

Simultaneous HPLC Determination of Costunolide and Dehydrocostuslactone in Xin-ke-shu Preparations

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Abstract: The objective of this study is to establish a quantitative method for simultaneous determination of costunolide and dehydrocostuslactone in Xin-ke-shu preparations. The HPLC quantitative analysis was established on a Hypersil BDS column (100× 4.6 mm, 2.4 μm, Thermo) with UV detection at 225 nm. The mobile phase was water and methanol (30:70, v/v), and used at a flow rate at 0.5 mL·min⁻¹. The established method showed a good linearity ($R^2 > 0.9999$) over the investigated concentration ranges (0.07-2.80 μg), good inter-day and intra-day precisions (less than 3%) and good recoveries (from 99.82% to 99.98%) for both target compounds. The method was found to be suitable for simultaneous determination of costunolide and dehydrocostuslactone in Xin-ke-shu preparations and can be used as a comprehensive approach to the previous reported quality control of Xin-ke-shu preparations.

Keywords: Xin-ke-shu preparations, costunolide, dehydrocostuslactone, HPLC, quantitation.

INTRODUCTION

Xin-ke-shu, a traditional Chinese medicine (TCM) preparation composed of *Crataegus pinnatifida* (Shan-Zha), *Radix Salviae Miltiorrhiae* (Dan-Shen), *Radix Puerariae* (Ge-Gen), *Radix Notoginseng* (San-Qi) and *Radix Aucklandiae* (Mu-Xiang), has demonstrated significant effects against coronary heart disease with few side-effects [1]. The main treatment for chest tightness, angina, high blood pressure, dizziness, headache, neck pain and arrhythmia, high blood lipids caused by the Qi stagnation and coronary artery disease is applied in clinical trials.

There are reports of puerarin [2], tanshinone IIA [3] and protocatechuic aldehyde [4] as an index of the quality control methods for Xin-ke-shu preparations. In order to quickly screen the chemical profiles and control the quality of Xin-ke-shu preparations, liquid chromatography coupled with electrospray ionization hybrid linear trap quadrupole orbitrap (LC-LTQ-Orbitrap) mass spectrometry was applied for simultaneous identification and quantitative determination of the constituents in complex chemical system [5]. A total of 51 compounds, including phenolic acids, isoflavone-C-glycosides, isoflavone-O-glycosides, were identified or tentatively deduced. However, none of constituents from the roots of *Radix Aucklandiae* (Mu-Xiang), one of the main herbs in Xin-ke-shu, were detected. Costunolide and dehydrocostuslactone (structures shown in Fig. 1), the main constituents of the roots of *Radix Aucklandiae* [6], have been

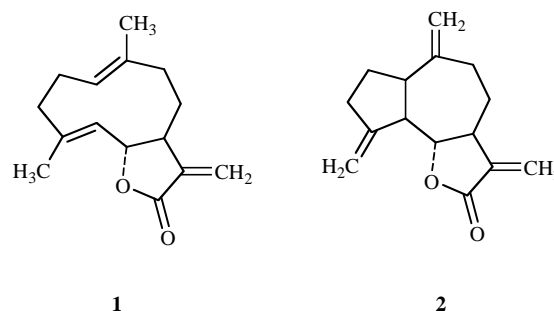


Fig. (1). The structures of costunolide (1) and dehydrocostuslactone (2).

usually used as chemical markers for quality control of this herb [7]. But both of them could not be detected by using our previously reported LC-MS conditions [5]. In order to provide a complementary strategy for quality control of Xin-ke-shu preparations, this paper described a validated and optimized HPLC method for simultaneous quantization of costunolide and dehydrocostuslactone in Xin-ke-shu preparations.

MATERIALS AND METHODS

Chemicals

HPLC grade methanol was purchased from Fisher (New Jersey, USA) and other reagents and solvents were of analytic grade. HPLC water was prepared with the Millipore Milli-Q SP water purification (Bedford, MA, USA) system.

Costunolide (1) and dehydrocostuslactone (2) were provided by the National Institutes for Food and Drug Control and their purities (>99%) were determined by HPLC.

