	2.93	0.62	0.22
39	O	1.69	1.21	0.58	7.29	3.07	0.73	0.10
40	O	1.37	1.27	0.40	0.12	3.04	0.70	0.13
41	P	1.62	1.37	0.67	2.29	3.28	0.65	0.10
42	P	1.60	1.32	0.64	1.93	3.31	0.64	0.26
43	P	1.62	1.39	0.64	1.90	3.30	0.72	0.28
44	Q	1.54	1.00	0.44	5.58	3.21	0.62	0.12
45	Q	1.59	0.98	0.43	5.68	3.25	0.62	0.12
46	Q	1.56	1.03	0.44	6.02	3.34	0.65	0.16
47	R	1.58	1.89	0.84	6.52	3.52	0.74	0.30
48	R	1.60	1.55	0.76	3.82	3.31	0.68	0.15
49	R	1.49	1.70	0.71	0.46	3.25	0.67	0.12
	Median	1.60	1.45	0.64	6.52	3.23	0.68	0.17
	Mean	1.58	1.45	0.62	5.88	3.18	0.70	0.08
	RSD (%)	9.7	19.9	18.1	57.2	7.9	21.0	47.5

3.5. Grade Evaluation of Niuhuang Qingwei Pills

To evaluate the "excellence or inferior grade" of Niuhuang Qingwei Pills, TCM Reference Drug (TCMRD), which derived from authentic high quality raw materials, and was produced strictly in accordance with the preparation and GMP specifications, was introduced to multi-component analysis of ready-made Chinese herbal medicines (RMCHM).

The approved or legislated sources, the harvesting periods/procedures and processing methods of the CMMs were confirmed by reviewing their statuary standards. The authentic habitat, medicinal resources, and current quality status of the herbs were investigated. Then the authentic raw materials from correct original plant, cultivated under good agriculture practice (GAP), were purchased. The selected CMMs were tested respectively in comply with their statutory standards. In addition, other tests were performed to rule out safety risks such as pesticide residues, heavy metals, mycotoxins, sulfur dioxide, adulteration, and illegal dyeing, etc. Finally, Niuhuang Qingwei Pills Reference Drug were produced in strict accordance with the provisions of GMP and the dosing of all the raw materials should comply with the amounts after shredding, breaking, or crushing, which is specified in the official procedure of the TCMRD's standard.

To cover the inevitable fluctuation of components in RMCHM, three batches of TCMRDs were prepared with authentic, high-quality and standardly processed CMMs from three different suppliers. Then the TCMRDs and their corresponding raw materials were determined by the same UPLC method for the NHQWP samples. As indicated in Table 6, variations were also found in contents of the seven markers in three batches of TCMRDs, especially for those of hesperidin, glycyrrhizic acid, and chrysophanol. Those might be due to naturally existed fluctuation of chemical components in Aurantii Immaturus Fructus and the different growth years of Glycyrrhizae Radix et Rhizoma and Rhei Radix et Rhizoma. Nevertheless, the transfer rates of all the analytes were stable, which offered valuable information for setting up the second-grade limits.

Table 6. Contents and transfer rates of seven components in three batches of Niuhuang Qingwei Pills Reference Drugs (n = 2).

Analyte		Geniposide	Forsythiaside A	3,5-*O*-dicaffeoyl Quinic Acid	Hesperidin	Baicalin	Glycyrrhizic Acid	Chrysofanol
Reference Drug 1	Contents (mg/g)	1.65	2.17	0.67	1.20	3.62	0.83	0.16
	Transfer rates (%)	92.6	98.7	85.8	97.2	94.2	97.4	98.3
Reference Drug 2	Contents (mg/g)	1.12	1.35	0.39	0.36	2.62	1.03	0.08
	Transfer rates (%)	91.5	97.5	88.2	95.4	93.1	96.5	93.8
Reference Drug 3	Contents (mg/g)	1.19	2.44	0.38	0.31	3.24	0.55	0.08
	Transfer rates (%)	93.4	98.5	86.6	94.7	94.7	95.1	95.2
Mean	Contents (mg/g)	1.32	1.99	0.48	0.62	3.16	0.80	0.11
	Transfer rates (%)	92.5	98.2	86.9	95.8	94.0	96.3	95.8
RSD (%)	Contents (mg/g)	21.8	28.6	34.3	80.2	16.0	30.0	43.3
	Transfer rates (%)	1.0	0.7	1.4	1.3	0.9	1.2	2.4

