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Qualitative and quantitative analysis of traditional Chinese medicine Niu Huang Jie Du Pill using ultra performance liquid chromatography coupled with tunable UV detector and rapid resolution liquid chromatography coupled with time-of-flight tandem mass spectrometry

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ABSTRACT

An ultra performance liquid chromatography coupled with tunable UV detector (UPLC-TUV) and rapid resolution liquid chromatography coupled with time-of-flight tandem mass spectrometry (RRLC-Q-TOF) method was developed for the quality assessment of Niu Huang Jie Du Pill (NHJDP), a commonly used traditional Chinese medicine (TCM). Ten compounds were simultaneously identified by electrospray ion mass spectrometry (ESI/MS) and comparison with reference standards and literature data. All of them were quantified by UPLC method. Baseline separation was achieved on an ODS-140HTP C_{18} column (2.3 µm, 100 mm × 2.1 mm I.D.) with linear gradient elution of acetonitrile–0.1% formic acid. This developed method provides good linearity ($r^2 > 0.9996$), repeatability (RSD < 3.63%), intra- and inter-day precisions (RSD < 0.86%) with accuracies (97.88–101.56%) and recovery (98.88–101.92%) of 10 major constituents, namely baicalin, baicalein, wogonoside, wogonin, glycyrrhizic acid, liquiritin, rhein, emodin, chrysophanol and physcion. In addition, the principal component analysis (PCA) coupled with the UPLC fingerprint was applied to classify the NHJDP samples according to their manufacture corporation. This proposed method with high sensitivity and selectivity was successfully utilized to analyze 10 major bioactive compounds in 30 batches of NHJDPs, and the results demonstrate that this analytical method is simple and suitable for the original discrimination and quality control of this TCM.
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1. Introduction

Ultra performance liquid chromatography (UPLC) method has become one of the most frequently applied approaches in the area of fast chromatographic separations, which holds excellent peak shapes, enhanced reproducibility and high-speed detection with complex biological samples and is valuable for the quality control of herbal medicines [1–4]. Certainly, a valuable and convincing UPLC chromatogram should have most of its peaks assigned. It is, however, usually difficult toward this end owing to the lack of reference standards necessary for the structure identification. Fortunately, quadrupole time-of-flight mass spectrometry (Q-TOF) is a rapid and sensitive technique with greater accuracy and precision for structural elucidation as long as the co-eluting compounds possess different m/z values [5]. Liquid chromatography coupled with mass spectrometry (LC–MS) could facilitate informative and high-throughput screening of chemical constituents in traditional Chinese medicines (TCMs), especially those trace components which are difficult to obtain by conventional isolation means [6,7].

Niu Huang Jie Du Pill (NHJDP), an ancient TCM, is comprised 5 g of *Calculus Bovis Artificialis*, 50 g of *Realgar*, 200 g of *Gypsum Fibrosum*, 200 g of *Radix et Rhizoma Rhei Palmati*, 150 g of *Radix Scutellariae Baicalensis*, 100 g of *Radix Platycodi*, 25 g of *Borneol* and 50 g of *Radix Glycyrrhizae* [8]. It has effect to antipyretic and detoxicate, and is widely used for swelling of throat and sore pain of gingival [8]. Baicalin is the marker compound for the quality control of this formula which was recorded in Chinese Pharmacopoeia [8].

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Several published papers have reported the determination of major active components in NHJDP, such as synthetic borneol, cholic acid and rhein, using analytical methods including capillary column gas chromatography (CGC) [9], thin layer chromatography (TLC) [10] and high performance liquid chromatography (HPLC) [11]. Apparently, only identification and quantitative determination of these compounds are not sufficient for the comprehensive quality control since TCM is a complex system containing tens or even hundreds of different chemical constituents which are responsible for the therapeutic effects. Recently, there was one published paper [12] reported the digitized HPLC fingerprints of Niu Hang Jie Du tablets. But the "common peaks", existed in all chromatograms of different batches of the same samples, were not identified and this method needs long analysis time. Therefore, an effective and reliable method, which can analyze as many bioactive constituents as possible in NHJDP to ensure its safety and efficacy, is necessary for qualitative and quantitative analysis of NHJDP.

