该方法操作简便,重现性好,可作为含白芍制剂控制质量的参考依据。

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Simultaneous determination of danshensu, protocatechuic aldehyde, caffeic acid, rosmarinci acid, salvianolic acid B, and salvianolic acid A in Xiangdan Injection by HPLC

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Abstract: Objective To establish an HPLC method to determine simultaneously the content of six water-soluble chemical constituents (danshensu, protocatechuic aldehyde, caffeic acid, rosmarinci acid, salvianolic acid B, and salvianolic acid A) in Xiangdan Injection, and to compare their content discrepancy of Xiangdan Injection from 20 manufacturers. **Methods** The HPLC method was established. The column was Alltima C_{18} (250 mm × 4.6 mm, 5 μm) at the gradient elution mode with the flow rate of 1.0 mL/min, column temperature was kept at 25 ℃ and the detector was set at 288 nm. **Results** Six components were separated clearly. The relationship between the concentration and the peak areas of the six compounds was all linear. The precision, stability, repetition, and average recovery were complied with the limit. Significant difference was found in contents of six chemical constituents in Xiangdan Injections from different manufacturers that the variation of salvianolic acid A content was the most distinct and danshensu was the most quietly. **Conclusion** The method has been successfully used to quanlify the six compounds in Xiangdan Injection, and then, can offer the reference for the quality control of Xiangdan Injections roundly.

Key words: Xiangdan (XD) Injection; water-soluble chemical constituents; HPLC

HPLC 法测定香丹注射液中丹参素、原儿茶醛、咖啡酸、迷迭香酸、丹酚酸 B 和丹酚酸 A

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摘 要:目的 建立用高效液相色谱同时测定香丹注射液中丹参素、原儿茶醛、咖啡酸、迷迭香酸、丹酚酸 B 和丹酚酸 A 6 个水溶性成分的方法,并对 20 个生产厂家香丹注射液进行测定。**方法** 采用 HPLC 色谱系统,Alltima C_{18} (250 mm × 4.6 mm, 5 μm);流动相为乙腈-0.026%磷酸,梯度洗脱;流速为 1.0 mL/min;柱温 25 ℃;检测波长为 288 nm。**结果** 本方法 6 个成分色谱峰之间具有良好的分离度,它们的浓度和各自峰面积之间有着良好的线性关系,精密度、稳定性、重复性及加样回收率均符合要求。不同厂家香丹注射液中化学成分的含量存在较大差异,其中丹酚酸 A 量差异最大,丹参素差异最小。**结论** 本方法能同时测定香丹注射液中 6 个主要化学成分,可为全面控制香丹注射液的质量提供参考。

关键词:香丹注射液;水溶性成分;HPLC

The Chinese herbal medicine compound preparation of Xiangdan (XD) Injection, made from the

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water extract of salvia and the rosewood saturated aqueous solution by distillation extraction method, which has shown obvious physiological effects in expanding blood vessel and increasing coronary blood flow, and has been widely used for the prevention and treatment of angina pectoris and myocardial infarction in clinic[1]. Salvia, whose main active component is the water-soluble phenolic acids, is the primary drug in XD Injection, and its content has intimately relevant with the whole clinical effect. Protocatechuic aldehyde was only regard as the crucial ingredient for the standard of quality control, though XD Injection was produced by many drug manufacturers[2]. In the domestic reports it was only some components content rather than the majority one[3-5], therefore, developing a multi-component assaying method is important for the quality control of Xiangdan Injection.

1 Materials and apparatus

1.1 Chemicals and reagents

Methanol (chromatography pure) was purchased from Merck Company. Acetonitrile (chromatography pure) from Tianjin Siyou Fine Chemicals Co., Ltd. Orthophosphoric acid (chromatography pure) from Tianjin Kermel Chemical Reagent Co., Ltd. Watsons water was purchased for chromatography. The reference substances of sodium Danshensu (LN 110855-200405), protocatechuic aldehyde (LN 110810-200506), salvianolic acid B (LN 111562-200504), and caffeic acid (LN 110885-200102) were provided by National Institute for The Control of Pharmaceutical and Biological Products. The reference substances of rosmarinic acid (LN-081111) and salvianolic acid A by Shanghai U-sea Biotech Co., Ltd.

1.2 Apparatus

HPLC system (Waters 2695) combined Waters 2996 photodiode Array Detector with Waters Empower data processing unit. The column was Alltima C_{18} (250 mm × 4.6 mm, 5 μm).

