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Simultaneous analysis of eight bioactive compounds in Danning tablet by HPLC-ESI-MS and HPLC-UV

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Abstract

A high performance liquid chromatography (HPLC) coupled with electrospray tandem mass spectrometry (ESI-MS) and ultraviolet detector (UV) has been developed for the simultaneous analysis of eight bioactive compounds in Danning tablet (including hyperin, hesperidin, resveratrol, nobiletin, curcumine, emodin, chrysophanol, and physcion), a widely used prescription of traditional Chinese medicine (TCM). The chromatographic separation was performed on a ZORBAX Extend C_{18} analytical column by gradient elution with acetonitrile and formate buffer (containing 0.05% formic acid, adjusted with triethylamine to pH 5.0) at a flow rate of 0.8 ml/min. The eight compounds in Danning tablet were identified and their MS^n fractions were elucidated by using HPLC-ESI-MS, and the contents of these compounds were determined by using HPLC-UV method. The standard calibration curves were linear between 5.0 and 100 μg/ml for hyperin, 10–200 μg/ml for hesperidin, 1.0–150 μg/ml for resveratrol, 2.0–120 μg/ml for nobiletin, 2.0–225 μg/ml for curcumine, 20–300 μg/ml for emodin, 2.0–200 μg/ml for chrysophanol, and 20–250 μg/ml for physcion with regression coefficient $r^2 > 0.9995$. The intra-day and inter-day precisions of this method were evaluated with the R.S.D. values less than 0.7% and 1.3%, respectively. The recoveries of the eight investigated compounds were ranged from 99.3% to 100.2% with R.S.D. values less than 1.5%. This method was successfully used to determine the 8 target compounds in 10 batches of Danning tablet.

Keywords: Danning tablet; HPLC-ESI-MS; HPLC-UV; Simultaneous analysis

1. Introduction

Danning tablet, as a composite prescription of traditional Chinese medicine (TCM), comprises extracts of seven medicinal materials, including *Radix et Rhizoma Rhei*, *Rhizoma Polygoni Cuspidati*, *Pericarpium Citri Reticulatee*, *Pericarpium Citri Reticulatae Viride*, *Radix Curcumae*, *Fruus Crataegi* and *Rhizoma Imperatae* [1]. In recent clinical application, these medicinal materials are widely used in TCM and pharmaceutical preparations. The versatile biomedical effects of them have been intensively investigated, for example, to treat cholecystitis, dyspepsia, pancreatitis, asthma, dysentery and thrombocytopenia [2]. According the theory of TCM, Danning tablet was prepared by these medicinal materials to cure the disease of the gallbladder and other diseases as hepatitis, fever, constipation, non-alcoholic fatty liver [3–5]. It was validated that these medicinal materials, used in one formula, had strong therapeutical effects in clinical application. However, on the other hand, it enhances the complexity of the constituents and preparation procedures, which makes it difficult to ensure the batch-to-batch uniformity of Danning tablet. Thus, the qualitative identification and quantitative measurement of its bioactive components are extraordinary necessary during the preparation and application of this prescription.

At present, the quality control of Danning tablet is mainly conducted according to China Pharmacopoeia (vol. 1, Edition 2005), in which only two anthraquinones, emodin and chrysophanol, are determined by high performance liquid chromatography-ultraviolet detector (HPLC-UV) method with isocratic elution [1]. This method has limitations and it is not enough to reveal the complexity and synergic effect of this formula, and sometimes leads to a certain biased assessment of the investigated systems. For a composite formula of Danning tablet, it seems necessary to determine not only two target com-

