Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 638–645

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Chemical fingerprinting of Liuwei Dihuang Pill and simultaneous determination of its major bioactive constituents by HPLC coupled with multiple detections of DAD, ELSD and ESI-MS

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ARTICLE INFO

Article history:
Received 2 September 2008
Received in revised form 4 December 2008
Accepted 9 December 2008
Available online 24 December 2008

Keywords:
Chemical fingerprinting
Simultaneous determination
HPLC-DAD-ELSD
PLS–DA
Liuwei Dihuang Pill

ABSTRACT

For quality control purpose, an approach of chemical fingerprinting of Liuwei Dihuang Pill (LDP) and simultaneous determination of its multiple bioactive components were established by using high performance liquid chromatograph (HPLC) coupled with multiple detection techniques. HPLC with ultraviolet detection (HPLC-UV) was used to acquire its fingerprint, and HPLC with combined detections of diode array detector and evaporative light scattering detector (HPLC-DAD-ELSD) was performed to simultaneously determine eight bioactive constituents: including gallic acid, 5-hydroxymethyl furfural, morroniside, sweroside, loganin, paeoniflorin, paeonol and alisol B-23 acetate. The detection limits and quantification limits ranged in 0.11–1.93 μg/mL and 0.38–3.85 μg/mL, respectively. The validation of the proposed approach was acceptable, with 93.47–104.62% accuracy in recovery test. The intra- and inter-day precisions of the method were evaluated and were less than 3.87%, with accuracy from 95.3% to 103.4%. In addition, the mass spectrometry of the investigated major constituents was also studied. Based on the chromatographic fingerprint data, partial least square (PLS) and discriminate analysis were utilized to visualize the quality information of 60 batches of LDP, and a partial least square–discriminate analysis (PLS–DA) model was constructed with acceptable predictive performance for the discrimination of various products. The proposed approach was expected to be developed as a powerful tool for the quality control of LDP.

1. Introduction

Liuwei Dihuang Pill (LDP), an ancient traditional Chinese medicine (TCM) widely used all over the world, which is prepared from *Radix Rehmannide Preparata*, *Rhizoma Dioscoreae*, *Fructus Corni*, *Cortex Moutan*, *Rhizoma Alismatis* and *Poria*, is applied for the treatment of various disorders such as backache, alopecia, menoxenia, sore waist and knees [1]. Previous pharmacological studies demonstrated its efficacy for anti-aging [2], regulating T lymphocytes and cytokines [3], treating diabetes type II disease [4] and modulating neutrals system [5]. Multiple constituents were responsible for the therapeutic effects of TCM [6], however, only two bioactive components of it, loganin (LG) and paenol (PN) were determined through officially conducted quality control of LDP presently [7]. Therefore, an integral quality control approach based on the multiple constituents of LDP is urgently needed to ensure the efficacy and safety of the drug.

Fingerprinting has been internationally accepted as an efficient technique for the quality control of complex analytes, especially for TCMs [8–12]. Multivariate data analysis [13–16], such as principal components analysis (PCA), partial least square (PLS) and discrimination analysis (PLS–DA), were often applied in combination with fingerprinting to reveal the quality information of TCMs. Wang et al. reported their exploring work of LDP fingerprinting by HPLC-UV method, and indicated that it could properly reflect the quality information of this TCM [17,18]. On the other hand, since the quality of the TCM is directly related to its major bioactive constituents, the quantitative analysis, in most cases, is also necessary in the practice of quality control. So far, quite a few approaches have been developed for the determination of the bioactive constituents of LDP, including high performance liquid chromatograph (HPLC) with ultraviolet (UV) detection [19–22], HPLC tandem mass

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doi:10.1016/j.jpba.2008.12.009

spectrometry (HPLC–MS/MS) [23], gas chromatography (GC) [24] and micellar electrokinetic capillary chromatography (MEKC) [25]. In LDP, 5-hydroxylmethyl furfural (5-HMF), gallic acid (GA), morroniside (MR), sweroside (SR), LG, paeoniflorin (PF) and PN were generally considered as the bioactive components, and their determination was respectively well-documented via above-mentioned methods. In recent years, alisol B-23 acetate (AB-23A) was reported as another important bioactive constituent for its predominant effect of anti-tumor [26], and it should also be investigated in the quality control of LDP. However, quality control approaches, which have been published elsewhere, were mainly focused on bioactive markers of monoterpenes and phenolics. To the best of our knowledge, the determination of AB-23A has not been reported yet.

AB-23A, a triterpene with poor UV absorption at terminal wavelength, exhibits good absorption in evaporative light scattering detector (ELSD) [27]. Consequently, two detection techniques of photodiode assay detector (DAD) and ELSD were proposed as a solution to simultaneously determine the contents of constituents in LDP. We reported here, for the first time, to apply the combination of chemical fingerprinting (DAD) and quantitative analysis (DAD and ELSD) of major bioactive constituents including 5-HMF, AB-23A, GA, MR, SR, LG, PF and PN in the quality control of LDP. The proposed approach could be readily utilized as a comprehensive quality control approach for the TCM formula.

2. Experimental

2.1. Reagents and materials

HPLC grade of acetonitrile and methanol were purchased from Merck Company Inc. (Merck, Darmstadt, Germany). Formic acid (HPLC grade) was purchased from Tedia Company Inc. (Tedia Way, Fairfield, USA). Ultrapure water was prepared by a Milli-Q50 SP Reagent Water System (Millipore Corporation, MA, USA). Other reagents were of analytical grade.