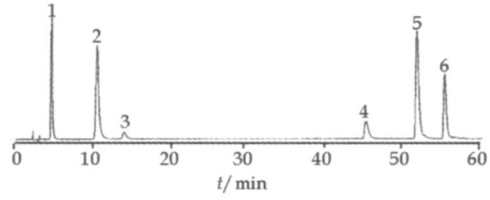
2 Methods and results

2.1 HPLC Analysis[6]

The HPLC analysis was carried out on a Waters high-performance liquid chromatography system. The mobile phase was acetonitrile-0.026% phosphoric acid solution mixture, gradient elution, with the flow rate of 1.0 mL/min, column temperature was kept at 25 ℃ and the detector was set at 288 nm. The injection volume was 20 μL. The standard curve of peak area was used to determine the sample contents, the number of theoretical plates is more than 3 000.

2.2 Sample preparation

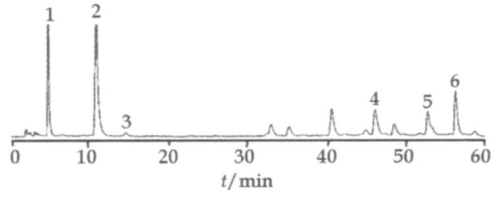
2.2.1 Preparation of control solution: Precisely weighed the authentic samples respectively, such as sodium danshensu 13.50 mg, protocatechuic aldehyde 2.59 mg, rosmarinic acid 1.88 mg, salvianolic A 15.45 mg, salvianolic B 2.55 mg, and dissolved with methanol to volume 10 mL. As well as the caffeic acid 2.27 mg to 10 mL, and then selected the diluted methanol 1 mL to 10 mL, that was the solution of caffeic acid reference solution. Mixing above reference solutions to prepare the mixture reference substance solution for injection (Fig. 1).



1-danshensu 2-protocatechuic aldehyde 3-caffeic acid 4-rosmarinic acid 5-salvianolic acid B 6-salvianolic acid A

Fig. 1 Chromatogram of reference substances

2.2.2 Preparation of test solution: Precisely weighed adequate Xiangdan Injection of various manufacturers respectively, which was diluted with methanol in the ratio of 1∶3 to the definitive volume, filtered by the microfiltration membrane with the 0.45 μm droplet size, finally taking the subsequent filtrate as the testing solution (Fig. 2).



1-danshensu 2-protocatechuic aldehyde 3-caffeic acid 4-rosmarinic acid 5-salvianolic acid B 6-salvianolic acid A

Fig. 2 Chromatogram of Xiangdan Injections (XD-18)

2.3 Standard curves studies

Precisely weighed the adequate reference solution, diluted with methanol to 60, 30, 15, 6, 3, 2, 1.5, 1.2, and 1 fold, respectively, then injected 20 μL, measured the peak areas. The linear regression equation of the reference solution was each chromatographic peak area of integral value (Y) to its concentration (X) for linear regression (Table 1).

Table 1 Linear relationship for peak area-concentration of six components

components	regression equation	linear range/ (μg · mL^{-1})	R^2
sodium danshensu	$Y = 9.282\ X \times 10^3 - 5.731 \times 10^4$	22.5—1350.0	0.999 3
protocatechuic aldehyde	$Y = 7.812\ X \times 10^4 - 2.809 \times 10^5$	4.32—259.35	0.999 4
caffeic acid	$Y = 8.116\ X \times 10^4 - 2.098 \times 10^4$	0.38 — 22.65	0.999 0
rosmarinic acid	$Y = 2.787\ X \times 10^4 - 6.726 \times 10^4$	3.13—187.5	0.999 6
salvianolic acid B	$Y = 1.585\ X \times 10^4 - 2.564 \times 10^5$	25.75—1545.0	0.999 8
salvianolic acid A	$Y = 4.814\ X \times 10^4 - 1.074 \times 10^5$	4.25 — 255.0	0.999 4

2.4 Precision studies

Injecting XD Injection (XD-18) continuously for 5 times, and then, determining the integral value of each peak area, the RSD of danshensu, protocatechuic aldehyde, caffeic acid, rosmarinic acid, salvianolic acid B, and salvianolic acid A was 1.16%, 0.94%, 1.94%, 1.12%, 0.53%, and 0.85%, respectively. The result illustrated that the precision was fit for the standard.

2.5 Stability studies

XD Injection (XD-18) was prepared to the testing solution in accordance with the above chromatographic conditions and was continuously injected for six times per 75 min in every 8 h, whose composition of the peak area value was determined, the RSD ($n = 6$) was as follows: sodium Danshensu 0.19%, protocatechuic aldehyde 0.23%, caffeic acid 3.38%, rosmarinic acid 0.56%, Salvianolic acid B 0.87%, and salvianolic acid A 4.89%, respectively. The results showed the various components of the sample solution was stable in 8 h.

