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A Rapid, Simple High Performance Liquid Chromatography Method for the Determination of Traditional Chinese Medicine Ointment Shiunko

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ABSTRACT

A rapid, simple and sensitive reversed-phase HPLC method with Ultraviolet (UV) absorbance measurement at 280 nm was applied for the simultaneous quantitative determination of marker substances in the traditional Chinese herbal ointment Shiunko. Shiunko consists of Lithospermum radix containing shikonin, acetylshikonin, β,β-dimethylacrylshikonin, isobutylshikonin, deoxyshikonin, and isovalerylshikonin, as well as Angelica radix containing ferulic acid. Specimens of Shiunko ointment were extracted with acetonitrile, separated on a reversed-phase C18 (ThermoQuest, 250 × 4.6 mm i.d.) column at ambient temperature, with a solvent system consisting of 47% acetonitrile and 53% acetic acid (v/v, pH 2.56) at a flow rate of 1.0 mL/min. Diclofenac was used as the internal standard. This procedure enabled a run time of within 35 min. In the validation of this analytical method, the RSD and RE were less than 7.0%, indicating satisfactory accuracy and repeatability. The linear relationships between the concentration of analyte in samples within a given range (ferulic acid 0.175- 3.50 μg/mL, shikonin 0.5-10.0 μg/mL, acetylshikonin 0.5-10 μg/mL, β,β-dimethylacrylshikonin 0.35-7 μg/mL, isobutylshikonin 0.4-0.8 μg/mL, deoxyshikonin 0.255-5.1 μg/mL, and isovalerylshikonin 0.25-5 μg/mL) and the corresponding peak area ratio were good indications that this analytical method could be used in quantitative analysis. The lower limit of detection of these marker substances under above conditions was 0.05 μg/mL.

Key words: Shiunko, shikonin, acetylshikonin, β,β-dimethylacrylshikonin, isobutylshikonin, deoxyshikonin, isovalerylshikonin, ferulic acid

INTRODUCTION

Shiunko is a traditional Chinese herbal ointment consisted of Angelica radix, Lithospermum radix, oleum sesame, cera flava and adeps suillus. It has been widely used for the acceleration of the proliferation of granulation tissue and for its anti-bacterial, anti-inflammatory and anti-tumor effects[1-13].

The therapeutic effects of traditional Chinese herbal ointment have been known to be strongly influenced by the source of the crude drugs and the variation of the manufacturing processes. Hence, quality control is an important issue for Chinese herbal products. Accordingly, the purpose of this study was to develop a simple and expedient analytical method for routine use. There have been some studies on individual or simultaneous analysis of Angelica radix and/or Lithospermum radix, but works regarding validated and quantitative manner are limited[14-19]. Following works on the reparability of seven different derivatives (including β-hydroxyisovalerylshikonin, acetylshikonin, β,β-dimethylacrylshikonin, deoxyshikonin, isobutylshikonin, and isovalerylshikonin as well as α-methyl-*n*-butyl-shikonin) by normal-phase, reversed-phase and ionic-pair HPLC, Nickel and Carroll[16] concluded that these shikonin derivatives could only be separated by the ion-pair method at 70˚C in a MicroPak MCH-10 column (Varian, 300 × 4 mm i.d.) with a 2 mL/min flow rate within 80 min. The chromatogram shows that isovalerylshikonin and α-methyl-*n*-butyl-shikonin were eluted as one peak, and the separation was poor for β-hydroxyisovalerylshikonin and acetylshikonin. The same situation was found for β,β-dimethylacrylshikonin and isovalerylshikonin. Fujita *et al*.[15] reported that they have separated seven of the shikonin derivatives (including shikonin, β-hydroxyisovalerylshikonin, acetylshikonin, β,β-dimethylacrylshikonin, deoxyshikonin, α-methyl-*n*-butylshikonin, isovalerylshikonin) at 40˚C using HPLC with a mobile phase consisting of acetonitrile/water/triethylamine/acetic acid (70/30/0.3/0.3) at 1.1 mL/min. Nickel and Carroll[17] experimentally proved that elution of these shikonin derivatives has never been accomplished by commercial columns. Lay *et al*.[19] claimed that they could simultaneous determine five constituents including ferulic acid, shikonin, acetylshikonin, β,β-dimethylacrylshikonin, and deoxyshikonin in "Tzyy-Yun-Gau" medicine. Lay *et al*.[19] used Inertsil ODS-2 250 × 4.6 mm i.d. column at 30˚C with a mobile phase consisting of methanol/acetonitril/2% acetic acid by a linear gradient elution at a flow rate of 1.0 mL/min. The detection wavelength varied with time during the 80-min running period. The mixture of *n*-hexane and methanol was used as extraction solvent system. The recoveries for these samples were about 100%. Exception

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for β,β-dimethylacrylshikonin that was greater than 100% indicated that there was interference at the same retention time. Therefore, it is still important to development a simple, rapid and sensitive quantitative method for the simultaneous determination of shikonin derivatives and ferulic acid in Shiunko.