In this paper, a combined ultra performance liquid chromatography coupled with tunable UV detector (UPLC-TUV) and rapid resolution liquid chromatography coupled with time-of-flight tandem mass spectrometry (RRLC-Q-TOF) method coupled with powerful chemometrics analysis for qualitation and quantification of the main constituents present in various NHJDP simultaneously was established, which was also compared with the conventional HPLC method. With this method, 10 compounds in the formula, including baicalin, baicalein, wogonoside, wogonin in *Radix Scutellariae Baicalensis* [13], glycyrrhizic acid, liquiritin in *Radix Glycyrrhizae* [14] and rhein, emodin, chrysophanol, physcion in *Radix et Rhizoma Rhei Palmati* [15] which were responsible for the therapeutic effects of antipyretic and detoxicate, have been identified and determined. Then based on the fingerprint data, principal component analysis (PCA) was utilized as a data reduction technique to generate a visual plot for qualitative evaluation on the resemblance and difference of tested samples.

2. Experiment

2.1. Chemicals and materials

HPLC-grade acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany) and Tedia (Fairfield, OH, USA). Deionized water was prepared by a Milli-Q_{50} SP Reagent Water System (Bedford, MA, USA) for preparing samples and mobile solution. Other reagents were of analytical grade. All solvents and samples were filtered through 0.22 μm membrane filters before analysis.

The reference standards of baicalin, baicalein, wogonin, glycyrrhizic acid, liquiritin, rhein, emodin, chrysophanol and physcion were obtained from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China, http://www.nicpbp.org.cn/CL0001/), wogonoside were purchased from Shanghai Winherb Medical Science Co., Ltd. (Shanghai, China, http://www.berbfine.com). The purities of all the standards (Fig. 1) were not less than 98%.

Thirty batches of NHJDP were collected from different pharmaceutical companies in China: Tongren Tang Pharmaceutical Co., Ltd. (samples 1–3 and 11–12), Ha Yao Shi Yi Tang Pharmaceutical Co., Ltd. (samples 5–10), Lei Yun Shang Pharmaceutical Co., Ltd. (samples 13–15), Pian Zi Huang Pharmaceutical Co., Ltd. (samples 16–18), Hui Ren Pharmaceutical Co., Ltd. (samples 19–22) and other manufacturers. These samples involved two dosage forms, including 7 batches of water-honeyed pills (marked as samples 1–8), 23 batches of tablets (marked as samples 9–30).