Reference compounds of 5-hydroxylmethyl furfural (5-HMF), gallic acid (GA), loganin (LG), paeoniflorin (PF) and paenol (PN) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and morroniside (MR), sweroside (SR), alisol B-23 acetate (AB-23A) were obtained from National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herb Medicine (Jiangxi Herbfine Hi-tech Co. Ltd., China). All the eight reference compounds have over 98% purity (see their chemical structures in Fig. 1).

Sixty batches of LDP samples (LDPs) were purchased from local drug stores. These samples involved three dosage forms, including 19 batches of water-honeyed pills (marked as samples 1–19), 37 batches of concentrated pills (marked as samples 20–57) and 4 batches of capsules (marked as samples 57–60). All the samples were collected from twenty-three Chinese medicine manufacturers: Tongren Tang Pharmaceutical Co., Ltd. (samples 1–10 and 20–24) Fengliaoxing Pharmaceutical Co., Ltd. (samples 11–12), Zhongyi Pharmaceutical Co., Ltd. (samples 13–14), Wanxi Pharmaceutical Co., Ltd. (samples 25–35), Jiuzhi Tang Pharmaceutical Co., Ltd. (samples 36–40), Fuoci Pharmaceutical Co., Ltd. (samples 41–45), Tonghanchun Tang Pharmaceutical Co., Ltd. (samples 46–48), Jinbao Pharmaceutical Co., Ltd. (samples 49–50) and other manufacturers.

2.2. Standard solutions

Each accurately weighed standard was dissolved in methanol, respectively, and various standard solutions were obtained through diluting the stock solution to a series of concentrations in order to make the calibration curves. All the standard solutions were stored in the refrigerator at 4 °C before analysis.

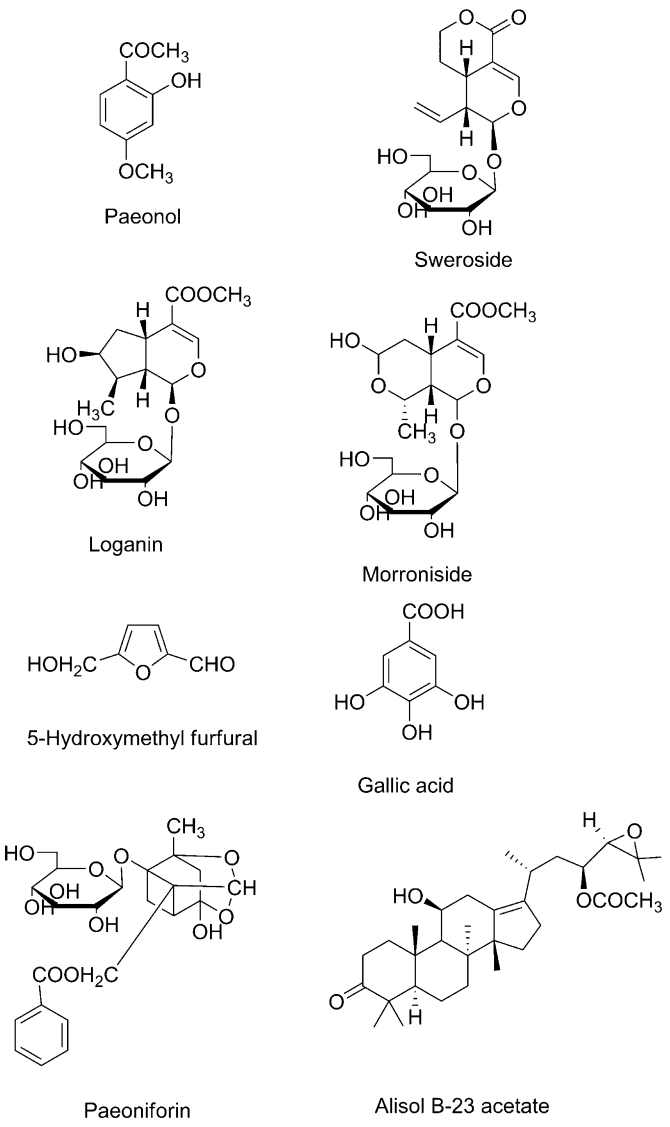

Fig. 1. Chemical structures of the bioactive compounds to be determined in LDP.

2.3. Sample solutions

The outer coats of capsules were removed. All the samples were cut into pieces and milled into the homogeneous size. 1.00 g pulverized powder was accurately weighed and ultrasonically extracted with 10 mL methanol for 60 min in a conical flask, and then cooled to room temperature. The supernatant filtrated through a syringe filter (0.45 μm) and aliquots (10 μL) were subjected to HPLC before analysis.

2.4. HPLC analysis

2.4.1. HPLC–ESI-MS analysis

An Agilent-1100 HPLC system (Agilent Technologies, MA, USA), equipped with quaternary pump, vacuum degasser, autosampler, column heater–cooler, was coupled with an LC/MSD Trap XCT electrospray ion trap mass spectrometer. A C_{18} TSKgel column (150 mm × 4.6 mm i.d., 5 μm, TOSOH, Japan) and a C_{18} guard column (7.5 mm × 4.6 mm i.d., 5 μm, Merck, USA) were used. The column temperature was maintained at 35 °C. Solvent A (water,

0.1% formic acid, v/v) and solvent B (acetonitrile, 0.1% formic acid, v/v) were used for gradient elution with program as follows: 1–12% B (0–20 min); 12–15% B (20–30 min); 15–50% B (30–70 min); 50–100% B (70–80 min); 100–100% B (80–85 min). Flow rate was set at 1.0 mL/min, and by solvent splitting, 0.2 mL/min portion of the column effluent was delivered into the ion source of mass spectrometry. Mass spectra were acquired in both positive and negative ion modes with conditions set as follows: drying gas (N_2) 10.0 L/min, temperature 350 °C, pressure of nebulizer 30 psi, HV voltage 3.5 kV and scan range from m/z 100 to 1000. Data acquisition was performed using Agilent ChemStation software (Agilent Technologies, MA, USA).