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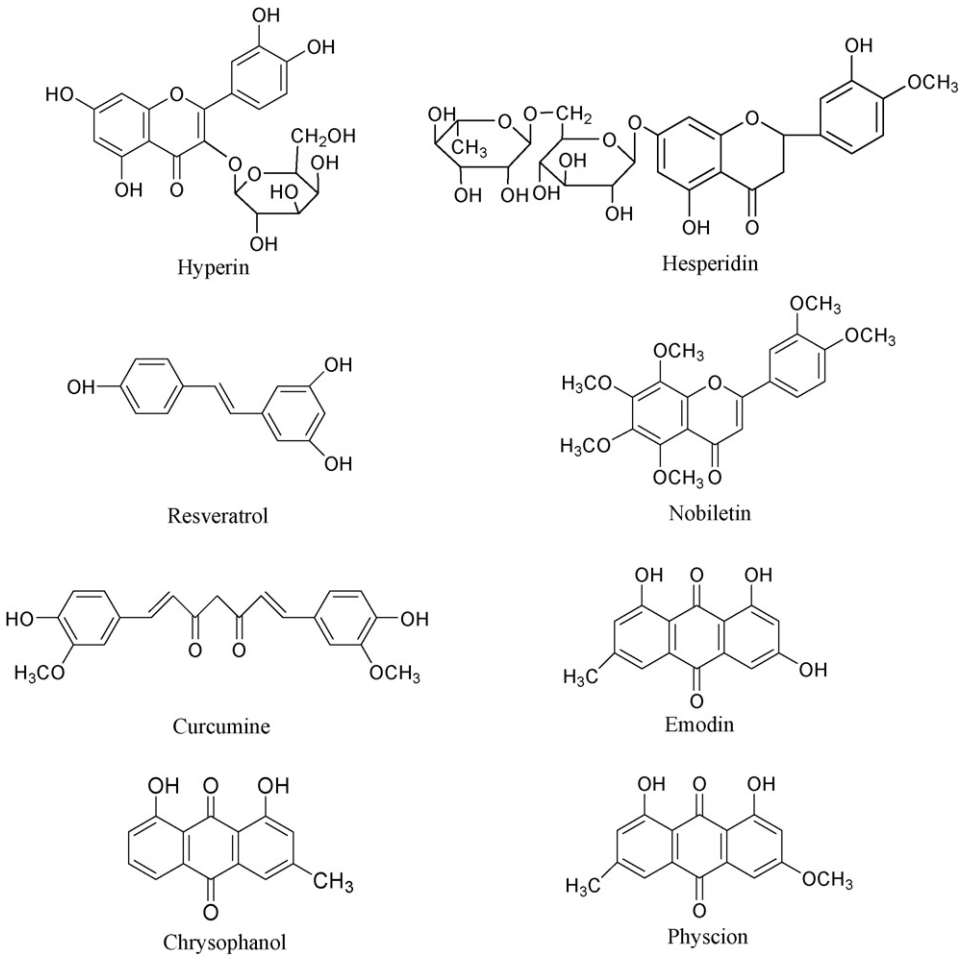

Fig. 1. Chemical structures of the eight investigated compounds from Danning tablet.

pounds, but also the bioactive compounds derived from various medicinal materials to ensure the uniformity of their extraction procedures. As reported previously [6–8], the eight components in Danning tablet, including hyperin, hesperidin, resveratrol, nobiletin, curcumine, emodin, chrysophanol and physcion, are derived from six different medical materials and have been considered as bioactive components contributing to the therapeutic effects of Danning tablet (structures shown in Fig. 1). Therefore, it is significant to determine the eight components to ensure the quality of Danning tablet. Up to now, several methods have already been developed for chemical fingerprinting and quantitative analysis of these compounds, such as HPLC method [9–11], gas chromatography (GC) [12,13], capillary electrophoresis (CE) [14,15], high performance thin layer chromatography (HPTLC) [16], etc. To our knowledge there was no analytical method for the simultaneous determination of bioactive compounds in Danning tablet.

In recent years, methods for multi-component analysis have increasingly been founded as a credible solution for the analysis of a complex system in TCM. Multi-component analysis of bioactive compounds in TCM has been reported with high resolution and wide application [17–19] and there were also a few reports about the multi-component analysis of composite prescription such as, Shexiang Baoxin Pill [20], Fufang Danshen Tablet [21], Qingkailing Injection [22], etc.

In this paper, a simple HPLC-ESI-MS-UV method was proposed for validation and quantification of the 8 bioactive compounds in 10 batches of Danning tablet, including hyperin, hesperidin, resveratrol, nobiletin, curcumine, emodin, chrysophanol and physcion. By using the described method, the eight compounds from 70% methanol extract of Danning tablet were identified and determined. The proposed method could be readily utilized to determine the bioactive compounds from each medicinal material in one run and was suggested to control the quality of Danning tablet during its preparation and application.