MATERIALS AND METHODS

I. *Materials*

Angelica radix, Lithospermum radix, cera flava and adeps suillus were purchased from the herbal market. Shikonin, acetylshikonin, β,β-dimethylacrylshikonin, isobutylshikonin, deoxyshikonin, and isovalerylshikonin were purchased from TCI (Japan). Ferulic acid, diclofenac sodium and sesame oil were purchased from Sigma (USA). All other chemicals and solvents were of analytical reagent grade. The crude drugs were identified by the fellow of Graduate Institute of Natural Products, Kaohsiung Medical University before preparation of Shiunko.

II. *Apparatus and Chromatographic Conditions*

An HPLC equipped with a Hitachi model L-700 pump, a L-7420 absorbance detector, a Jasco 855-AS autosampler and a ThermoQuest C18, HYPURITY advanced column (250 × 4.6 mm i.d., 5μ) was used. The mobile phase was a mixture of 47% acetonitrile and 53% of pH 2.56 acetic acid solution (v/v), and the flow rate was 1.0 mL/min. The effluent from the column was monitored at 280 nm.

III. *Preparation of Standard Solution*

To prepare a standard solution (containing shikonin, acetylshikonin, β,β-dimethylacrylshikonin, isobutylshikonin, deoxyshikonin, isovalerylshikonin, and ferulic acid), an appropriated amount of internal standard solution (diclofenac) was added to an solution containing accurately weighed amount of all marker substances that were dissolved in the HPLC mobile phase. Twenty microliter of standard solution was injected into the HPLC for analysis. Calibration graphs were plotted subsequently for linear regression analysis of the peak area ratios against concentrations.

IV. *Preparation of Shiunko*

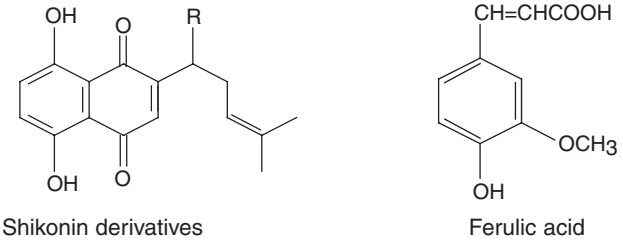
Angelica radix and Lithospermum radix were pulverized and sieved through 12 mesh. Fifty gram of sesame oil, 12.5 g of lard oil and 7.5 g of yellow wax were mixed and melted at 130-140˚C, then the Angelica radix was added while the temperature was kept at same condition. While the color of the Angelica radix became brownish and the color sustained for more than 15 min, 5 g of Lithospermum radix was added. The mixture was then filtered while hot to provide the product.

V. *Recovery Studies*

Three different quantities of markers were spiked into 100 mg of blank Shiunko ointment base prepared by the method described above but without the two radices, respectively. Two millilter of acetonitrile or tetrahydrofuran was added and the mixture was shaken for 30 min followed by centrifugation at 3,000 rpm for 10 min. The supernatant solution (0.5 mL) transferred to another tube containing 0.5 mL of the HPLC mobile phase followed by vortex for 30 sec. The samples were filtered through a 0.45 μm syringe filter and 0.1 mL of the filtrate was admixed with 0.1 mL of the internal standard solution. An aliquot of 20 μL of the final solution was injected into the HPLC for analysis.

RESULTS AND DISCUSSION

Shiunko is a traditional Chinese herbal preparation consisted of Angelica radix and Lithospermum radix. The marker substances of shikonin, acetylshikonin, β,β-dimethylacrylshikonin, isobutylshikonin, deoxyshikonin, and isovalerylshikonin in Angelica radix and ferulic acid in Lithospermum radix were analyzed in this study. The chemical structures of these marker substances of Angelica radix were similar, as shown in Figure 1. There were many studies[15-19] on the isolation of marker substances of Lithospermun radix and Angelica radix. These works described that these radices, relevant analytical aspects in a quantitative manner are extremely limited such as running with a linear gradient elution, detection wavelength varied with time, for a long running time and at higher temperature. In this study, a simple, efficient and accurate HPLC method was developed for the determination of these marker substances in Shiunko. The HPLC chromatograms in Figure 2 shows that these 7 markers and the internal standard were well separated by the chromatographic condition within 35 min running time. In comparison of the features between the current and previous methods[15-19], it was



Shikonin derivatives

	R
deoxyshikonin	H
shikonin	OH
acetylshikonin	$OCOCH_3$
b,b-dimethylacrylshikonin	$OCOCHC(CH_3)_2$
isobutylshikonin	$OCOCH(CH_3)_2$
isovalerylshikonin	$OCOCH_2CH(CH_3)_2$

Ferulic acid

Figure 1. Structure of marker constituents.