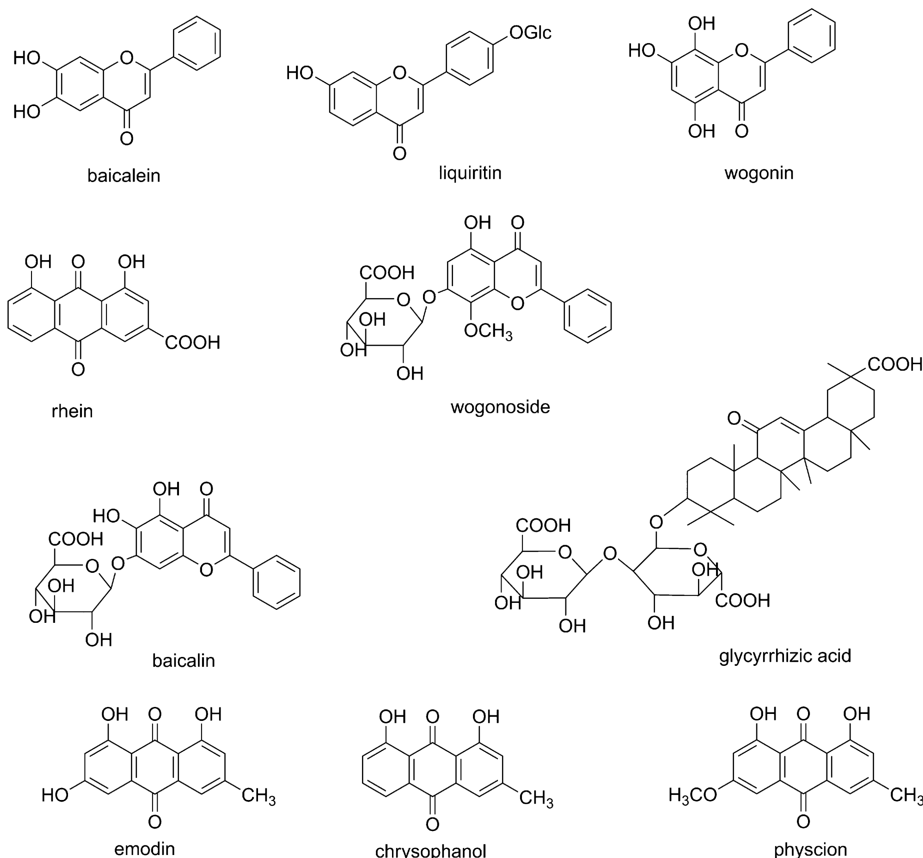

Fig. 1. Structures of the constituents identified from NHJDP.

2.2. Standard solutions and sample preparation

Each accurately weighed standard was dissolved in methanol, respectively, and diluted to provide a series of standard solutions with gradient concentration in order to make the calibration curve. And the mixture of the reference compounds stock solution was also prepared. All the solutions were stored at 4 °C in refrigerator.

The pills of NHJDP were smashed into power. 0.5 g of pulverized samples was accurately weighed into 25 mL volumetric flask, ultrasonic extracted at room temperature with 70% ethanol for 1 h and then made up to volume. Prior to use all samples were filtered through a 0.22 μm membrane filters.

2.3. Analytical method

Waters ACQUITY UPLC^{TM} system (Waters Co., MA, USA) equipped with TUV detector was used to acquire chromatograms. The separation was performed on a TSKgel ODS-140HTP C_{18} column (2.3 μm, 100 mm × 2.1 mm I.D., TOSOH Co., Tokyo, Japan). The mobile phase was consisted of acetonitrile (A) and 0.1% (v/v) formic acid (B). The linear gradient was as follows: 0–0.03 min, 1–4% A; 0.03–2.81 min, 4–12% A; 2.81–6.29 min, 12–12% A; 6.29–9.41 min, 12–20% A; 9.41–14.62 min, 20–20% A; 14.62–22.96 min, 20–50% A; 22.96–26.43 min, 50–100% A at a flow rate of 0.9 mL/min. The column temperature was 40 °C, detection wavelength was at 254 nm and the injection volume was 10 μL.

HPLC analysis was performed on a LC2010 AHT HPLC system coupled with DAD detector and LC solution workstation (Shimadzu, Kyoto, Japan). The sample were separated on a TSKgel ODS-100V C_{18} column (3.0 μm, 150 mm × 4.6 mm I.D., TOSOH Co., Tokyo, Japan) at a column temperature of 25 °C and flow rate of 1.0 mL/min with a linear gradient: 0–8 min, 4–22.5% A; 8–17 min, 22.5–22.5% A; 17–26 min, 22.5–25.6% A; 26–40 min, 25.6–25.6% A; 40–64 min, 25.6–50% A; 64–76 min, 50–100% A; 76–80 min, 100–100% A.

An Agilent-1200 RRLC/6510 Q-TOF system (Agilent, MA, USA) with ESI ion source in the positive ion mode was used for qualitative analysis of 10 compounds. The separation was performed on a TSKgel ODS-140HTP C_{18} column (2.3 μm, 100 mm × 2.1 mm I.D., TOSOH Co., Tokyo, Japan). The linear gradient conditions were the same as that used for UPLC-UV analysis. Elution was performed at a solvent flow rate of 0.4 mL/min, and a portion of the column effluent (0.2 mL/min) was delivered into the ion source of mass spectrometry. The conditions of MS analysis were as follows: drying gas N_2 flow rate 8 L/min, gas temperature 330 °C, pressure of nebulizer 35 psi; HV voltage 3.9 kV and scan range of 50–1000 *m*/*z*.