2. Experimental

2.1. Reagents and materials

HPLC-grade acetonitrile, methanol, triethylamine and formic acid were purchased from Merck Company Inc. (Merck, Darmstadt, Germany). Ultrapure water was prepared by a Milli-Q50 SP Reagent Water System (Millipore Corporation, MA, USA) for the preparation of samples and buffer solutions. Other reagents were of analytical grade.

The eight reference standards (hyperin, hesperidin, resveratrol, nobiletin, curcumine, emodin, chrysophanol, and physcion) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and their chemical structures (shown in Fig. 1) were identified by the ESI-MS and previous reports [12–19]. Ten batches of Danning tablet were friendly provided by Shanghai Hutchison Pharmaceuticals (Shanghai, China) at March 2006.

2.2. Preparation of standard solutions

Each standard substance (including hyperin, hesperidin, resveratrol, nobiletin, curcumine, emodin, chrysophanol and physcion) was accurately weighted, then dissolved in methanol and diluted to appropriate concentration, respectively. A set of standard solutions were prepared by the appropriate dilution of the stock solution with methanol, containing 5.0–100 μg/ml for hyperin, 10–200 μg/ml for hesperidin, 1.0–150 μg/ml for resveratrol, 2.0–120 μg/ml for nobiletin, 2.0–225 μg/ml for curcumine, 20–300 μg/ml for emodin, 2.0–200 μg/ml for chrysophanol, and 20–250 μg/ml for physcion. All the solutions were stored in the refrigerator at 4 °C before analysis.

2.3. Preparation of samples

After removing the coating, Danning tablets were dried at 60 °C until constant weight. Each dried material was ground to 100 mesh. Approximately 100 mg pulverized powder was accurately weighted and then extracted with 8 ml 70% methanol by ultrasound for 45 min in a 10 ml volumetric flask. The solution was cooled down to the ambient temperature, and 70% methanol was added into the volumetric flask to 10 ml. The supernatant solution was filtrated through a syringe filter (0.45 μm) and aliquots (20 μl) were subjected to HPLC analysis.

2.4. HPLC-DAD-ESI-MS analysis

An Agilent-1100 HPLC system with photodiode array detector (DAD) was coupled with an LC/MSD Trap XCT electrospray ion mass spectrometer, equipped with quaternary pump, vacuum degasser, auto sampler, column heater-cooler (Agilent Corporation, MA, USA). The chromatographic separation was performed on a ZORBAX Extend C_{18} analytical column (250 mm × 4.6 mm, 5 μm, Agilent Corporation, MA, USA) with the column temperature set at 25 °C. A linear gradient elution of A (formate buffer, consisting of 0.05% formic acid, adjusted to pH 5.0 with ammonia) and B (100% acetonitrile) was used with the gradient elution as followed (v/v): 0 min, B 15%; 40 min, B 35%; 50 min, B 50%; 60 min, B 100%, hold for 10 min. The flow rate was 0.8 ml/min, and the injection volume was 20 μl. By solvent splitting, 0.2 ml/min portion of the column effluent was delivered into the ion source of mass spectrometry. The ESI-MS spectra were acquired in positive and negative ion mode to produce $[M+Na]^+$, $[M+H]^+$ and $[M-H]^-$ ions. The conditions were as follows: drying gas N_2 8 l/min, temperature 350 °C, pressure of nebulizer 30 psi, HV voltage 3.5 kV and scan range 100–800 u. Data acquisition was performed using Chemstation software (Agilent Corporation, MA, USA).

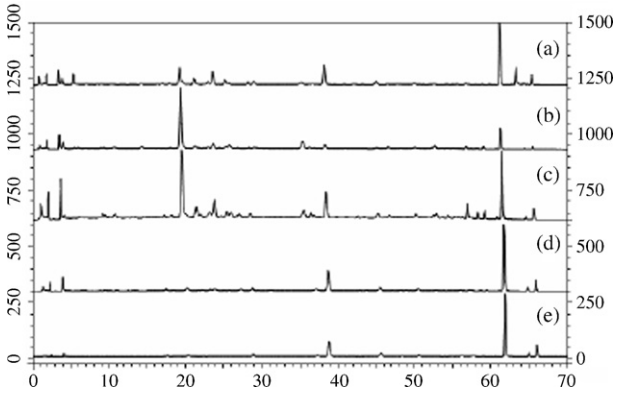

Fig. 2. HPLC chromatograms of Danning tablet (lot no. 040608) by DAD detector at the wave length of (a) 280 nm, (b) 314 nm, (c) 346 nm, (d) 375 nm and (e) 412 nm.

