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Simultaneous Determination of Six Active Ingredients of Sanhuang Tablet by HPLC

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An HPLC method for the simultaneous determination of six active ingredients present in famous Chinese clinical prescription has been established. HPLC conditions included Agilent Hypersil-C_{18} column (4.6 mm × 250 mm, 5 µm) and the mobile phase was a mixture of acetonitrile and 0.1 % phosphoric acid for gradient elution. The flow rate was 1.0 mL/min and the column temperature was at 30 ºC. Six active ingredients had good linear relationships: berberine in the range of 0.0081-0.4045 µg (R^2 = 0.9997), aloe-emodin in the range of 0.0076-0.3820 µg (R^2 = 0.9999), rhein in the range of 0.0104-0.5175 µg (R^2 = 0.9998), emodin in the range of 0.0122-0.6110 µg (R^2 = 0.9999), chrysophanol in the range of 0.0214-1.0690 µg (R^2 = 0.9999) and physcion in the range of 0.0057-0.2830 µg (R^2 = 0.9994). Their average recoveries were 104.73 % (RSD = 1.6 %), 95.19 % (RSD = 2.3 %), 104.31 % (RSD = 2.0 %), 101.72 % (RSD = 2.7 %), 95.50 % (RSD = 2.2 %) and 103.08 % (RSD = 2.7 %), respectively. This method was steady with high precision and good repeatability and could be used for the determination of the six active ingredients and quality control of Sanhuang tablet.

Key Words: HPLC, Sanhuang tablet, Berberine, Aloe-emodin, Rhein, Emodin, Chrysophanol, Physcion.

INTRODUCTION

Sanhuang Tablet was developed based on the Xiexin Decoction in the book called Medical Treasures of the Golden Chamber written by the famous medical scientist Zhang Zhongjing of Han Dynasty[1], composing of three traditional Chinese medicine, including *Rhizoma coptidis*, Radix et *Rhizoma Rhei* and *Radix Scutellariae* with bitter cold properties, possessing the functions of clearing away heat and removing toxicity, reducing fire and relaxing the bowels[2]. In 1958, Handan Pharmaceutical Industry developed the prescription into Sanhuang Tablet, simultaneously, *Rhizoma coptidis* was replaced by berberine hydrochloride, which not reinforced the function of antibacterial anti-inflammatory effect, but also strengthened the effect of reducing fire of *Radix et Rhizoma Rhei*. Mainly used against redness and pain of the eye, swelling and pain in throat, swelling and aching of gum, dark urine, constipation acute gastroenteritis and dysentery[3], caused by excessive heat in three jiao. Clinically, it can be used to treat conjunctivitis, infantile acute canker, acute tonsillitis acute and chronic gastroenteritis, jaundice, hypertension *etc*.[4]. Modern pharmacology has proved that the active ingredients in Sanhuang tablet are mainly berberine and anthraquinones, such as baicalin has the function of antiinflammatory and antiviral, emodin and chrysophanol has the function of dredging and regulating stasis and antibacterial anti-inflammatory effect[5-8]. In addition, the metabolin of rhein called rhein anthrone has the effect of purging excessive heat. By referring to the method of detection of rheum on Chinese pharmacopoeia 2010 Version[8], the experiment established the method for the simultaneous determination of the six active ingredients (berberine, aloe-emodin, rhein, emodin, chrysophanol and physcion) in Sanhuang Tablet. This method was stable with high precision, which could be used for the determination of the six ingredients and quality control of Sanhuang tablet.

EXPERIMENTAL

Agilent 1100 series HPLC system (Angilent Technologies, USA) was equipped with quaternary pump (G1311A), automatic sampler (G1313A), UV variable-wavelength detector (1314A-UV) and column oven (CTO-10ASVP); Pa2251 electronic analytical balance from Sartorius Group of Germany; Tw20 constant temperature bath box from Julabo Labortechnik GmbH company of Germany.

Standards of berberine, aloe-emodin, rhein, emodin, chrysophanol and physcion were supplied by National Institute for Food and Drug Control (NIFDC) (Beijing, China) with the batch number of 110713-200911, 110795-200203, 110757-200206, 110756-200210, 110796-200309, 110758-200307, respectively. Sanhuang tablet was purchased from three different company - Fusen Pharmaceutical Co., Ltd. (Henan, China), Bainian Kangxin Pharmaceutical Co., Ltd. (Henan), Hua tuo traditional Chinese medicine Co., Ltd. (Anhui, China), respectively, with the batch number of 110303, 111102, 120303. Acetonitrile was of HPLC grade and other reagents used were of analytical grade. Deionized water was prepared using a Millipore water purification system.