2.4.2. HPLC-DAD-ELSD analysis

A Shimadzu LC 2010A liquid chromatograph system (Shimadzu Corporation, Kyoto, Japan) was coupled with double detectors of DAD and Sedex 85 ELSD (Sedere, Alfortville, France). The system was operated by LC Solution software (Shimadzu, Kyoto, Japan). The chromatographic conditions were the same as noted in Section 2.4.1. The drift tube temperature of ELSD was 40 °C, the nebulization gas was air with a pressure of 3.5 bar and the gain (sensitivity) of the detector was 7.

2.5. Method validation

2.5.1. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

The linearity study was achieved by diluting stock solution into a series of concentrations. The calibration curves were constructed for at least six concentrations in triplicate. Calibration curves for all the compounds except for AB-23A were constructed by plotting the integrated chromatography peak areas (Y) versus the corresponding concentration of the injected standard solutions (X) using a $1/x^2$ weighted linear least-squares regression model. While for AB-23A, its calibration curve was constructed using a logarithmic conversion for both peak area (y) and the concentration (x). LOD and LOQ were calculated by diluting the standard solution when the signal-to-noise ratios (S/N) of analytes were almost 3, 10, respectively.

2.5.2. Precision, repeatability and accuracy

Intra- and inter-day precision and accuracy tests were performed by analyzing standard solutions during a single day ($n = 5$) and on 5 different days ($n = 3$), respectively. For repeatability test, five independent working solutions were prepared in same procedures noted in Section 2.2. Known amount of standard solutions were spiked into a LDP sample solution (sample 21), and three concentration levels of solutions were prepared. Recovery tests were performed by comparatively analyzing spiked and unspiked samples. Relative standard deviation (R.S.D.) was used to describe precision, repeatability and recovery.

2.6. PLS and PLS–DA analysis

In order to reveal the quality information of LDPs, PLS and discrimination analysis (DA) were conducted here on the data of fingerprints. PLS analysis was applied to distinguish the products of the same dosage form from the different commercial factories. PLS–DA analysis was performed to cluster and to make a recognition system of two major dosage forms (water-honeyed pills group and concentrated pills group). Before PLS and DA analysis, the original variables were commonly standardized as expressed by the following formula:

$$x_{ij}^{std} = \frac{x_{ij} - \bar{x}_j}{\sqrt{\sum_{i=1}^{p}(x_{ij} - \bar{x}_j)^2 / p}}$$

where $\bar{x}_j$ is the average of variable j, p is variable number. All programs were coded in MATLAB 7.0 for windows.

3. Result and discussion

3.1. Optimization of extraction procedure

The extraction time (30, 60 and 90 min) and solvents including the solution of ethanol (20%, 60%, 100%, v/v) and methanol (50%, 100%, v/v) were investigated. Finally, the procedure of 60 min and 100% methanol was adopted because it produced much more peaks with higher response, little interference and better peak shape.

3.2. Optimization of HPLC-DAD-ELSD conditions

Optimized chromatographic condition was achieved after several trials with gradient elution systems of methanol–water, acetonitrile–water, adding formic acid or acetic acid in both acetonitrile and water in various proportions. A linear gradient elution of acetonitrile–water with 0.1% (v/v) formic acid was selected since it permitted the best separation ability for all the samples investigated. The DAD detector was employed at the wavelength range from 190 nm to 400 nm for obtaining a sufficient number of detectable peaks. As a result, 254 nm and 238 nm were selected by comparing all the chromatograms and the UV characteristic spectra of referenced compounds (Fig. 2a and b). The ELSD detector was employed to determine AB-23A for better response (Fig. 2c). The optimal conditions were shown in details in Section 2.4.2.