2.5. HPLC-UV analysis

A LC2010AHT HPLC system coupled with UV detector was used (Shimadzu Corporation, Kyoto, Japan) for quantitative determination of the 10 batches of samples and the 8 standard substances. The chromatographic conditions were performed as described above except that the formate buffer was adjusted to pH 5.0 by triethylamine instead of ammonia so as to obtain better retention feature of the target compounds during analysis. The detection wavelength was set at 346 nm. Data acquisition was performed using a CLASS-*VP* workstation (Shimadzu Corporation, Kyoto, Japan).

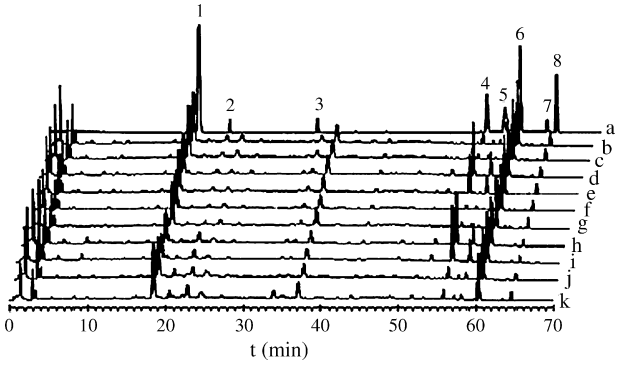

Fig. 3. HPLC chromatograms of (a) standard mixture and 10 batches of Danning tablet (b–k). The eight standard substances are: (1) hyperin, (2) hesperidin, (3) resveratrol, (4) nobiletin, (5) curcumine, (6) emodin, (7) chrysophanol, and (8) physcion. The batch numbers (lot no.) were (b) 050205, (c) 050204, (d) 050111, (e) 050110, (f) 050108, (g) 050107, (h) 041209, (i) 041208, (j) 040608 and (k) 040106, respectively. Chromatographic conditions: analytical column, ZORBAX Extend C_{18} column (250 mm × 4.6 mm, 5 μm); mobile phase, linear gradient elution of A (formate buffer consisting of 0.05% formic acid, adjusted to pH 5.0 with triethylamine) and B (100% acetonitrile) with gradient procedure as followed (v/v): 0 min, B 15%; 40 min, B 35%; 50 min, B 50%; 60 min, B 100%, hold for 10 min; flow rate, 0.8 ml/min; column temperature, 25 °C and injection volume, 20 μl.

3. Result and discussion

3.1. Optimization of chromatographic separation

The optimization of experimental conditions was guided by the requirement of obtaining chromatograms with better resolution of adjacent peaks, especially when numerous similar components were to be analyzed. Because the ingredients in sample could not be separated with isocratic HPLC elution, gradient elution was carried out. Optimized chromatographic conditions were achieved after several trials with elution systems of acetonitrile–water, methanol–water, acetonitrile–formate buffer and methanol–formate buffer in various proportions. It was found that the presence of formate buffer (0.05% formic acid adjusted to pH 5.0 with triethylamine or ammonia) in mobile phase lead to a significant improvement on the retention behavior of the different component in Danning tablet, and otherwise, the peaks were rather broad with poor separation. The optimal mobile phase, consisting of acetonitrile–0.05% formate buffer (adjusted to pH 5.0 with triethylamine), was subsequently employed, which leads to good resolution and satisfactory peak shape. To avoid the interference of $[M+H]^+$ (m/z 102) from triethylamine during LC–MS analysis, the formate buffer was adjusted to pH 5.0 with ammonia instead of triethylamine, which made the target peaks broader but still separated well. It was observed that separation could not be affected by column temperature obviously, so the column temperature was set at 25 °C during analysis. DAD detection was employed at the wavelength range of 200–600 nm and the UV spectra of 70% methanol extract from Danning tablet were investigated. It was found that the chromatogram at 346 nm could properly represent the profile of the constituents (shown in Fig. 2), and showed good separation and high sensitivity. Under the proposed conditions, the 10 batches of samples were analyzed and their chromatograms are shown in Fig. 3.

