Phytochemical Analysis
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Simultaneous Quantification of Twelve Bioactive Components in San-Huang-Xie-Xin-Tang by HPLC

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Abstract: A simple and reliable high-performance liquid chromatographic method with ultraviolet detection (280 nm) has been developed for the simultaneous analysis of 12 bioactive components in San-huang-xie-xin-tang (SHXXT), a traditional Chinese medicine containing Rhei rhizome, Coptidis rhizome and Scutellariae radix. A relatively simple extraction procedure was employed and optimised, and separation of the components was obtained within 1 h using a reversed-phase column under gradient elution with acetonitrile and a buffer containing 0.01 M sodium 1-pentanesulphonate (pH 3). The lower limit of detection for the analytes ranged from 25 to 75 ng/mL. The correlation coefficients associated with each calibration curve were greater than 0.99. The precision and accuracy of the method ranged from 1.0 to 10.5% at low concentration levels, 0.8 to 8.7% at medium levels and 1.2 to 5.8% at high levels. In commercial products of SHXXT, baicalin and berberine were present in the highest amounts with levels up to 4.0 and 3.3%, respectively, in one sample. The HPLC method was able rapidly and efficiently to analyse constituents in crude herb and traditional Chinese medicinal preparations containing Rhei rhizome, Coptidis rhizome and Scutellariae radix. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: HPLC; herbal medicine; San-huang-xie-xin-tang, Rhei rhizome; Coptidis rhizome; Scutellariae radix.

INTRODUCTION

The traditional Chinese herbal medicine San-huang-xie-xin-tang (SHXXT) is a combinatorial formula including Rhei rhizome, Coptidis rhizome and Scutellariae radix. Moreover, a number of concentrated herbal preparations that also contain these herbs have been used in traditional Chinese herbal medicines to treat diseases (Bi *et al.*, 1982; Kubo *et al.*, 1984; Hirano *et al.*, 2001). A number of studies have been carried out to analyse the components of natural products that could determine the amount of active ingredients in herbal medicines. A suitable assay method for the combinatorial formula is an important and necessary issue in quality control for medicine manufacturers. There are many studies in which marker substances from the single constituent herbs have been determined using various analytical methods (Springolo and Coppi, 1988; Chen and Chang, 1995; Liang *et al.*, 1995; Chen *et al.*, 2002; Lai *et al.*, 2003; Lee *et al.*, 2003). In previous studies, several HPLC methods have been reported for the particular components in Rhei rhizome (Sagara *et al.*, 1987; Zhang *et al.*, 1988; Springolo and Coppi, 1988), Coptidis rhizome (Chu and Sheu, 1996; Chuang *et al.*, 1996; Ji *et al.*, 1999) and Scutellariae radix (Lee *et al.*, 1999; Akao *et al.*, 2000; Chen *et al.*, 2000).

Recently, SHXXT has been used clinically to relieve the symptoms of hot flush, stomach disorders, constipation and irritability. Some research has been focused on the role of gastric muscle relaxation since SHXXT is also used to treat gastritis, gastric bleeding and peptic ulcers (Takase *et al.*, 1989, 1991; Lin and Tan, 1994; Zhang *et al.*, 1997). The hypotensive effect of SHXXT on the theophylline-induced increase in arterial blood pressure has also been investigated (Sanae *et al.*, 2001). Hence, the development of a simple and expedient analytical method for simultaneously assaying the constituents of SHXXT is important. In this study, a total of 12 compounds in Rhei rhizome, Coptidis rhizome and Scutellariae radix were separated using an HPLC method. Sennoside B (**1**), sennoside A (**2**), aloe-emodin (**8**), rhein (**9**), emodin (**11**) and chrysophanol (**12**) are the active components in Rhei rhizome, whilst coptisine (**4**), palmatine (**5**) and berberine (**6**) are the active components in Coptidis rhizome, and baicalin (**3**), baicalein (**7**) and wogonin (**10**) are the active components in Scutellariae radix (Fig. 1). A simple, sensitive, reliable HPLC-UV method using a gradient solvent system has been developed to quantify simultaneously the 12 major bioactive indicators in Rhei rhizome, Coptidis rhizome and Scutellariae radix. Moreover, the validated HPLC method was then successfully applied to the quantification of these crude herbs and commercial products of SHXXT.