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Table 1. A Summary of the Tested Samples

Sample No.	Preparation Form	Source	Batch No.
Sh1	Tablet	Shandong Wohua Pharmaceutical Technology Co., Ltd, China	090629
Sh2	Tablet	Shandong Wohua Pharmaceutical Technology Co., Ltd, China	100537
Sh3	Tablet	Shandong Wohua Pharmaceutical Technology Co., Ltd, China	100645
Sh4	Tablet	Shandong Wohua Pharmaceutical Technology Co., Ltd, China	100104
Sh5	Tablet	Shandong Wohua Pharmaceutical Technology Co., Ltd, China	100307
S	Capsule	Sichuan Longren Pharmaceutical Co., Ltd, China	100301
Ch1	Capsule	Chongqing Xieran Pharmaceutical Co., Ltd, China	100301
CH2	Capsule	Chongqing Xieran Pharmaceutical Co., Ltd, China	090901
H1	Capsule	Hebei Guojin Pharmaceutical Co., Ltd, China	1001051
H2	Capsule	Hebei Guojin Pharmaceutical Co., Ltd, China	0904051
X	Capsule	Xian Tianyi Pharmaceutical Co., Ltd, China	100404-029
Y	Capsule	Deyuantang Pharmacy Group, China	90003004-076
J	Pill	Jilin Jichun Pharmaceutical Co., Ltd, China	100101

Xin-ke-shu Samples

A total of 13 samples of Xin-ke-shu preparations produced by different pharmaceutical companies in China were collected from pharmacy (Table 1). The blank sample was the extract of Xin-ke-shu prescription without Mu-Xiang provided by Shandong Wohua Pharmaceutical Technology Co., Ltd, China.

Chromatographic Conditions

All sample analyses were carried out on a HPLC system consisted of a Waters 600 HPLC with a 486 UV detector and 717 Autosampler. Data were acquired and processed with the Empower® software system. Chromatographic separation was performed on a Thermo BDS HYPERSIL C₁₈ column (4.6 mm × 100 mm, 2.4 μm). The mobile phase was delivered at a flow rate of 0.5 mL/min consisting methanol–water solution (70:30, v/v). The column temperature was maintained at 25°C and the effluent was monitored at 225 nm.

Preparation of Standard Solutions

A stock solution containing costunolide and dehydrocostuslactone was prepared in methanol at the same concentration of 2.8 mg/mL for each of them and stored at -20°C prior to use. Stock solutions were diluted with methanol to give a series of working solutions. The range of the concentration was 7–280 μg/mL.

Preparation of Sample Solutions

The dried powders of Xin-ke-shu preparation (1.0 g) were accurately weighed and transferred into a 50 mL Erlenmeyer flask. They were extracted with 25 mL chloroform in an ultrasonic bath for 20 min. After recruitment the weight, aliquots of 5 mL of supernatant was

precisely taken out, and evaporated to dryness under reduced pressure to give a residue. The residue was dissolved in methanol, quantitatively transferred into a volumetric flask and adjusted to a final volume (5 mL) with methanol. The obtained solution was filtered through 0.45 μm syringe filter before HPLC analysis and 10 μL was injected. Each sample solution was injected in triplicate.

The blank sample, having the same amount of raw herb equivalent to 1 g of Xin-ke-shu preparation except Mu-Xiang was prepared and analyzed identically to Xin-ke-shu samples.

Method Validation

Specificity

The specificity was determined by injecting the reference standards, the blank and Xin-ke-shu samples to evaluate possible interferences in samples. The sample preparation and chromatographic condition were optimized to guarantee that no interferences incurred at the retention time of the tested compounds.

Calibration Curves

The calibration curves were prepared at seven different concentration levels. The range of the concentration was 7–280 μg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting dilute solutions of the standards with known concentrations. The LOD and LOQ were defined as the signal-to-noise ratio equal to 3 and 10, respectively.

Precision, Reproducibility, and Stability

Precision of the method was determined by preparing and assaying 6 replicate samples of Xin-ke-shu (batch No. 100537) on the same day; the same procedure was repeated

on three consecutive days. The intra-day and inter-day precisions were determined by comparing variations within the same days and the 3 days, respectively.

Recovery

The spiked samples were analyzed and the recoveries were calculated by comparing the peak area of measured concentration to the peak area of the spiked concentration.

RESULTS AND DISCUSSION

Selection of Detection Manner and Optimization of Chromatographic Conditions

First, the MS condition for detection of costunolide and dehydrocostuslactone was optimized. However, the optimized conditions do not suitable for quantization of other constituents in Xin-ke-shu preparations. In addition, both costunolide and dehydrocostuslactone have their maximum absorbance at 225 nm [7]. Thus, in order to provide a complementary strategy for quality control of Xin-ke-shu preparations, a simple HPLC method with UV detection was established for simultaneous determination of costunolide and dehydrocostuslactone in Xin-ke-shu preparations.

Various compositions of mobile phase such as acetonitrile–water, methanol–water and acetonitrile–aqueous formic acid, were tried to obtain chromatograms with good resolution of adjacent peaks. The mixture of methanol and water (70:30, v/v) were found to give the desired separation in a relatively short analytical time for the HPLC quantization of the two markers.

Optimization of Sample Preparation

Optimization of the extraction solvents and procedures was essential in the development of analytical methods for herbal extracts because different solvents and procedures

will influence the extraction efficiency of different compounds, as well as the overall sensitivity and selectivity of the method.