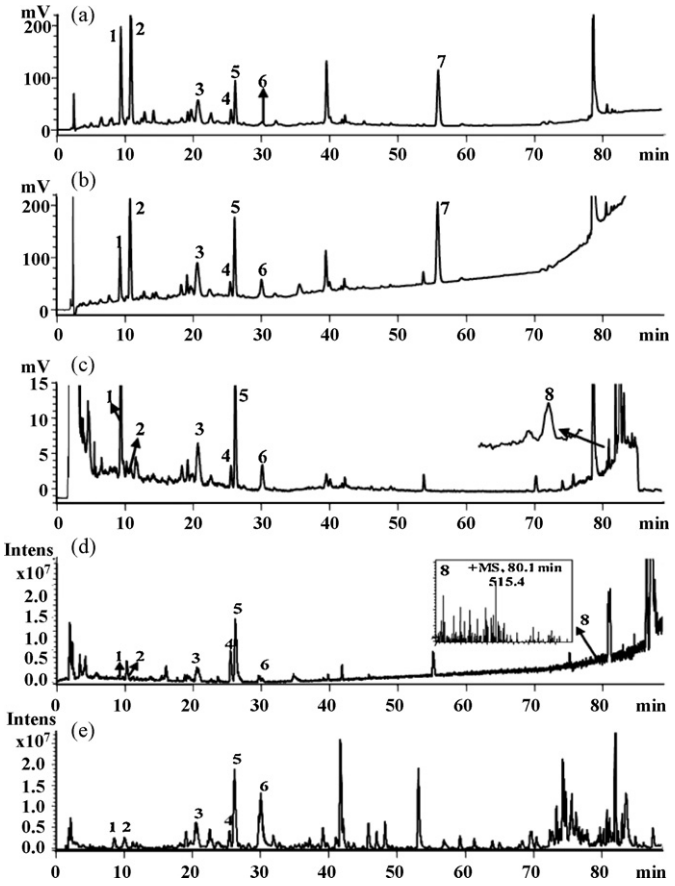

Fig. 2. Typical HPLC-ESI-MS and HPLC-DAD-ELSD chromatograms of LDP sample (sample 21): (a) BPC profile in positive ion mode; (b) BPC profile in negative ion mode; (c) HPLC-UV chromatogram at 254 nm; (d) HPLC-UV chromatogram at 238 nm; (e) HPLC-ELSD chromatogram. Validated components: (1) GA, (2) 5-HMF, (3) MR, (4) SR, (5) LG, (6) PF, (7) PN, (8) AB-23A.

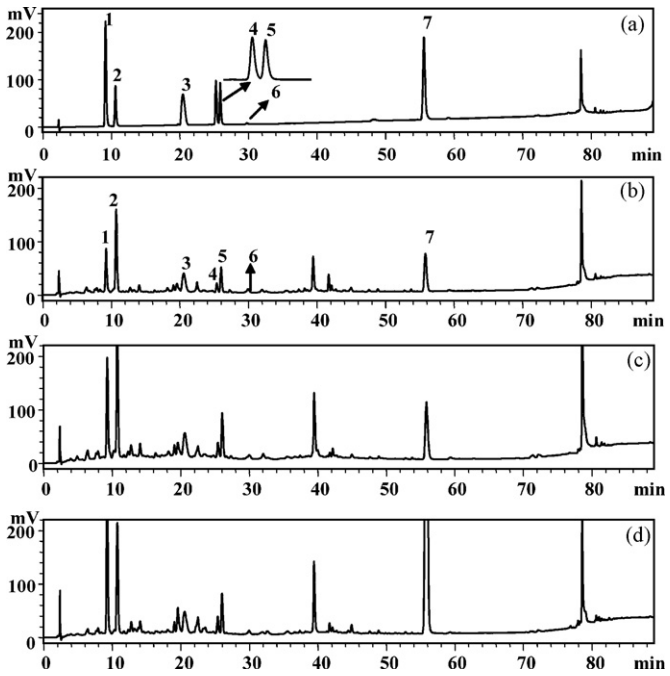

Fig. 3. Typical HPLC-UV fingerprints of LDPs (254 nm): (a) standard mixture, (b) water-honeyed pill (sample 1), (c) concentrated pill (sample 21) and (d) capsule (sample 58).

Under the optimized conditions, all the analytes of the samples were separated with good resolution. The representative chromatograms of standard mixture and HPLC-UV fingerprints of LDPs in three dosage forms are shown in Fig. 3.

3.3. Qualitative analysis of eight bioactive compounds in LDPs

The base peak chromatograms (BPC) of LDP were shown in Fig. 2d and e. The mass spectra data of the investigated components in both negative and positive ion modes were listed in Table 1. In positive ion mode, three groups of ions were produced, i.e. protonated ions, sodium adduct ion, and ions with neutral loss of H_2O or glucose. The quasi-molecular ions $[M+H]^+$ were the most abundant ions, while for MR and PF, the most abundant ions were the sodium adduct ions $[M+Na]^+$ at m/z 429 and 503, respectively. GA, 5-HMF and PF tended to eliminate H_2O to produce the fragmentation peaks at m/z 153, 109 and 463, respectively, and PF also had a fragment ion at m/z 375 $[M+H\text{-}C_7H_5OH]^+$. MR, SR and LG belonged to glucosides, and they were apt to loss the glucose to produce aglycone ions of m/z 229 $[M+H+H_2O\text{-glu}]^+$, m/z 197 $[M+H+H_2O\text{-glu}]^+$ and m/z 227 $[M+H\text{-glu}]^+$, respectively. While under the proposed conditions, PN and AB-23A only exhibited quasi-molecular ions. In negative ion mode, the quasi-molecular ion $[M-H]^-$, adducted ions of $[2M-H]^-$, $[M+Cl]^-$ or $[M+HCOO]^-$ could be observed. The $[M-H]^-$ ions were the most abundant ions for most compounds except for GA and LG, whose most abundant ions were $[2M-H]^-$ at m/z 339 and 779, respectively. A signal of m/z 287 $[2M+Cl]^-$ could also be detected for 5-HMF. The fragments of PN and AB-23A, unfortunately, could not be detected in the negative ion mode.

An HPLC-ESI-MS analysis was performed both on standards and samples, which could be utilized for the identification of the target compounds. Finally, eight investigated analytes in the extracts of LDP were comprehensively determined by comparing their retention times, UV and MS data with those of reference standards (Figs. 2 and 3).

3.4. Validation of the chromatographic method

3.4.1. Linearity, LOD and LOQ

As shown in Table 2, acceptable results of the regression analysis, the correlation coefficients (r^2), LOD and LOQ were obtained for all the analytes. The LOD and the LOQ were in the range of 0.11–0.54 μg/mL and 0.38–1.94 μg/mL for all the analytes except AB-23A in DAD, while for AB-23A in ELSD were recorded as 1.93 μg/mL and 3.85 μg/mL, respectively. Both DAD and ELSD detections have acceptable linearity, with over 0.9995 and 0.9953, respectively.