2.4. UPLC method validation

After the optimum conditions had been established, the method validation was performed. The linearity calibration curves were constructed by at least six different concentrations of chemical markers. Each concentration was analyzed in triplicate. The lim-

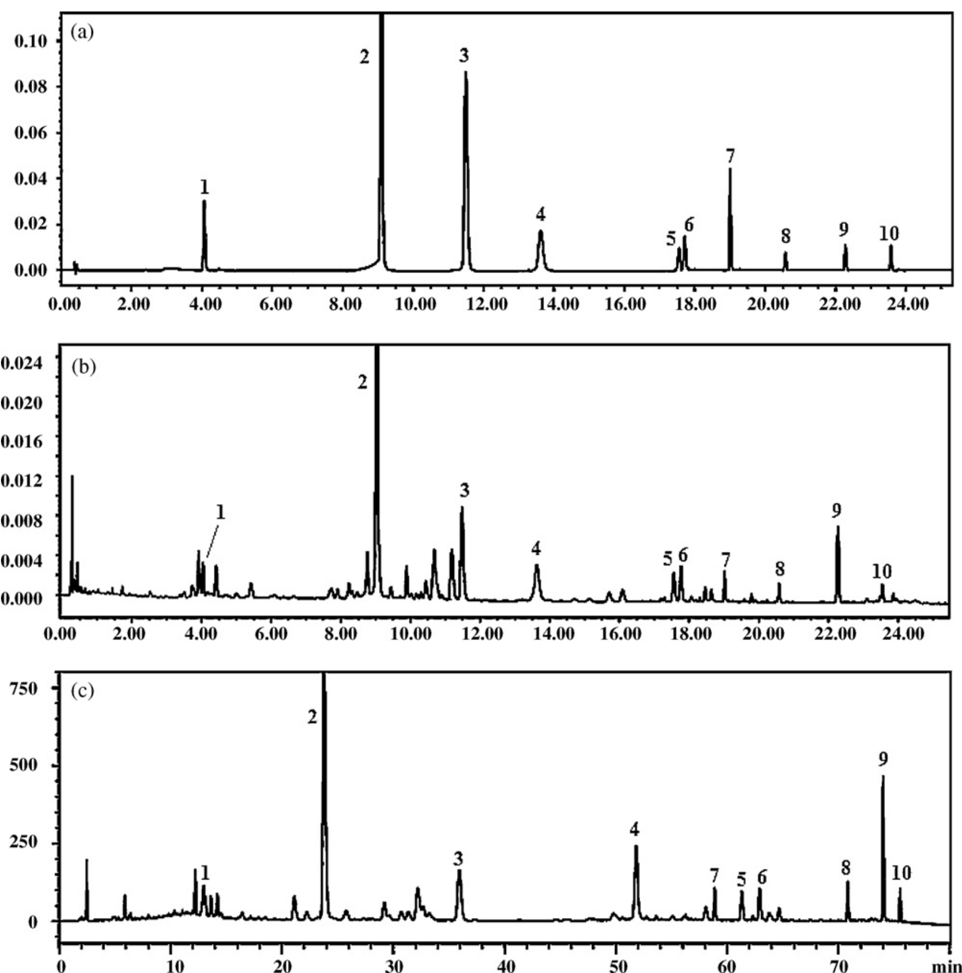

Fig. 2. (a and b) UPLC-UV chromatograms of (a) 10 mixed bioactive markers and (b) NHJDP (S4), and (c) HPLC-UV chromatogram of NHJDP (S4) with the detection at 254 nm: (1) glycyrrhizin, (2) baicalin, (3) wogonoside, (4) baicalein, (5) rhein, (6) wogonin, (7) glycyrrhizic acid, (8) emodin, (9) chrysophanol, (10) physcion.

Table 1
The identification of the 12 peaks.

No.	Observed mass $[M+H]^+$	Calculated mass	Assignment	Error (ppm)	Compound
1	419.1339	418.1264	$C_{21}H_{22}O_9$	0.5	Liquiritin
2	447.0941	446.0849	$C_{21}H_{18}O_{11}$	4.3	Baicalin
3	461.1097	460.1007	$C_{22}H_{20}O_{11}$	4.1	Wogonoside
4	271.0610	270.0528	$C_{15}H_{10}O_5$	3.5	Baicalein
5	285.0402	284.0321	$C_{15}H_8O_5$	2.8	Rhein
6	285.0771	284.0685	$C_{16}H_{12}O_5$	4.9	Wogonin
7	823.9445	822.9321	$C_{42}H_{62}O_{16}$	3.7	Glycyrrhizic acid
8	271.0605	270.0528	$C_{15}H_{10}O_5$	1.5	Emodin
9	255.0652	254.0579	$C_{15}H_{10}O_4$	0.2	Chrysophanol
10	285.0759	284.0685	$C_{16}H_{12}O_5$	0.5	Physcion
11	271.0607	270.0538	$C_{15}H_{10}O_5$	2.1	Aloe-emodin
12	375.1086	374.1002	$C_{19}H_{18}O_8$	3.0	Skullcapflavone II