3.2. Validation of eight components from Danning tablet

The MS spectra of major components from Danning tablet were acquired in positive and negative ion mode under the conditions mentioned above, and Table 1 listed their retention times (t_R), MS and MS^2 fragmentation ions. In MS spectra, the eight components exhibited their quasi-molecular ions $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$. As listed in Table 1, other fragmentations, for example, losing –CO, $–CH_3O$, $–CH_3$, etc., could also be observed from their MS^2 spectra. Their fragmentation patterns were well matched with their chemical structures. From the m/z values and retention features, the 8 components were identified from 70% methanol extract in 10 batches of Danning tablets. The results further revealed that the eight investigated

Table 1
Identification of the eight compounds by HPLC-ESI-MS

Peak	Compound	t_R (min)	Fractions in positive ion mode (m/z)		Fractions in negative ion mode (m/z)	
			MS	MS^2	MS	MS^2
1	Hyperin	20.3	–	–	462.1 $[M-H]^-$	301.0 $[M-$H-glucose$]^-$
2	Hesperidin	24.5	633.4 $[M+Na]^+$	–	609.3 $[M-H]^-$	–
3	Resveratrol	35.4	229.0 $[M+H]^+$	–	227.0 $[M-H]^-$	186.0 $[M-H\text{-}CH_3O]^-$
4	Nobiletin	56.6	403.4 $[M+H]+$	388.1 $[M+H\text{-}CH_3]^-$	402.3 $[M-H]^-$	387.3 $[M-CH_3]^-$ 325.3 $[M-CH_3\text{-}2CH_3O]^-$
5	Curcumine	58.8	391.3 $[M+Na]^-$ 369.2 $[M+H]^+$	245.1 $[M+H\text{-}C_7H_7O_2]^+$	367.1 $[M-H]^-$	217.0 $[M-H\text{-}C_9H_{10}O_2]^-$
6	Emodin	60.9	–	–	269.0 $[M-H]^-$	241.0 $[M-H\text{-}CO]^-$
7	Chrysophanol	64.0	255.4 $[M+H]^+$	199.5 $[M+H\text{-}C_3H_4O]^-$	253.4 $[M-H]^-$	–
8	Physcion	65.0	–	–	283.4 $[M-H]^-$ 255.5 $[M-H\text{-}CO]^-$ 227.4 $[M-H\text{-}2CO]^-$	199.4 $[M-H\text{-}3CO]^-$

Table 2
Statistical results of linear regression equation analysis in the determination of the eight compounds

Compound	Regression equation					
	Linear range (μg/ml)	Slope (a) (mean ± deviation, $n=3$)	Intercept (b) (mean ± deviation, $n=3$)	r^2 ($n=6$)	LOD (μg/ml)	LOQ (μg/ml)
Hyperin	5.0–100	$2.233\times10^4 \pm 7.781\times10^1$	$-2.395\times10^3 \pm 5.458\times10^2$	0.9996	0.21	0.53
Hesperidin	10–200	$4.923\times10^3 \pm 2.068\times10^1$	$-3.174\times10^4 \pm 9.225\times10^3$	0.9995	0.27	0.81
Resveratrol	1.0–150	$2.034\times10^4 \pm 6.124\times10^1$	$-2.768\times10^3 \pm 5.584\times10^2$	0.9998	0.25	0.80
Nobiletin	2.0–120	$8.292\times10^4 \pm 3.375\times10^2$	$-6.972\times10^3 \pm 1.981\times10^3$	0.9997	0.06	0.25
Curcumine	2.0–225	$2.242\times10^4 \pm 6.476\times10^1$	$-1.502\times10^2 \pm 1.721\times10^1$	0.9996	0.22	0.87
Emodin	20–300	$6.096\times10^3 \pm 2.640\times10^1$	$1.495\times10^4 \pm 2.952\times10^3$	0.9999	0.18	0.68
Chrysophanol	2.0–200	$3.632\times10^3 \pm 1.324\times10^1$	$6.902\times10^2 \pm 1.145\times10^2$	0.9997	0.32	0.93
Physcion	20–250	$4.088\times10^3 \pm 1.622\times10^1$	$1.533\times10^3 \pm 3.310\times10^2$	0.9999	0.30	0.84

In the regression equation $y=ax+b$, y refers to the peak area (A), x concentration of the reference standard substances (μg/ml), r^2 the correlation coefficient of the equation, LOD (the limit of detection, S/N = 3) and LOQ (limit of quantitation, S/N = 10) were expressed in concentration unit with injection volume 20 μl.