3.4.2. Precision, repeatability and recovery

Table 3 lists the data of precision and repeatability tests, and Table 4 displays the results of recovery test. For precision test, statistic data showed that the R.S.D.s of eight compounds were in the range of 0.43–3.26% for intra-day variation and 0.30–3.87% for inter-day variation, with accuracy of 95.4–102.5% and 95.3–103.4%, respectively. The R.S.D.s of repeatability were less than 7.26. For recovery test, mean recoveries of the standard substances were between 93.5% and 104.6%, with R.S.D.s less than 5.40% ($n = 3$). The results described above showed that the developed method was reliable for the quality control of LDP.

3.5. Quantitative analysis of eight components in LDPs

The proposed HPLC-DAD-ELSD method was subsequently applied to simultaneous determination of eight predominant bioactive ingredients in 60 batches of LDPs produced by various manufacturers. The results summarized in Table 5 revealed that the investigated contents were obviously different among various samples. The total content of PN was in the range of 20.99–28.35 mg/g in capsules, but unfortunately, only a range of 0.76–3.96 mg/g could be detected in concentrated pills and water-honeyed pills except that the data of sample 35 was 18.34 mg/g. In capsules, although the mean content of PN was abundant, the mean content of PF was low oppositely, only ranged from 0.66 mg/g to 1.06 mg/g. On the other hand, the mean contents of GA, 5-HMF, SR, MR and LG in both concentrated pills and capsules were much higher than those in water-honeyed pills, whereas the contents of LG in samples 35 and 59 were hardly detected. The mean contents of GA, 5-HMF and LG in both concentrated pills and capsules were dramatically higher than that in water-honeyed pills, whilst the mean contents of MR and SW in concentrated pills were slight higher than that in water-honeyed pills and capsules. On the contrary, the total content of AB-23A was the lowest among all the eight bioactive analytes, and its mean content in water-honeyed pills was higher than that in concentrated pills and capsules, but it could hardly be detected in samples 24 and 54.

According to the results listed in Table 5, it was suggested that dosage form was one of the key factors affecting the contents of ingredients. The procedure of capsule would comparably yield high extraction of most of its bioactive components. The results also indicated that quality had marked variations, and quality control of six medicinal materials of LDP was necessary. Multiple factors for the six raw medicinal materials such as various regions, source, different harvesting time and various manufacturing procedures, would accordingly result in the difference on their qualities of products. From the fingerprint data of the same dosage form produced by the same manufacturers, for instance, samples 20–24 or samples 25–34, it was found that their eight quantitative contents were similar from batch to batch. Thus, the selection of the stable source of the medicinal materials, especially the authentic medicinal herbs for the six medical materials, is quite important and meaningful for quality evaluation of this medicine.

Table 1
MS fragmentation of the investigated compounds by HPLC-ESI-MS.

No.	t_R (min)	Analyte	Fractions in (m/z)		M.W.
			Positive ion mode	Negative ion mode	
1	9.2	GA	171 $[M+H]^+$, 153 $[M+H\text{-}H_2O]^+$	169 $[M-H]^-$, 339$[2M-H]^-$, 205 $[M+Cl]^-$	170
2	10.4	5-HMF	127 $[M+H]^+$, 109 $[M+H\text{-}H_2O]^+$, 149 $[M+Na]^+$, 253 $[2M+H]^+$	143 $[M+H_2O\text{-}H]^-$, 161 $[M+Cl]^-$, 287 $[2M+Cl]^-$	126
3	20.8	MR	429 $[M+Na]^+$, 227 $[M+H\text{-glu}]^+$	405 $[M-H]^-$, 451 $[M+HCOO]^-$	406
4	25.8	SR	359 $[M+H]^+$, 197 $[M+H+H_2O\text{-glu}]^+$	357 $[M-H]^-$, 393 $[M+Cl]^-$, 403 $[M+HCOO]^-$	358
5	26.4	LG	391 $[M+H]^+$, 413 $[M+Na]^+$, 229 $[M+H+H_2O\text{-glu}]^+$	779 $[2M-H]^-$, 425 $[M+Cl]^-$, 435 $[M+HCOO]^-$	390
6	30.4	PF	503 $[M+Na]^+$, 463 $[M+H\text{-}H_2O]^+$, 375 $[M+H\text{-}C_7H_5OH]^+$	479 $[M-H]^-$, 515 $[M+Cl]^-$, 525 $[M+HCOO]^-$	480
7	55.9	PN	167 $[M+H]^+$	–	166
8	80.1	AB-23A	515 $[M+H]^+$	–	514

Table 2
Statistics results of linear regression equation analysis in the determination of eight bioactive components.

Analyte[a]	Detection	Regression equation $Y(y)=aX(x)+b$[b]	r^2 ($n=6$)	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
1	254 nm	$Y=16388X-41048$	0.9996	6.98–698.0	0.14	0.50
2	254 nm	$Y=11329X+2337.9$	0.9999	19.4–1164	0.54	1.94
3	254 nm	$Y=10383X-31921$	0.9996	8.60–430.0	0.43	1.56
4	254 nm	$Y=12657X+7615.6$	1.0000	7.68–192.0	0.11	0.38
5	254 nm	$Y=7970.5X+30052$	0.9998	21.0–420.0	0.13	0.63
6	238 nm	$Y=10205X-57099$	0.9997	4.03–645.0	0.25	0.55
7	254 nm	$Y=10255X+245270$	0.9995	0.31–3095	0.28	0.55
8	ELSD	$y=1.4341x+2.8722$	0.9953	3.85–154.0	1.93	3.85

[a] The notation for analyte refers to Table 1.
[b] For DAD, the regression equation $Y=aX+b$, Y is the peak area while X is the concentration (μg/mL); for ELSD, the regression equation $y=ax+b$, y, x are the logarithmic values of area and concentration (μg/mL), respectively.