its of detection (LOD) and limits of quantification (LOQ) were measured with the signal-to-noise ratio of 3 and 10 as criteria, respectively.

The mixture standard solution was analyzed under the optimal conditions six times both in 1 day for intra-day variation and in a day on 3 successive days for inter-day variation to evaluate the precision and accuracy. In order to check the repeatability, five different solution made by the same sample (S4) were determined.

The recovery of this method was using the standard addition method. Three different concentration levels (approximately equivalent to 0.8, 1.0 and 1.2 times of the concentration of the matrix) of the reference standards were added into the sample S4 in triplicate. The solutions were extracted and quantified as described before.

3. Results and discussion

3.1. Optimization of the chromatographic conditions and extraction

Because of the existence of acidic ingredients in NHJDP extraction, a small amount of acid was added into the mobile phase which could inhibit the ionization of these components to improve the peak shape and restrain the peak tailing. Zero%, 0.1% and 0.2% aqueous formic acid and acetic acid solutions were compared. The results showed that 10 compounds could be baseline separated when 0.1% aqueous formic acid solution was selected.

DAD detection was set at the wavelength range of 190–400 nm. Compared all chromatograms and the UV characteristic spectra of 10 reference compounds, it was found that 10 active compounds had higher absorbance, better separation and steady baseline at 254 nm.

Prior to sample analysis the optimal process of extraction had to be investigated. 0.5 g samples were accurately weighted and extracted with different volumes and percentages of methanol and ethanol, respectively. The result showed that 25 mL 70% ethanol was more effective and had better response. 0.5 g of accurately weighted samples with 25 mL 70% ethanol was extracted for 30 min, 60 min and 90 min, respectively. It was obvious that 10 compounds were almost completely extracted within 60 min.

3.2. Comparison of HPLC and UPLC

The NHJD sample was analyzed by using UPLC and HPLC system, respectively (Fig. 2). In order to obtain the similar separation of the complex constituents of this sample, HPLC needed 80 min while UPLC only needed 25 min. Therefore, UPLC method had its advantages over HPLC in terms of analytical speed, time saving, solvent saving, high performance and high efficiency. The reduced solvent consumption is also friendly to environment and financial expense. This method might be a powerful tool for the analysis of complex system such as Chinese herbal prescription.

In addition, the elution orders of peaks 5–7 in HPLC chromatogram and those in UPLC chromatogram are different since

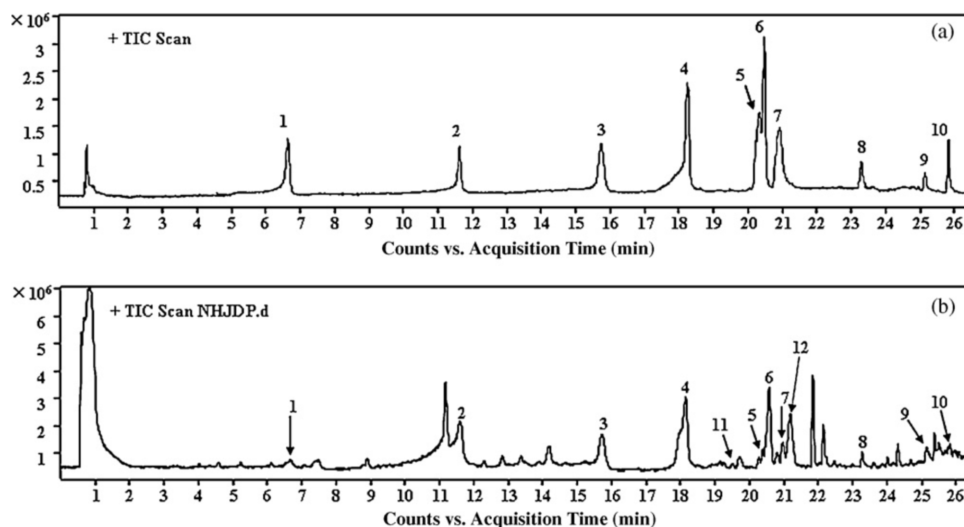

Fig. 3. RRLC-Q-TOF-MS total ion chromatogram (TIC) in positive ion mode of (a) 10 mixed bioactive markers and (b) NHJDP: (11) aloe-emodin, (12) skullcapflavone II.