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found that the current HPLC procedure with a mobile phase consisting of 47% acetonitrile and 53% of pH 2.56 acetic acid solution at a flow rate of 1.0 mL/min and the detection wavelength fixed at 280 nm was very simple and fast.

In this analytical method, marker substances could be measured reliably over a wide range of concentration from

Table 1. Accuracy, repeatability and linearity for the determination of marker substance of Shiunko

	Intra-day (n = 6)			Inter-day (n = 6)		
Concentration Know (μg/mL)	Concentration found (μg/mL)	RE(%)	RSD (%)	Concentration found (μg/mL)	RE(%)	RSD (%) Ferulic acid
0.175	0.18 ± 0.008	2.78	4.49	0.18 ± 0.007	2.78	4.40
0.350	0.36 ± 0.008	2.78	2.23	0.36 ± 0.020	2.78	4.34
0.700	0.69 ± 0.003	1.44	1.81	0.69 ± 0.020	-1.44	3.15
1.750	1.75 ± 0.040	0.01	2.18	1.74 ± 0.003	-0.58	1.53
3.500	3.51 ± 0.010	0.28	0.35	3.51 ± 0.001	0.28	0.31
		Y = 0.2453 X + 0.0009			Y = 0.2425 X – 0.0032	
		r = 0.9998, RSS[a] = 0.000268			r = 0.9999, RSS = 0.000141	
Shikonin						
0.5	0.51 ± 0.020	1.96	4.48	0.50 ± 0.020	0.01	4.47
1.0	1.05 ± 0.030	4.76	2.69	1.04 ± 0.020	0.99	2.77
2.0	2.01 ± 0.010	0.49	0.49	1.99 ± 0.030	-0.50	1.45
5.0	4.98 ± 0.030	-0.40	0.64	4.99 ± 0.040	-0.20	0.78
10.0	10.00 ± 0.001	0.01	0.01	10.01 ± 0.060	0.09	0.59
		Y = 0.1246 X + 0.0003			Y = 0.1265 X + 0.0034	
		r = 0.9998, RSS= 0.000159			r = 0.9999, RSS = 0.000302	
Acetylshikonin						
0.5	0.48 ± 0.010	-4.17	2.69	0.49 ± 0.020	-0.02	3.44
1.0	1.04 ± 0.020	3.85	2.09	1.04 ± 0.020	3.85	2.03
2.0	1.97 ± 0.030	-1.52	1.48	1.98 ± 0.040	-0.01	1.84
5.0	5.05 ± 0.030	0.99	0.69	5.00 ± 0.060	0.01	1.24
10.0	10.01 ± 0.040	0.09	0.37	10.02 ± 0.020	0.19	0.24
		Y = 0.1431 X – 0.0007			Y = 0.1459 X +0.0009	
		r = 0.9999, RSS = 0.000449			r = 0.9999, RSS = 0.000413	
β,β-dimethylacrylshikonin						
0.35	0.36 ± 0.020	2.78	6.39	0.37 ± 0.020	5.40	5.07
0.70	0.74 ± 0.010	5.41	1.59	0.74 ± 0.020	5.40	2.41
1.40	1.37 ± 0.030	-2.19	1.93	1.39 ± 0.040	0.72	2.93
3.50	3.48 ± 0.060	-0.58	1.68	3.50 ± 0.050	0.01	1.29
7.00	7.02 ± 0.020	0.28	0.27	7.01 ± 0.020	0.14	0.27
		Y = 0.0436 X – 0.0026			Y = 0.0416 X + 0.0009	
		r = 0.9994, RSS = 0.0000381			r = 0.9999, RSS = 0.000253	
Isobutylshikonin						
0.4	0.43 ± 0.010	6.97	3.17	0.42 ± 0.003	4.76	0.89
0.8	0.81 ± 0.020	1.23	1.89	0.83 ± 0.020	3.61	1.97
1.6	1.56 ± 0.020	-2.56	1.31	1.56 ± 0.010	-2.56	0.92
4.0	4.01 ± 0.050	0.25	1.18	4.02 ± 0.040	0.49	0.99
8.0	8.01 ± 0.010	0.12	0.16	8.02 ± 0.060	0.25	0.81
		Y = 0.1005 X – 0.0009			Y = 0.1034 X – 0.0032	
		r = 0.9997, RSS = 0.000153			r = 0.9999, RSS = 0.000222	
Deoxyshikonin						
0.255	0.26 ± 0.010	1.92	3.82	0.26 ± 0.010	1.92	3.09
0.51	0.52 ± 0.010	1.92	2.07	0.52 ± 0.020	1.92	3.32
1.02	1.00 ± 0.020	-0.02	1.57	1.01 ± 0.020	- 0.09	2.33
2.55	2.56 ± 0.004	0.39	0.18	2.56 ± 0.030	0.39	1.29
5.10	5.10 ± 0.080	0.01	1.54	5.14 ± 0.010	0.78	0.21
		Y = 0.1407 X - 0.0023			Y = 0.1379 X – 0.0038	
		r = 0.9998, RSS = 0.000315			r = 0.9999, RSS = 0.0000973	
Isovalerylshikonin						
0.25	0.25 ± 0.010	0.00	2.57	0.25 ± 0.010	0.01	2.65
0.50	0.53 ± 0.010	5.66	2.68	0.54 ± 0.020	7.40	4.02
1.0	1.01 ± 0.020	0.99	1.68	0.99 ± 0.030	- 0.01	2.72
2.5	2.54 ± 0.030	1.57	1.05	2.51 ± 0.040	0.39	1.53
5.0	5.00 ± 0.010	0.00	0.22	5.01 ± 0.020	0.19	0.39
		Y = 0.0984 X – 0.0048			Y = 0.0942 X + 0.0048	
		r = 0.9997, RSS = .0000683			r = 0.9998, RSS = 0.000444	