Table 3
Results of intra-, inter-day, precision, accuracy and repeatability.

Analyte[a]	Accuracy and precision						Repeatability ($n=5$)	
	Intra-day ($n=5$)			Inter-day ($n=5$)			Mean (μg/mL)	R.S.D. (%)
	Mean (μg/mL)	R.S.D. (%)	Accuracy[b] (%)	Mean (μg/mL)	R.S.D. (%)	Accuracy[b] (%)		
1	198.78	0.43	101.4	200.12	0.43	102.1	184.81	1.41
2	109.32	0.50	102.5	110.31	0.48	103.4	393.79	2.07
3	218.60	0.54	100.2	220.16	1.98	100.9	175.50	1.96
4	105.99	0.56	100.0	106.39	0.30	100.4	42.63	0.97
5	153.53	3.26	95.4	153.42	0.86	95.3	175.03	0.69
6	34.50	0.84	99.1	34.59	0.39	100.6	90.82	2.41
7	360.05	1.50	98.3	366.12	0.90	99.9	381.52	7.26
8	43.89	2.15	97.5	44.82	3.87	99.6	18.58	1.89

[a] The notation for analyte refers to Table 1.
[b] Concentration detected/concentration spiked × 100%.

3.6. PLS analysis on the fingerprint data of LDP

Partial least squares is a commonly used multivariate data analysis approach. Classification models were constructed by PLS, leading to show the variance of the formula prescriptions. PLS was utilized to classify the chromatographic fingerprint data of 19 water-honeyed pills, 37 concentrated pills and 4 capsules under 254 nm. As shown in Fig. 4, 60 samples in three dosage forms were well-separated by this method. Data standardization was processed, then a 60 (object) × 501 (variable) data matrix containing the absolute peak areas was submitted to PLS and the principal components (PCs) were calculated. In Fig. 4, the results of 2D-projection plot of PLS, where PC1 and PC2 altogether, accounted for 74.1% variance. It was suggested that three dosage forms of water-honeyed pills, concentrated pills and capsules, marked as "whp", "cp" and "c" respectively in the 2D-projection plot, were clearly clustered and separated each other. Various products with the same dosage form, despite their different manufacturers, were clustered closely, which suggested that PLS might be viewed as an effective tool for making discrimination successfully. It can be concluded that although some inevitable reasons, such as cultivation regions, harvesting times, storing processes and preparation pro-

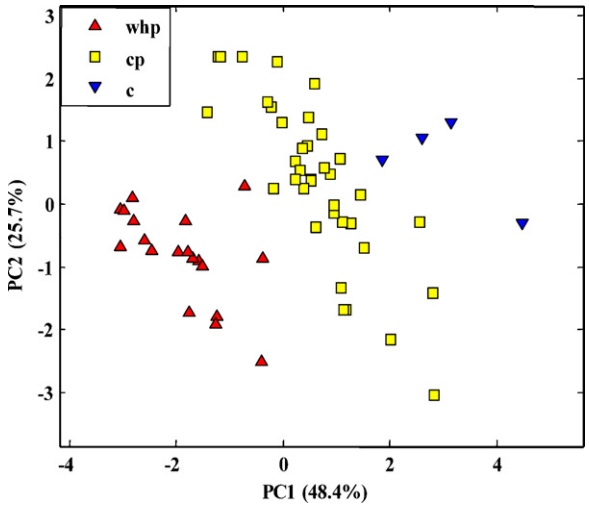

Fig. 4. PLS projection plot on the fingerprints of 60 batches of LDPs with various dosage forms: 'whp', 'cp' and 'c' represent water-honeyed pills, concentrated pills and capsules, respectively. Peak areas of 501 chemical markers are feed to PLS.

Table 4
Statistic results of recovery for extraction of analytes in LDP.

Analyte[a]	Spiked amount (mg)	Recorded amount (mg)	RSD (%)[b]	Calculated recovery (%)	Mean recovery (%)
1	0.196	0.198	0.67	101.5	100.2
	0.392	0.390	0.27	99.4	
	0.588	0.586	0.13	99.6	
2	0.107	0.107	1.55	99.6	96.8
	0.214	0.203	1.33	95.0	
	0.321	0.308	1.08	95.0	
3	0.218	0.220	1.26	101.5	100.4
	0.436	0.436	0.72	99.9	
	0.654	0.652	0.03	99.7	
4	0.106	0.109	1.19	103.5	101.3
	0.212	0.213	0.19	100.2	
	0.318	0.320	0.11	100.2	
5	0.161	0.162	0.58	100.6	104.6
	0.322	0.347	1.09	107.8	
	0.483	0.509	0.24	105.4	
6	0.035	0.033	5.40	95.5	93.5
	0.069	0.065	1.05	94.2	
	0.105	0.095	1.48	90.7	
7	0.401	0.403	0.25	100.5	97.3
	0.802	0.774	0.60	96.5	
	1.203	1.140	2.38	94.8	
8	0.045	0.045	1.03	100.5	100.8
	0.090	0.092	2.37	102.5	
	0.134	0.133	0.73	99.5	

[a] The notation for analyte refers to Table 1. Triplicate assay at each concentration level.
[b] Calculated recovery (%) = (amount found − original amount)/amount spiked × 100%.

cedures of six raw medicinal materials, influence the contents of ingredients seriously, its dosage form could be considered as one of the most important affect factors.