packing materials in two chromatographic columns which used in two analytical methods are different.

3.3. RRLC-TOF-MS analysis

RRLC-Q-TOF-MS method was employed to verify the peaks found in UPLC chromatogram of NHJDP. In ESI-TOF-MS experiment, accurate molecular mass of the components can be obtained. As ESI was a "soft" ionization technique, the interface produces little fragmentation of analytes and generally forms protonated molecular ions $[M+H]^+$ for positive ionization mode. Comparing the mass spectra of the compounds with the standards and those in the literature [13–16], 10 compounds were unequivocally identified and 2 compounds (peaks 11 and 12) were tentatively assigned (Fig. 3 and Table 1).

The molecular formula of peak 11 was determined as $C_{15}H_{10}O_5$ by ESI (+)-TOF-MS (found $[M+H]^+$ 271.0607, calculated 270.0528), which consistent with aloe-emodin isolated from *Rhizoma Rhei Palmati* [15]. Similarly, peak 12 showed $[M+H]^+$ at m/z 375.1086 (calculated for $C_{19}H_{18}O_8$ 374.1002, found 374.1013), thus was deduced as skullcapflavone II which has been reported in *Radix Scutellariae Baicalensis* [16]. However, owing to the unavailability of authentic compounds, the peaks could only be tentatively assigned. For unambiguous identification further studies are required by using authentic compounds.

3.4. Validation of the quantitative analysis

3.4.1. Linearity, limits of detection and limits of quantification

Good linear correlation and high sensitivity at these chromatographic conditions were confirmed by the correlation coefficients ($r^2 > 0.9996$), LOD (1.2–2.6 ng), and LOQ (3.8–8.6 ng) (Table 2).

3.4.2. Accuracy, precision, repeatability and recoveries

The RSD of both relative retention time and relative peak area were less than 0.66%. The intra- and inter-day precisions were within 0.78% and 0.86%, respectively, with accuracies from 97.88% to 101.56%. The RSD of repeatability was less than 3.63%. The mean recoveries were from 98.88% to 101.92% with RSD less than 3.61% for 10 components. These results indicated that the UPLC fingerprint chromatograms had a good repeatability, precision, accuracy, and recovery (Table 2) and the developed assay was a reliable and useful method for assessment of the quality of NHJDP.

3.5. Sample analysis

The described UPLC method was subsequently applied to analysis and quality evaluation of 30 batches NHJDP, through simultaneous determination of 10 marker compounds. The quantitative analytical results (Table 3) indicated that the variations of their contents were great, even in the samples from the same company. Chinese medicine preparation with different pretreatment processes, manufacturing procedure, and dosage forms will be of different quality. Meanwhile, the content of bioactive markers was also affected by plant origins, sources, cultivated year, harvest time, geographical climate and environment. All of these could result in significant differences in quality of NHJDP.

Although the differences of the content between the samples from different companies were obvious, it is difficult to distinguish the sources of the samples. The PCA classification using original data obtained from 55 common components as input data instead of the full chromatogram of fingerprints without any preprocessing could discriminate the samples according to different manufacture corporation. All programs were performed using Matlab7.0. In the scatter plot (Fig. 4), the majority of samples were classified into

Table 2
Statistical results of the validation of the 10 compounds.