HPLC conditions: The separation of the six ingredients was carried out on an Agilent Hypersil-C_{18} (250 mm × 4.6 mm, 5 μm). The solvents used for HPLC separation were acetonitrile (A) and 0.1 % phosphoric acid (B) at a flow rate of 1.0 mL/min with gradient elution (0-15 min, 25-29 % A; 15-17 min, 29-42 %A; 17-35 min, 42-45 %A; 35-45 min, 45-87 %A; 45-50 min, 87 %A) and the analysis was monitored at 270 nm with the column temperature of 30 ºC and the injection volume was 10 μL.

Preparation of standard solution: Certain amounts of the six standard compounds were dissolved with methanol into volumetric flasks, respectively. Then measured 1.7 mL berberine, 0.4 mL aloe-emodin, 0.7mL rhein, 1.1mL emodin, 1.9 mL physcion and 1.4 mL chrysophanol standard solution, respectively, into a 10 mL volumetric flask and diluted to the concentrations of 275.4, 206.4, 81.4, 155.5, 115.6 and 40.48 μg/mL.

Preparation of sample solution: Thirty Sanhuang tablets were pulverized into fine powder, *ca.* 0.3 g sample was accurately weighted, then added into a 150 mL conical flask. Accurate 20 mL 10 % hydrochloric acid and 30 mL trichloromethane were added into the flask and placed in constant temperature bath box for 2 h circulation reflux at 75 ºC. After refrigerating, the suspension was moved to a separating funnel for extraction with trichloromethane solvent 2 times, each time for 30 mL and merged the $CHCl_3$ layer into suspension instrument for recycling $CHCl_3$ and then dissolved the residue with methanol into a 50 mL volumetric flask and metered the volunm. The solution was ready for chromatographic analysis after passing through a 0.45 μm membrane filter.

RESULTS AND DISCUSSION

Linear range: Linear regression analysis for each component was performed by the external standard method. The above six compound solution was accurately injected 1, 2, 5, 10, 20 and 30 μL, respectively. The linearity of each compound was calculated by plotting the peak area (Y) *vs*. concentration (X). (Table-1). All the 6 components showed good linearities in wide concentration ranges.

Precision: The standard mixture solution of six components was injected into HPLC six times continuously and the area of each peak was used for the calculation of precision. The results showed that relative standard deviation (RSD) of peak area of each standard was 1.6, 0.4, 1.2, 0.6, 0.9 and 2.8 %, respectively (Fig. 1).

TABLE-1
LINEAR REGRESSION EQUATION AND LINEAR RANGES

Components	Regression equation	Correlation coefficient (R^2)	Linear range (μg mL^{-1})
Berberine	Y=3.67X+2.84	0.9997	0.0185-0.9225
Aloe-emodin	Y=2.93X+0.09	0.9999	0.0076-0.3820
Rhein	Y=3.47X-5.76	0.9998	0.0104-0.5175
Emodin	Y=4.25X-3.43	0.9999	0.0122-0.6110
Chrysophanol	Y=3.07X-10.42	0.9999	0.0214-1.0690
Physcion	Y=4.13X-4.34	0.9994	0.0057-0.2830

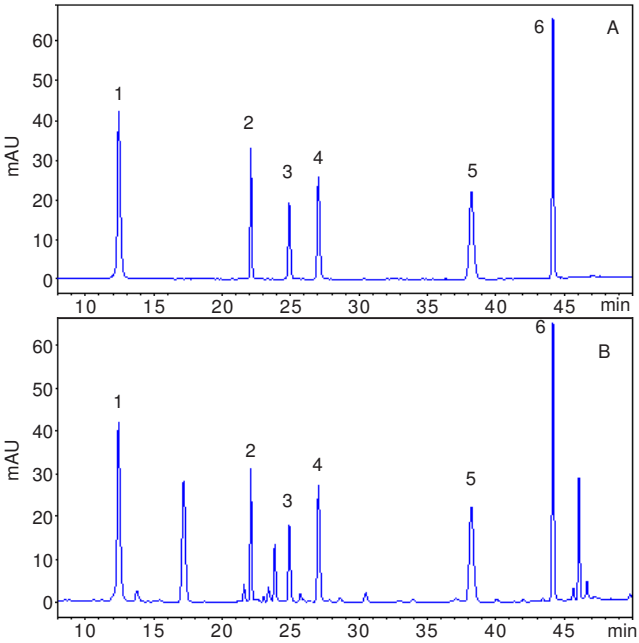

Fig. 1. HPLC chromatograms of the six ingredients mixture (A) and Sanhuang tablet (B). 1: Berberine; 2: Aloe-emodin; 3: Rhein; 4: Emodin; 5: Chrysophanol; 6: physcion

Stability: For stability test, the same sample solution was analyzed at designated time points in 24 h. The results showed that RSDs of peak area were 1.9, 0.8, 0.8, 0.8, 0.6 and 1.8 % and found to be stable for the experiment.