On the other hand, PLS was used to discriminate LDPs of the same dosage form from five noted manufacturers. After the data standardization, a 29 (object) × 501 (variable) data matrix yielded a result of 71.5% variance from the PC1 versus PC2 biplot (PC1 = 36.3%, PC2 = 35.2%) (Fig. 5). In our study, the within-class scatter of samples manufactured by Tongren Tang Pharmaceutical Co. Ltd. was small, which indicated qualities of its products were much more consistent and reliable compared with other four manufacturers.

To investigate the model performance, 26 samples of water-honey pills group and concentrated pills group (the 'training set') were randomly selected to construct a PLS–DA model, and the remaining 30 samples (the 'test set') were tested. Fig. 6 shows that these two dosage forms have been successfully classified. The discrimination equation with prediction accuracy of 93.33% was obtained and it was calculated as

$$F = -2.416484\,t1 - t2 + 7381122$$

where $t1$ (refers to peak area of GA), $t2$ (refers to peak area of PN) were the first two PCs. If the F value of an unknown LDP was positive, it was classified into the concentrated pills group; otherwise, it belonged to the water-honeyed pills group. The classification

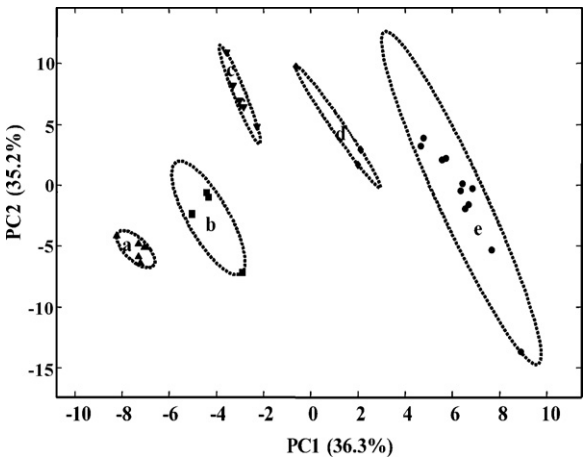

Fig. 5. PLS projection plot on the fingerprints of concentrated pills from five different manufacturers: (a) Tongren Tang Pharmaceutical Co., Ltd., (b) Jiuzhi Tang Pharmaceutical Co., Ltd., (c) Fuoci Pharmaceutical Co., Ltd., (d) Tonghanchun Tang Herb factory and (e) Wanxi Pharmaceutical Co., Ltd.

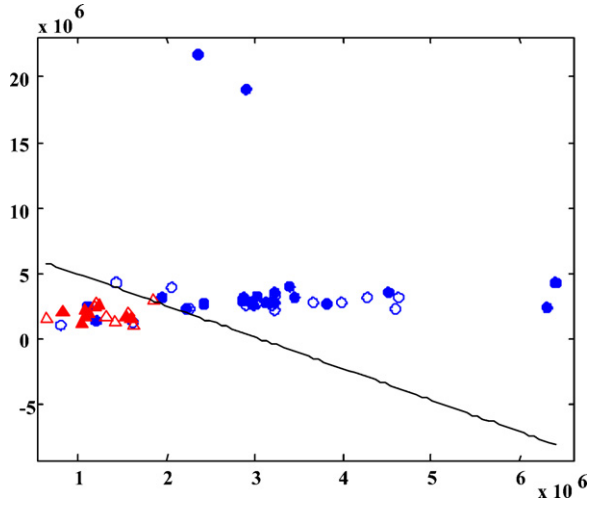

Fig. 6. PLS–DA model for the prediction of water-honeyed pills and concentrated pills: discrimination line was constructed and the discrimination equation was "$F = -2.416484 \times t1 - t2 + 7381122$"; peak areas were feed to PLS–DA as input data; solid circle, hollow circle, solid diamond and hollow diamond denote water-honeyed pill for test set and training set, concentrated pill for test set and training set, respectively.

Table 5
Contents (mg/g) of eight bioactive components from 60 batches of LDP.