Investigated compound	Relative retention time (RSD, %)	Relative peak area (RSD, %)	Regression equation				LOD (ng)	LOQ (ng)	Intra-day ($n=6$)		Inter-day ($n=3$)		Repeatability ($n=5$) RSD (%)	Recovery	
			Linear range (μg/mL)	Slope, a	Intercept, b	r^2 ($n=6$)			Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)		Mean recovery (%)	RSD (%)
Liquiritin	0.05	0.65	0.77–480	535.70624	960.9488	0.9999	2.3	7.7	99.92	0.78	100.40	0.86	1.31	99.57	1.31
Baicalin	0.02	0.10	5.9–1475	1512.8983	−1890.4226	0.9997	2.6	8.6	98.61	0.44	99.34	0.46	2.95	98.88	3.61
Wogonoside	0.04	0.15	2.98–745	1678.4636	4388.2141	0.9997	2.6	8.4	99.85	0.34	99.95	0.28	3.10	99.02	2.98
Baicalein	0.04	0.22	1.03–206	1569.2880	−2419.2795	0.9998	2.6	8.6	100.48	0.34	100.33	0.68	3.21	101.92	2.26
Rhein	0.02	0.66	0.4–50	2014.0799	−906.3501	0.9996	1.7	4.0	101.49	0.23	101.56	0.79	3.63	99.73	1.79
Wogonin	0.02	0.64	0.8–80	1422.9838	−675.6798	0.9996	1.2	3.8	99.40	0.13	100.07	0.24	2.87	100.87	1.94
Glycyrrhizic acid	0.01	0.55	4.14–414	515.3500	−529.0243	1.0000	2.2	7.3	98.52	0.60	97.88	0.35	2.82	101.00	3.08
Emodin	0.01	0.45	0.42–42	1120.5036	−274.1374	0.9998	1.1	4.2	99.65	0.45	99.64	0.37	3.03	100.60	2.51
Chrysophanol	0.02	0.39	0.54–54	1419.5279	131.5038	1.0000	1.4	4.6	99.63	0.31	100.23	0.52	2.14	99.67	2.34
Physcion	0.02	0.57	0.5–62.5	1100.4286	−177.0100	0.9998	1.8	5.0	99.84	0.36	98.35	0.36	2.35	100.06	2.84

In the regression equation $y=ax+b$, y refers to the peak area (A), x concentration of the reference (μg/mL), r^2 the correlation coefficient of the equation.

Table 3
Contents (mg/g) of the 10 compounds in the 30 samples.

Dosage form	Sample no.	Content of each compound in 30 samples (mg/g)									
		Liquiritin	Baicalin	Wogonoside	Baicalein	Rhein	Wogonin	Glycyrrhizic acid	Emodin	Chrysophanol	Physcion
Water-honeyed pills	1	0.20	3.04	0.61	0.52	0.13	0.27	0.46	0.13	0.44	0.17
	2	0.27	3.08	0.54	0.61	0.134	0.28	0.40	0.13	0.41	0.20
	3	0.23	2.94	0.56	0.49	0.13	0.29	0.37	0.12	0.37	0.13
	4	0.16	1.57	0.25	0.49	0.13	0.25	0.35	0.23	0.57	0.17
	5	0.05	1.54	0.26	0.64	0.18	0.25	0.37	0.26	0.48	0.17
	6	0.05	1.82	0.34	0.85	0.19	0.30	0.52	0.29	0.66	0.19
	7	0.06	1.51	0.29	0.75	0.14	0.27	0.31	0.22	0.61	0.18
	8	0.06	1.62	0.33	0.78	0.15	0.26	0.42	0.24	0.52	0.15
Tablets	9	0.57	4.07	0.80	0.77	0.78	0.33	0.69	0.86	2.23	0.48
	10	0.60	2.77	0.61	0.79	0.33	0.48	0.48	0.39	1.00	0.24
	11	0.86	3.48	0.68	0.68	0.34	0.37	1.07	0.33	1.25	0.36
	12	0.72	3.61	0.73	0.57	0.38	0.26	0.77	0.31	1.16	0.36
	13	0.33	2.60	0.41	0.45	0.71	0.24	0.53	0.84	1.97	0.49
	14	0.45	2.91	0.54	0.40	0.82	0.23	0.57	0.94	2.38	0.52
	15	0.35	3.32	0.62	0.34	0.68	0.22	0.68	1.00	1.82	0.50
Tablets	16	0.49	2.62	0.51	0.63	0.30	0.28	0.59	0.36	0.87	0.25
	17	2.14	3.14	0.68	0.59	0.34	0.26	0.41	0.24	0.43	0.17
	18	1.95	2.76	0.61	0.59	0.33	0.28	0.46	0.27	0.43	0.18
	19	0.98	3.36	–	0.59	0.13	0.24	0.33	0.26	1.49	0.44
	20	0.62	2.56	–	0.46	0.19	0.21	0.25	0.28	1.08	0.31
	21	1.31	1.98	0.22	0.61	0.08	0.26	0.36	0.26	1.28	0.37
	22	1.26	2.01	0.22	0.55	0.12	0.29	0.41	0.21	1.08	0.32
	23	1.35	3.30	0.62	0.65	0.29	0.36	0.36	0.31	0.62	0.20
	24	0.37	2.45	2.88	0.39	0.19	0.54	0.40	0.24	0.80	0.19
	25	0.97	1.78	0.24	0.33	0.14	0.16	0.21	0.20	1.10	0.46
	26	0.96	2.82	0.29	0.35	0.47	0.18	0.32	0.38	0.98	0.41
	27	1.54	2.24	0.44	0.53	0.43	0.32	0.48	0.17	0.39	0.13
	28	0.29	3.18	–	0.18	0.08	0.13	0.89	0.19	0.78	0.40
	29	0.66	1.50	–	–	0.16	0.14	0.34	0.19	0.46	0.17
	30	0.04	2.65	0.48	0.80	0.23	0.44	0.56	0.46	0.99	0.40