Variables involved in the extraction such as solvents (ethyl acetate, methanol and chloroform), extraction methods (sonication and refluxing) and extraction time (10, 20 and 40 min) were evaluated for the extraction efficiency of the two target compounds. Compared with refluxing extraction, the sonication method was found to be more efficient for both of the components. The sonication with chloroform exhibited similar extraction efficiency to extraction with methanol, but more impurities were observed in methanol extract. Consequently, the samples were extracted with chloroform by sonication for 10, 20, and 40 min to screen optimal extraction time. The results showed that two markers compounds could be entirely extracted within 20 and 40 min [8]. Therefore, 20 min was selected as an appropriate extraction time.

Method Validation

Specificity

Specificity of the method was evaluated through the analysis of the blank sample. Possible interference present in the blank sample solution was analyzed by the described HPLC method and compared with the chromatograms of costunolide and dehydrocostuslactone reference sample (Fig. 2). No interfering peaks could be detected at the retention time of costunolide and dehydrocostuslactone.

Linearity, Limits of Detection and Limits of Quantification

Linear calibration plots were obtained over seven concentration levels. The results showed a good linear correlation ($R^2 > 0.9999$) between the peak area and concentration (Table 2) over the investigated concentration ranges (7–280 $\mu\text{g/mL}$).

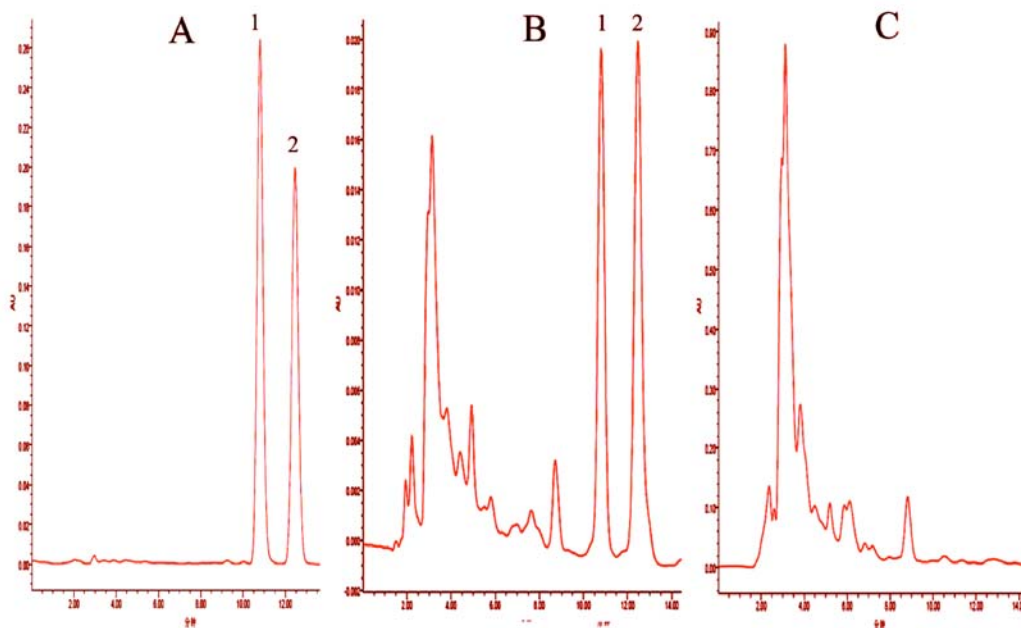


Fig. (2). HPLC of reference substances (A), Xin-ke-shu sample (B), sample of Xin-ke-shu without Radix Aucklandiae (C), 1. costunolide, 2. dehydrocostus lactone.

Table 2. Regression Equations, Linearity Range, LODs, LOQs, Precision, Repeatability, and Stability Tests (RSD %) for 2 Analytes Analyzed with the Established HPLC Method

Analytes	Regression Equation	Correlation Coefficient (r ²)	Linearity Range (μg)	LOD (μg)	LOQ (μg)	Precision		Repeatability (n = 6)	Stability (n = 6)
						Intra-day (n = 6)	Inter-day (n = 3)		
Costunolide	$Y = 1.9044 \times 10^6 X + 1781.7$	1	0.07-2.8	0.007	0.07	0.5%	2.68%	2.32%	2.32
Dehydrocostuslactone	$Y = 1.5593 \times 10^6 X + 2006.7$	1	0.07-2.8	0.014	0.07	0.65%	2.16%	2.45%	2.13

Note: in the regression equation, X is the peak area, Y is the concentration of each analyte (μg), and r is the correlation coefficient, LOD is the limit of detection, LOQ is the limit of quantification.