Dosage form	Batch no.	Contents (mg/g)							
		GA	5HMF	MR	SR	LG	PF	PN	AB-23A
Water-honey pill	1	0.83	2.06	1.54	0.28	0.98	0.99	1.37	0.13
	2	2.10	3.50	2.49	0.59	1.84	1.21	4.00	0.12
	3	0.69	1.41	1.53	0.29	0.92	0.83	1.90	0.22
	4	0.78	1.65	1.46	0.30	0.94	1.40	2.29	0.26
	5	0.53	1.38	1.22	0.19	0.68	0.94	1.82	0.18
	6	0.76	1.95	1.58	0.30	1.00	1.38	2.41	0.30
	7	0.75	1.07	1.47	0.26	0.91	0.89	2.14	0.19
	8	0.68	3.69	1.36	0.36	0.98	1.32	2.43	0.27
	9	0.70	1.28	1.61	0.31	1.08	0.75	1.34	0.11
	10	0.58	2.66	1.32	0.43	0.92	1.39	2.05	0.17
	11	0.42	2.12	0.83	0.17	0.54	1.17	1.24	0.11
	12	0.71	1.49	1.05	0.23	0.76	0.97	1.33	0.12
	13	0.55	0.57	1.78	0.38	1.17	0.89	1.09	0.23
	14	0.48	0.70	1.70	0.40	1.32	1.40	1.64	0.22
	15	1.00	10.08	1.37	0.33	1.11	0.74	1.21	0.18
	16	0.56	3.25	1.51	0.38	1.17	1.06	1.71	0.18
	17	1.02	3.29	1.21	0.32	0.83	0.80	0.85	0.20
	18	1.74	2.26	1.46	0.34	1.15	1.28	2.32	0.21
	19	0.66	3.57	1.50	0.26	0.89	0.63	0.86	0.08
Concentrated pill	20	1.99	4.91	2.31	0.58	1.99	0.77	2.90	0.10
	21	1.82	3.72	2.44	0.64	2.14	0.98	2.63	0.14
	22	2.13	4.13	2.35	0.59	1.82	0.94	2.83	0.08
	23	1.78	3.94	2.36	0.53	2.05	1.01	2.65	0.06
	24	2.06	3.54	2.38	0.79	2.01	0.91	3.20	N.D.[a]
	25	2.00	5.75	2.00	0.50	1.86	1.32	2.46	0.11
	26	1.97	4.92	2.04	0.49	1.78	1.51	2.41	0.11
	27	1.78	6.43	1.65	0.44	1.55	0.89	2.12	0.11
	28	1.22	3.92	0.99	0.29	0.93	1.14	2.59	0.10
	29	1.80	5.38	0.92	0.51	1.82	1.37	2.21	0.06
	30	1.85	5.14	1.83	0.46	1.67	1.22	2.28	0.09
	31	1.98	4.65	1.92	0.50	1.75	1.61	2.27	0.13
	32	1.80	5.04	2.08	0.49	1.89	1.39	2.31	0.10
	33	1.95	5.46	1.96	0.56	1.85	1.55	2.83	0.13
	34	1.68	5.45	1.88	0.71	1.87	1.18	2.84	0.11
	35	0.68	0.64	0.23	0.19	N.D.	0.06	18.34	0.04
	36	2.64	3.11	2.06	0.46	1.63	0.69	2.78	0.07
	37	1.95	6.29	1.61	0.57	1.59	1.83	2.84	0.15
	38	1.85	3.75	1.50	0.38	1.39	1.40	2.43	0.11
	39	1.02	3.68	0.70	0.14	0.69	0.19	0.96	0.05
	40	0.90	3.93	0.90	0.17	0.89	0.15	1.66	0.06
	41	1.29	1.33	2.99	0.50	2.14	0.61	2.42	0.06
	42	1.87	2.86	2.32	0.56	2.38	0.71	2.48	0.11
	43	1.52	0.56	2.77	0.62	2.24	0.90	3.53	0.09
	44	0.52	0.39	0.93	0.13	0.68	0.22	0.76	0.04
	45	0.70	0.66	1.28	0.22	0.90	0.45	1.03	0.05
	46	1.40	5.46	0.59	0.21	0.95	1.22	1.94	0.10
	47	1.25	3.25	1.50	0.54	1.17	1.10	2.41	0.11
	48	0.76	2.48	0.54	0.15	0.50	0.45	1.04	0.06
	49	3.88	3.51	2.29	0.81	2.61	2.04	3.34	0.16
	50	3.29	3.86	2.04	0.75	2.48	1.31	3.96	0.20
	51	1.50	4.21	1.88	0.52	1.94	0.61	2.35	0.06
	52	1.28	4.43	1.89	0.52	2.26	0.06	1.85	0.13
	53	2.13	1.34	2.12	0.59	2.01	1.05	2.04	0.14
	54	1.88	1.27	2.67	0.52	2.40	0.65	2.87	N.D.[a]
	55	2.26	2.81	2.59	0.49	2.05	1.61	2.47	0.11
	56	1.39	3.94	2.52	0.51	2.06	0.11	2.06	0.13
Capsule	57	1.50	6.33	1.63	0.67	1.89	0.80	28.35	0.09
	58	2.31	2.65	2.06	0.58	1.65	0.82	23.83	0.11
	59	0.07	1.46	0.33	0.25	N.D.	0.17	20.99	0.04
	60	2.13	5.66	1.99	0.36	1.55	0.86	26.37	0.15

[a] Not detected.

results indicate that constructed PLS–DA model can make good prediction of two dosage forms (water-honeyed pills and concentrated pills) and it thus could be applied for the discrimination of LDPs.

4. Conclusion

An HPLC-DAD-ELSD combined with HPLC-ESI-MS method has been firstly established for qualitative and quantitative analysis of eight bioactive components in 60 batches of LDP. PLS and PLS–DA approaches applied on chromatographic fingerprint data obtained using HPLC-UV techniques allow to cluster LDPs, classify different manufacturers and able to lead us to make prediction between water-honeyed pills and concentrated pills. The results remind us that not only the strictly quality regulation during the preparation procedure is important, but also the quality control of Chinese medical materials is indispensable. Compared to the method previously

reported, this study could be utilized to the alternative approach for the quality evaluation of LDPs.

Acknowledgements

This work was supported by program for Changjiang Scholars and Innovative Research Team in University (PCSIRT), NCET Foundation, NSFC (30725045), National 863 Program (2006AA02Z338), China Postdoctoral Science Foundation (20070410711), "973" program of China (2007CB507400), Shanghai Leading Academic Discipline Project (B906) and in part by the Scientific Foundation of Shanghai China (07DZ19728, 06DZ19717, 06DZ19005).

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