[a]RSS: residual sum of squares.

0.175 to 3.50 μg/mL for ferulic acid, 0.5 to 10.0 μg/mL for shikonin and acetylshikonin, 0.35 to 7.0 μg/mL for β,β-dimethylacrylshikonin, 0.4 to 0.8 μg/mL for isobutylshikonin, 0.255 to 5.1 μg/mL for deoxyshikonin, and 0.25 to 5.0 μg/mL for isovalerylshikonin. The accuracy and repeatability of this method for each analyte expressed as the RSD and relative error (RE) were calculated for both inter- and intra-day analysis for each standard concentration. As shown in Table 1, the RSD and RE were less than 7.0%, indicated that the analytical method had good accuracy and repeatability. The linearity of the spiked concentrations of each analyte and the corresponding peak area ratios was determined using the least-square linear regression. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares (RSS) of each marker are listed in Table 1. The result showed good linear relationships between the corresponding peak area ratio and the concentration of analyte in samples within a given range. The lower limit of detection of these marker substances under above conditions was 0.05 μg/mL.

For the determination of extraction efficiency of marker substances from Shiunko, three different amounts for each marker substances were spiked into 0.1 g of the blank Shiunko ointment base (Table 2). The marker substances were extracted from the ointment base by acetonitrile and tetrahydrofuran. It was found that efficiency of the extraction from acetonitrile was slightly higher than from tetrahydrofuran. Except for β,β-dimethylacrylshikonin and isovalerylshikonin, all marker substances were completely extracted from the Shiunko base as shown in Figure 3. The chromatographic results showed that there was interference between β,β-dimethylacrylshikonin and isovalerylshikonin, and ferulic acid, shikonin, acetylshikonin, isobutylshikonin as well as deoxyshikonin can be effectively separated by the present method. β,β-Dimethylacrylshikonin was only partly resolved from a mixture containing itself and isovalerylshikonin. These findings concur with the result of Lay and his co-workers[17,19] for the determination of the marker substance for 'Tzyy-Yun-Gau'.

Table 3 shows the results of the content assay for the contents of each marker substance in two different Shiunko ointments, including one commercial preparation and our synthetic product. The contents of each marker substance in these two Shiunko preparations are markedly different. This is probably due to the variation of the source of the corresponding crude drugs and different preparation process.