Table 3
Statistical results of recovery of the eight compounds ($n = 5$)

Compound	Added amount (mg)	Recorded amount (mg)	Recovery (%)	R.S.D. (%)
Hyperin	3.20	3.20 ± 0.05	99.7 ± 1.5	1.5
Hesperidin	3.21	3.19 ± 0.03	99.3 ± 1.0	1.0
Resveratrol	0.200	0.200 ± 0.003	99.7 ± 1.3	1.3
Nobiletin	1.71	1.70 ± 0.02	99.6 ± 1.2	1.2
Curcumine	0.402	0.402 ± 0.006	99.8 ± 1.4	1.4
Emodin	9.01	9.03 ± 0.04	100.2 ± 0.5	0.5
Chrysophanol	0.499	0.498 ± 0.006	99.9 ± 1.2	1.2
Physcion	1.69	1.69 ± 0.02	99.8 ± 1.3	1.3

compounds were the main constituents in Danning tablet. So it was extremely necessary to establish a feasible determination method to control its quality.

3.3. Method validation

3.3.1. Linearity

The linearity calibration curves were constructed by six concentration assays of each reference compound in triplicate. The regression equation was calculated in the form of $y = ax + b$, where y and x were the values of peak area and concentration of each reference compound, respectively. Results of the regression analyses and the correlation coefficients (r^2) were listed in Table 2. The high correlation coefficient values ($r^2 > 0.9995$) indicated good linearity between their peak areas (y) and investigated compound concentrations (x, μg/ml) in relatively wide concentration ranges. As listed in Table 2, the limits of detection (LOD) were determined with a signal-to-noise ratio of 3 and ranged from 0.06 to 0.32 μg/ml, while the limits of quantitation (LOQ) with signal-to-noise ratio of 10 ranged from 0.25 to 0.93 μg/ml. It showed a high sensitivity at these chromatographic conditions.

3.3.2. Precision

The reproducibility (relative standard deviation, R.S.D.) of the proposed method in terms of the peak-area in five replicate extraction samples (lot no. 040608) was detected in intra-day and inter-day ($n = 5$) for eight reference standards. The results showed that the intra-day and inter-day reproducibility (R.S.D.) of the eight investigated components were less than 0.7% and 1.3%, respectively.

3.3.3. Accuracy

The recoveries of the eight compounds were determined by the method of standard addition. Suitable amounts (about 50% of the content) of the eight standard substances were spiked into a sample of Danning tablet (lot no. 040608), which were determined previously. The mixture was extracted and analyzed by using the proposed procedure. For comparison, an unspiked sample was prepared and analyzed simultaneously. As shown in Table 3, the mean recoveries of the compounds were 99.3–100.2%, with R.S.D. values ranged from 0.5% to 1.5% ($n = 5$).

Table 4
Contents (mg/g) of the 8 compounds in 10 batches of Danning tablet (mean ± deviation, $n = 3$)