Repeatability: Repeatability was carried out using six samples solution after the same treatment procedure. The results showed that RSD of each peak area was 1.7, 1.3, 1.9, 1.7, 2.1 and 1.9 %, respectively.

Recovery test: The sample with known targeted contents was spiked with certain amounts of the 6 standards. Then the spiked sample was processed in accordance with the established method for the HPLC detection. The average recoveries for berberine, aloe-emodin, rhein, emodin, chrysophanol and physcion determined were 95.19-104.73 % (Table-2).

Application of the HPLC method for quantitation studies: The experiment determined three samples of different batches of the same manufacturer by the above method. The contents of the 6 components were shown in Table-3.

Optimization of HPLC separation conditions: In order to get a separation with better resolution of targeted components with shorter analytical time, we compared with three different column temperature: 20, 30 and 40 ºC, then we found that the higher temperature, the shorter retention time with almost the same resolution, so we chosen 30 ºC finally for protecting the lifespan of the column.

TABLE-2
RECOVERY RESULTS OF THE SIX ACTIVE INGREDIENTS

Component	Contents in samples (mg)	Added (mg)	Found (mg)	Recovery (%)	Mean (%)	RSD (%)
Berberine	0.4843	0.5508	1.049	102.52	104.73	1.6
	0.4766	0.5508	1.0469	103.54		
	0.4876	0.5508	1.0793	107.43		
	0.5614	0.5508	1.1617	108.98		
	0.5059	0.5508	1.111	109.32		
	0.4986	0.5508	1.0973	108.7		
Aloe-emodin	0.1798	0.2064	0.3713	92.77	95.19	2.3
	0.1770	0.2064	0.372	94.49		
	0.1811	0.2064	0.3768	94.83		
	0.2085	0.2064	0.4134	99.27		
	0.1890	0.2064	0.3847	94.8		
	0.1851	0.2064	0.3744	91.71		
Rhein	0.2842	0.2958	0.59	103.37	104.31	2.0
	0.2797	0.2958	0.5825	102.38		
	0.2862	0.2958	0.6063	108.2		
	0.3295	0.2958	0.6374	104.09		
	0.2987	0.2958	0.6227	109.53		
	0.2926	0.2958	0.5964	102.72		
Emodin	0.3103	0.3333	0.647	101.01	101.72	2.7
	0.3054	0.3333	0.6305	97.54		
	0.3124	0.3333	0.6647	105.69		
	0.3597	0.3333	0.6995	101.95		
	0.3260	0.3333	0.671	103.5		
	0.3194	0.3333	0.6548	100.64		
Chrysophanol	0.5753	0.5063	1.0524	94.25	95.5	2.2
	0.5662	0.5063	1.0306	91.74		
	0.5792	0.5063	1.0448	91.98		
	0.6669	0.5063	1.1549	96.39		
	0.6045	0.5063	1.0812	94.17		
	0.5923	0.5063	1.072	94.76		
Physcion	0.1567	0.1215	0.2821	103.19	103.08	2.7
	0.1542	0.1215	0.2737	98.39		
	0.1577	0.1215	0.2862	105.78		
	0.1816	0.1215	0.3096	105.35		
	0.1646	0.1215	0.2874	101.08		
	0.1613	0.1215	0.2876	103.95		

TABLE-3
CONTENTS DETERMINATION OF THE SIX ACTIVE INGREDIENTS IN 3 BATCHES

Batch	Berberine (mg/g)	Aloe-emodin (mg/g)	Rhein (mg/g)	Emodin (mg/g)	Chrysophanol (mg/g)	Physcion (mg/g)
111102	4.7	1.14	3.22	2.59	3.75	1.7
110303	3.23	1.47	1.94	2.1	1.98	1.07
120303	6.54	0.74	3.41	2.67	2.42	1.32

Besides, reflux extraction and ultrasonic extraction were investigated in the experiments. Considering the advantages of high efficiency, we chose reflux extraction as the way to prepare sample solution. In the meantime, we compared the efficiency of different elution solvents, namely methanol-phosphoric acid and acetonitrile-phosphoric acid, the results indicated that the latter was slightly better. We also tried different reflux time: 1, 2 and 3 h. Finally, taken the determination of targeted components into consideration, 2 h reflux extraction was better.

Six chromatographic columns of the same specification were compared in the experiment: Zorbax SB-C_{18}, Kromasil 100-5C18, Eclipse-C18, SymmetryShield RP18, Hypersil - C18 and Extend-C18, according to the effect of separation, Hypersil-C18 (250 mm × 4.6 mm, 5 μm) was used for the further research of the methodology.

Conclusion

In this study, 6 active components were quantitatively determined in Sanhuang tablet. On the whole, this developed method was simple, accurate for the determination of berberine, aloe-emodin, rhein, emodin, chrysophanol and physcion simultaneously.

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