In general, the grading of quality of the products refers to the minimum limits for the first- and second-grade products should be specified. In principle, the second grade referred to the quality level that should be achieved by using qualified raw materials with predefined quantity (charge quantity) and standardized manufacturing processes. The first-grade referred to the quality level that should be achieved by using high-quality raw materials, predefined quantity, and standardized manufacturing processes [33]. In this study, geniposide, 3,5-*O*-dicaffeoyl quinic acid, baicalin, and glycyrrhizic acid were selected as the markers for quality evaluation of Gardeniae Fructus, Chrysanthemi Flos, Scutellariae Radix, and Glycyrrhizae Radix et Rhizoma in Niuhuang Qingwei Pills (NHQWP), respectively. The minimum limits of these four markers and the maximum limits of water in the corresponding CMMs are specified in ChP 2020. The second-grade limits of geniposide,

3,5-*O*-dicaffeoyl quinic acid, baicalin, and glycyrrhizic acid in NHQWP were calculated as following: minimum limit of the marker in the corresponding CMM% × (100% − maximum limit of water in the corresponding CMM%) × proportion of the corresponding CMM in NHQWP% × average transfer rate of the marker in the NHQWP RDs%. On the other hand, the first-grade limits of geniposide, 3,5-*O*-dicaffeoyl quinic acid, baicalin, and glycyrrhizic acid in NHQWP were specified after comparison between their median contents in 49 batches of samples and their mean contents in 3 batches of reference drugs, based on dispersion of the data. If the mean contents were larger than the median contents, the first-grade limits were specified as 80% of the mean contents. On the contrary, the first-grade limits were specified as the mean contents.

As for forsythiaside A, hesperidin, and chrysophanol, their second-grade limits in NHQWP were specified only since their contents in the corresponding CMMs (Forsythiae Fructus, Aurantii Immaturus Fructus and Rhei Radix et Rhizoma, respectively) varied dramatically [34–40]. For forsythiaside A, its minimum limit in Forsythiae Fructus and the maximum limit of water are specified in ChP 2020. So the second-grade limit of the marker in NHQWP was calculated as the same way of those of geniposide, 3,5-*O*-dicaffeoyl quinic acid, baicalin, and glycyrrhizic acid, which was elaborated in the paragraph above. For hesperidin and free chrysophanol, their minimum limits in the corresponding CMMs are not specified. So the second-grade limits of the two markers in NHQWP were specified as 60% of their mean contents in three batches of reference drugs.

Based on the comprehensive analysis mentioned above, the quality grade specifications of the seven markers in NHQWP are specified in Table 7. Accordingly, 49 batches of samples from 18 manufacturers were primarily classified into three quality grades: 13 of first grade, 28 of second grade, and 8 unqualified.

Table 7. Quality grade specifications of the seven markers and quality rating results of Niuhuang Qingwei Pills.

Analyte	First Grade			Second Grade		
	Specification	Batches Qualified	Batches Qualified All 7 Specifications	Specification	Batches Qualified	Batches Qualified All 7 Specifications
Geniposide	≥1.32 mg/g	48	13	≥0.40 mg/g	1	28
Forsythiaside A	N/A	N/A		≥0.06 mg/g	49	
3,5-*O*-dicaffeoyl quinic acid	≥0.48 mg/g	44		≥0.22 mg/g	5	
Hesperidin	N/A	N/A		≥0.37 mg/g	48	
Baicalin	≥3.16 mg/g	29		≥1.93 mg/g	20	
Glycyrrhizic acid	≥0.64 mg/g	34		≥0.43 mg/g	15	

4. Conclusions

In conclusion, a new principle of multi-component analysis was presented for quality grade evaluation of ready-made Chinese herbal medicines (RMCHM) in this paper. A sensitive and accurate method for simultaneous determination of seven markers in Niuhuang Qingwei Pills (NHQWP) was developed using UPLC combined with wavelength switching. The method was validated for specificity, linearity, LOD, LOQ, precision, and accuracy and was utilized in assays of samples and NHQWP reference drugs (RD). With comprehensive consideration of the determination results and the current quality status of the corresponding raw materials, specifications for the first grade and the second grade were proposed, based on which the samples were classified. With combination of results obtained from former reported safety examinations, whole-ingredient identification, fingerprint, and determinations on other ingredients [18–24], the marketed samples were rated to distinguish "good" from "bad" in accordance with a holistic strategy. Challenges brought by large variability of a few markers in reference drugs warrant further and deeper research.

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