Compound	Content of each compound in 10 batches of Danning tablet (mg/g)										Average content
	040106	040608	041208	041209	050107	050108	050110	050111	050204	050205	
Hyperin	8.70 ± 0.21	6.35 ± 0.12	4.29 ± 0.15	4.70 ± 0.13	7.68 ± 0.13	7.92 ± 0.24	6.05 ± 0.11	6.21 ± 0.15	7.50 ± 0.22	7.36 ± 0.23	6.68
Hesperidin	9.03 ± 0.15	6.50 ± 0.09	7.65 ± 0.19	6.53 ± 0.18	4.12 ± 0.09	3.89 ± 0.12	3.75 ± 0.14	3.80 ± 0.09	4.21 ± 0.15	5.57 ± 0.21	5.50
Resveratrol	1.53 ± 0.04	0.42 ± 0.02	0.62 ± 0.03	0.59 ± 0.03	0.40 ± 0.02	0.41 ± 0.03	0.27 ± 0.01	0.50 ± 0.04	0.45 ± 0.03	0.30 ± 0.01	0.55
Nobiletin	2.70 ± 0.06	3.29 ± 0.07	16.37 ± 0.37	13.01 ± 0.29	1.33 ± 0.03	1.37 ± 0.08	9.37 ± 0.18	14.07 ± 0.27	1.29 ± 0.05	2.68 ± 0.07	6.55
Curcumine	0.57 ± 0.03	0.72 ± 0.05	2.51 ± 0.08	1.99 ± 0.04	0.29 ± 0.01	0.30 ± 0.02	1.54 ± 0.10	2.25 ± 0.11	0.29 ± 0.01	0.51 ± 0.02	1.10
Emodin	16.60 ± 0.31	18.15 ± 0.42	19.46 ± 0.41	15.19 ± 0.29	18.78 ± 0.28	19.13 ± 0.32	17.42 ± 0.34	17.98 ± 0.34	18.03 ± 0.34	19.58 ± 0.35	18.03
Chrysophanol	1.21 ± 0.03	0.95 ± 0.03	1.06 ± 0.07	1.23 ± 0.04	0.92 ± 0.03	0.99 ± 0.04	0.96 ± 0.03	0.94 ± 0.04	0.89 ± 0.03	0.95 ± 0.03	1.01
Physcion	4.30 ± 0.17	3.46 ± 0.13	3.49 ± 0.15	2.81 ± 0.05	5.56 ± 0.11	5.80 ± 0.16	4.68 ± 0.21	4.56 ± 0.15	5.45 ± 0.16	5.63 ± 0.18	4.57

Lot number of each batch was expressed according to the manufacture date by the form of year-month-day.

3.3.4. Determination of eight components in Danning tablet

The 8 components in 10 batches of Danning tablet were simultaneously determined by the proposed HPLC-UV method at the conditions described above. The quantitative analyses were performed by means of the external standard methods. Data of the quantitative analyses were expressed as mean ± deviation (listed in Table 4).

The results showed that the content of hesperidin in four batches (lot no. 040106, lot no. 040608, lot no. 041208, lot no. 041209) was much higher than the others; the content of resveratrol in lot no. 040106 was higher than the other nine batches; the contents of nobiletin showed the most difference from batch to batch. Even there were obvious differences among batches, the eight investigated compounds were determined out in each of Danning tablet. It was obvious that the pharmacological activities would make distinctions for the differences of their contents in batches. Further researches might be carried on to define the limit of content for each compound in Danning tablet according to pharmacological tests so as to validate its therapeutic effects.

The variance of contents for the investigated compounds had no relationship with store time (lot number shows the manufacture date by the form of year-month-day), and their difference might come from the variance of medicinal material. For example, according to the manufacture record, the contents of nobiletin in lot nos. 041208 and 041209 were varied for the different medicinal materials used to prepare them. It also could conclude from the similar contents of the eight compounds in closely manufactured batches (lot nos. 050110 and 050111) prepared with similar material. The results strongly reminded us that it was important to strictly control the quality of these medicinal materials firstly.

As we all known, the quality control of Danning tablet is mainly conducted according to the official criterion recorded in China Pharmacopoeia (vol. 1, Edition 2005) [1] and only two anthraquinones (emodin and chrysophanol) are determined by HPLC method with isocratic elution. This method has limitations to reveal the complexity of this formula. By the proposed method, the bioactive compounds, mainly belonging to two categories: anthraquinone and flavone, are quantitatively determined and the batch to batch uniformity is analyzed in one run. It elucidates the bioactive compounds derived from various medicinal materials and ensures the efficacy of clinical application.

4. Conclusion

The proposed quantification method could determinate the eight bioactive compounds in Danning tablet simultaneously. Compared to the method previously reported, it could be applied as a convenient, effective technique to control the quality of Danning tablet. This study provided an approach to develop a bioactive chromatographic profile of major compounds to ensure the quality of commercial Danning tablet. Since multiple constituents are responsible for the therapeutic effects of TCM and its prescriptions, the ingredients and their contents in TCM may affect therapeutic effect extremely. It strongly reminded us to systematically control the content of bioactive compounds in TCM so as to insure its therapeutic effects during preparation and application.

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