five groups, which could be marked as groups I–V according to different corporations, respectively. Other individual samples from other corporations were distributed dispersedly, such as the samples 4 and 24 were closed to group I, the sample 30 was closed to group II, the samples 23, 27 and 29 were closed to group IV, which suggested that these products were similar as those from these companies. However, the samples 25, 26 and 28 were different from those produced by any other companies. Moreover, the plot showed the group V was far from the groups I–IV, which demonstrated the samples in group V were different significantly from those in other groups, and the samples in groups I–IV had a closer relationship.

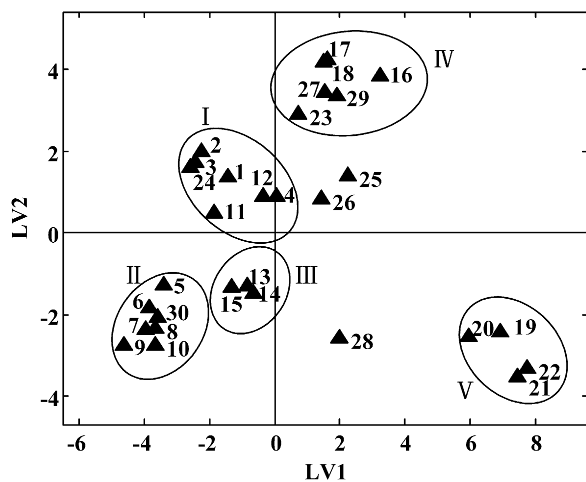

Fig. 4. Scores plot of PCA of 30 NHJDPs from different origin: (I) Tongren Tang Pharmaceutical Co., Ltd., (II) Ha Yao Shi Yi Tang Pharmaceutical Co., Ltd., (III) Lei Yun Shang Pharmaceutical Co., Ltd., (IV) Pian Zi Huang Pharmaceutical Co., Ltd., and (V) Hui Ren Pharmaceutical Co., Ltd.

4. Conclusion

A rapid UPLC-TUV and RRLC/Q-TOF-MS method was established for the comprehensive analysis of NHJDP. This method could separate the complex constituents in a shorter time. Furthermore, TOF-MS/MS would give accurate mass of the protonated molecule which is helpful in the identification of the compounds. In this work it has been shown that PCA is able to classify samples objectively and successfully in accordance with the origin. The method was successfully applied for simultaneous determination of 10 bioactive compounds in NHJDP. This readily available, rapid and reliable method is fit for routine analysis, the original discrimination and effective quality control of this Chinese herbal prescription. The amount of 10 compounds varied considerably raised the question of whether the various NHJDP would exhibit equivalent efficacy. Further work should be focus on finding out the influence of the compounds' content differences on the therapeutic effect of this TCM with the help of pharmacodynamic test and clinical test.

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