Table 2. Recovery of each marker substance from Shiunko using acetonitrile

Compound	Spiked concentration (μg/mL)	Recovery (n = 6)
Ferulic acid	0.175	113.19 ± 0.06
	0.700	98.95 ± 0.03
	3.500	103.26 ± 0.04
Shikonin	0.5	108.83 ± 0.05
	1.0	118.42 ± 0.03
	2.0	98.69 ± 0.05
Acetylshikonin	0.5	98.08 ± 0.02
	1.0	106.26 ± 0.09
	2.0	100.17 ± 0.01
β,β-Dimethylacrylshikonin	0.35	142.54 ± 0.01
	0.70	123.99 ± 0.21
	1.40	125.04 ± 0.04
Isobutylshikonin	0.4	102.41 ± 0.09
	0.8	94.22 ± 0.05
	1.6	95.49 ± 0.04
Deoxyshikonin	0.255	102.06 ± 0.08
	0.51	97.96 ± 0.05
	1.02	101.56 ± 0.09
Isovalerylshikonin	0.25	126.03 ± 0.13
	0.50	104.63 ± 0.03
	1.0	113.21 ± 0.09

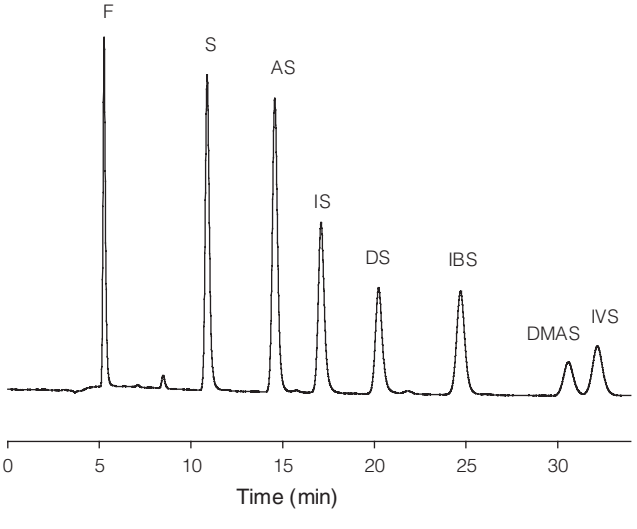

Figure 2. HPLC profile of each marker substance and internal standard.
F = ferulic acid (5 μg/mL); S = shikonin (5 μg/mL); AS = acetylshikonin (2 μg/mL); IS = diclofenac (5 μg/mL); DS = deoxyshikonin (1 μg/mL); IBS = isobutylshikonin (2 μg/mL); DMAS = β,β-dimethylacrylshikonin (1μg/mL); IVS = isovalerylshikonin (1 μg/mL).

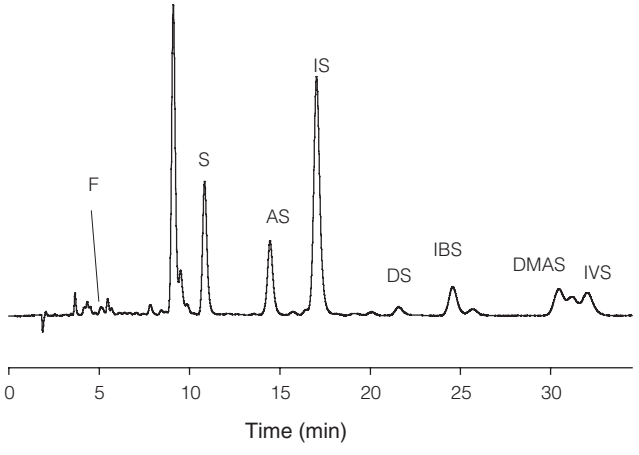

Figure 3. HPLC profile of each marker substance and internal standard in commercial Shiunko preparation. F = ferulic acid; S = shikonin; AC = acetylshikonin, IS = diclofenac; DS = deoxyshikonin; IBS = isobutylshikonin; DMAS = β,β-dimethylacrylshikonin; IVS = isovalerylshikonin.

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Table 3. Contents of each marker substance in two different Shiunko preparations

Compound	Commercial product (μg / 0.1g)	Our synthetic preparation (μg / 0.1g)
Ferulic acid	2.19 ± 0.11	7.61 ± 0.24
Shikonin	90.54 ± 0.85	57.66 ± 1.96
Acetylshikonin	54.39 ± 0.37	8.71 ± 0.71
β,β-dimethylacrylshikonin	141.09 ± 1.29	30.72 ± 0.84
Isobutylshikonin	50.31 ± 0.58	10.35 ± 0.59
Deoxyshikonin	11.37 ± 0.52	4.72 ± 0.19
Isovalerylshikonin	59.43 ± 0.46	13.63 ± 0.33

Data are represented mean ± SD, n = 6.

CONCLUSIONS

A rapid, simple and accurate HPLC method was developed with a capability of separating ferulic acid, shikonin, acetylshikonin, isobutylshikonin, and deoxyshikonin effectively within 35 min. Application of this method can be extended to the quality control of Shiunko.

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