Table 3. The Recovery of Determination of Costunolide and Dehydrocostuslactone

Analyte	Initial Amount/mg	Added Amount/mg	Detected Amount/mg	Recovery/%	RSD/%
		0.437	0.985	100.4	0.78
Costunolide	0.544	0.541	1.075	99.1	1.56
		0.649	1.202	100.8	0.49
		0.615	1.394	100.1	0.71
Dehydrocostuslactone	0.778	0.769	1.542	99.7	1.28
		0.922	1.707	100.4	0.49

The stock solutions were diluted to a series of working reference solutions with methanol, and an aliquot of the diluted solutions were injected into HPLC for analysis. LOD and LOQ under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of around 3 and 10, respectively. As shown in Table 2, LOD and LOQ of the analytes were no more than 0.014 μg and 0.07 μg, respectively.

Precision, Reproducibility and Stability

The precision study used the same sample (batch number: 100537) and followed the validated procedure for sample preparation. The precision of the assay method was evaluated by carrying out six independent assays on three consecutive days. The % RSD of two analytes was determined to be within the acceptable limit of 5.0%. Multiple injections illustrated that the results were highly reproducible and had a low standard deviation. The RSD of assay results obtained in inter-day and intra-day study (Table 2) was within 3% and showed a maximum of 2.68% for costunolide in inter-day, and 0.65% for dehydrocostuslactone in intra-day studies, confirming a good precision of the developed method.

Recovery

The accuracy of the method was determined by adding different concentrations of costunolide and dehydrocostuslactone into the sample with a known amount of two standard compounds. The percentage recovery ranged from 99.1% to 100.8%. The RSD values of each concentration level were not more than 1.56% (Table 3).

Determination of Sample Content

The validated HPLC method was subsequently applied to simultaneous determination of costunolide and dehydrocostuslactone in 13 samples of Xin-ke-shu preparations. The concentrations of the two markers were calculated by external standard method. The results (Table 4) showed that the total concentrations of two markers were among 0.088-1.317mg/g. It may due to the difference of the content of *Radix Aucklandiae* in the preparations and operation of production process. The content of dehydrocostuslactone was always higher than that of costunolide in all samples even eight times higher in sample Sh5. However, in samples Sh1, Ch1, Sh4, H2, and J, there were remarkable differences among the contents of the chemical markers analyzed in different samples, even the contents of costunolide and dehydrocostuslactone varied significantly in different batches from the same pharmaceutical manufacturer. For example, costunolide was abundant in the batches of Sh2 and Sh3, but lower in the batches of Sh1, Sh4, and Sh5. Moreover, the content of costunolide in batch Sh2 is three times higher than that in batch Sh1, both of which produced from the same company. This variation might be due to the quality of *Radix Aucklandiae*. Contents of constituents from the roots of *Radix Aucklandiae* will be different because of the weather and geographical location, and even production process will result in the loss of the constituents in *Radix Aucklandiae*.

CONCLUSIONS

An analytical reversed-phase HPLC method was developed and validated thoroughly for simultaneous

Table 4. The Contents of Costunolide and Dehydrocostuslactone in Xin-ke-shu Preparations (n=3)

Sample No.	Costunolide (mg/g)	RSD (%)	Dehydrocostuslactone (mg/g)	RSD (%)	Total of Two Analytes (mg/g)
Sh1	0.180	2.17	0.355	2.97	0.535
Sh2	0.542	2.32	0.775	2.45	1.317
Sh3	0.449	1.63	0.664	2.65	1.113
S	0.458	0.95	0.674	0.91	1.132
Ch1	0.130	2.03	0.259	1.68	0.389
Ch2	0.202	2.16	0.378	1.61	0.580
Sh4	0.143	2.42	1.100	2.23	1.243
Sh5	0.096	1.81	0.819	0.56	0.915
H1	0.451	0.22	0.584	0.91	1.035
H2	0.030	3.13	0.058	4.56	0.088
Y	0.206	2.22	0.328	2.61	0.534
X	0.238	2.91	0.401	0.89	0.639
J	0.045	3.85	0.124	2.79	0.169

determination of costunolide and dehydrocostuslactone in Xin-ke-shu preparations. The developed method is sensitive, simple and specific and can be employed for monitoring the quality of the roots of *Radix Aucklandiae* (Mu-Xiang). This established method, combined with our previous established procedure [5], provided a comprehensive strategy for quality control of Xin-ke-shu preparations.

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CONFLICT OF INTEREST

None declared

ABBREVIATIONS

HPLC	=	High Performance Liquid Chromatography
LC-LTQ-Orbitrap	=	Liquid Chromatography Coupled with Electrospray Ionization Hybrid Linear Trap Quadrupole orbitrap

LOD	=	Limit of Detection
LOQ	=	Limit of Quantification
TCM	=	Traditional Chinese Medicine

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