2.6 Repetition studies

Under the same conditions, six samples of XD Injection (XD-18) were taken to prepare to the test solution, and to determine the content of each composition. The average concentration showed danshensu 429.64 μg/mL, protocatechuic aldehyde 111.54 μg/mL, caffeic acid 3.58 μg/mL, rosmarinic acid 78.13 μg/mL, Salvianolic acid B 135.85 μg/mL, and salvianolic acid A 156.21 μg/mL, respectively. The RSD of danshensu, protocatechuic aldehyde, caffeic acid, rosmaric acid, salvianolic acid B and salvianolic acid A was 0.29%, 0.30%, 3.03%, 1.18%, 4.43%, and 4.88%, respectively. The result illustrated that the repetition was fit for the standard.

2.7 Average recovery rate studies

Six Xiangdan Injection (XD-18) samples were precisely taken, (1.0 mL per one sample), into the volumetric flask of 10 mL, respectively, added the corresponding control solution 1.0 mL, and diluted to volume 10mL by methanol. Filtered by the microfiltration membrane with the 0.45 μm droplet size, injected 20 μL subsequent filtrate into the chromatography, the recovery rate of each component was finally determined and calculated (Table 2).

2.8 Sample determination and results

Each Xiangdan Injection sample was taken to prepare the testing solution. Injected 20 μL, recorded the peak area, then added the result to each regression equation, respectively. Finally the average content of the six constituents was calculated and, transfered to the original content (Table 3).

Table 2 Reset of recovery rate (n = 6)

composition	sample contents/ μg	addition/ μg	average determination/ μg	recovery rate/ %	RSD/ %
danshensu	1 324.6	1 350.0	1 362.70	100.90	0.62
protocatechuic aldehyde	334.9	259.0	258.41	99.77	1.11
caffeic acid	11.4	22.7	22.59	99.52	1.67
rosmarinic acid	237.7	188.0	190.49	101.30	2.26
salvianolic acid B	424.7	1545.0	1585.19	102.60	1.18
salvianolic acid A	495.5	255.0	260.28	102.10	1.11

Table 3 Results of sample determination (μg/mL, n = 3)

samples	lot numbers	sodium danshensu	protocatechuic aldehyde	caffeic acid	rosmarinic acid	salvianolic acid B	salvianolic acid A
XD-1	080312	1992.43	388.68	9.57	208.39	584.45	389.90
XD-2	200806155	2202.25	449.41	13.96	493.11	806.18	104.13
XD-3	20090201	2009.24	409.87	11.62	311.02	341.06	174.99
XD-4	080702	1776.43	259.11	11.78	648.96	394.62	200.00
XD-5	07080142	1674.79	672.69	31.03	455.56	510.88	200.41
XD-6	20080104	1652.06	286.93	11.42	361.13	913.24	19.81
XD-7	0809221	2858.21	230.24	6.22	148.01	771.76	203.18
XD-8	080711-1	1705.67	307.04	20.49	216.77	885.90	227.57
XD-9	0809042	1949.90	277.92	7.18	21.36	128.55	144.83
XD-10	081104	1632.94	563.67	33.20	649.96	689.36	160.77
XD-11	08052704	2267.06	237.40	6.01	16.43	123.60	22.50
XD-12	20089302	1962.26	395.68	9.60	346.45	376.96	287.46
XD-13	070812	2600.85	342.56	8.02	106.35	198.21	361.91
XD-14	0809050	3792.18	555.00	15.80	578.54	854.34	924.24
XD-15	0712031	2324.69	426.54	7.08	294.53	397.98	244.30
XD-16	080709-1	3163.35	560.84	11.24	407.74	493.07	603.09
XD-17	081002	1800.52	147.09	8.79	101.75	621.06	267.92
XD-18	0702085	1766.05	446.55	15.14	316.90	566.22	660.64
XD-19	081101	1867.70	331.75	8.84	312.32	337.78	485.74
XD-20	080307	1495.88	468.16	27.66	481.15	799.47	85.43

3 Discussion

Protocatechuic aldehyde was the only indicators of the quality control component in the original ministerial standard of Xiangdan Injection, and its content was defined no less than 0.17 mg per 1.0 mL. According to this standard, twenty manufacturers' products were sub-standard except the XD-17, and the highest content (672.69 μg) and the lowest content (147.09 μg) were nearly 4.6-fold difference. At the same time, by calculating the ratio between the highest content and the lowest one, the sample of the content of protocatechuic aldehyde was not the most different, however, the ratio of the rosmarinic acid and rosemary salvianolic acid A had reached to 39.55 and 46.67 respectively that was bound to lead to the differences efficacy and adversed reactions in clinical. Therefore, the components including the multiple indicators should be measured for the quality control of Xiangdan Injection at the same time.

Salvianolic acid A had a strong free radical scavenging and antioxidant effects[7], however, its content was gradual lower in line with the requirements of the methodology in 8 h in the stability test. As for the conversion factors and circumstances about the stability of salvianolic acid A, it had a good further investigation.

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