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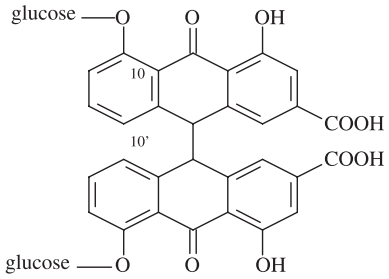
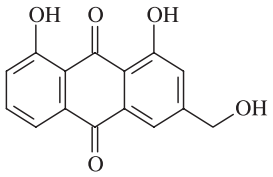
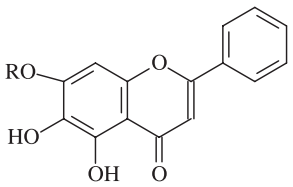
Sennoside B (10-10' meso) (**1**)
Sennoside A (10-10' trans) (**2**)



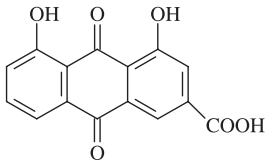
Aloe-emodin (**8**)



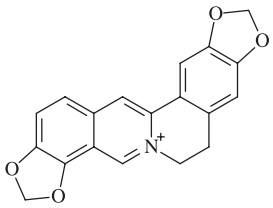
Baicalin (**3**): R = glucopyranuronosyl
Baicalein (**7**): R = H



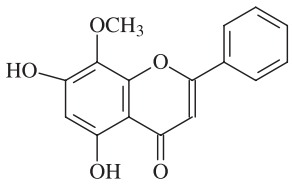
Rhein (**9**)



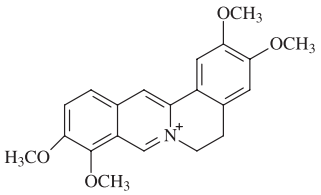
Coptisine (**4**)



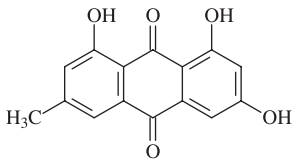
Wogonin (**10**)



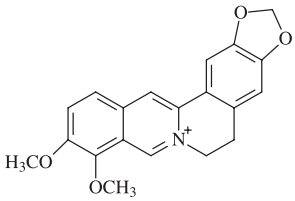
Palmatine (**5**)



Emodin (**11**)



Berberine (**6**)



Chrysophanol (**12**)

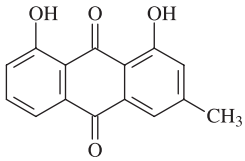


Figure 1 The chemical structures of the 12 bioactive components of SHXXT.

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EXPERIMENTAL

Chemicals and materials

The standards of **1–5**, **7** and **10** were purchased from Wako (Osaka, Japan), of **6**, **8**, **9**, **11** and indomethacin (IS) from Sigma (St. Louis, MO, USA), and of **12** from Extrasynthése (Genay Cedex, France). Rhei rhizome, Coptidis rhizome and Scutellariae radix were all obtained from a local drug store in Kaohsiung. The commercial products of SHXXT were manufactured by GMP medicinal companies. Methanol and acetonitrile for sample preparation and liquid chromatography were of HPLC grade and were obtained from Tedia (Fairfield, OH, USA). All other chemicals and reagents were of analytical reagent grade.

Apparatus and chromatographic conditions

The analyses were carried out on a Hitachi (Tokyo, Japan) HPLC system consisting of an L-7100 pump, an L-7200 auto-sampler and a L-7455 photodiode array detector. An Atlantis™ from Waters (Milford, MA, USA) dC_{18} reversed-phase column (5 μm, 150 × 4.6 mm i.d.; 5 μm) and a Supelguard Discovery from Supelco (Bellefonte, PA, USA) C_{18} guard column (20 × 4.0 mm i.d.; 5 μm) were used and UV absorption was measured either with a full spectrum (200–600 nm) or at 280 nm.

The separations were obtained by gradient elution using solvent A (acetonitrile) and solvent B (0.01 M sodium 1-pentanesulphate buffer adjusted with phosphoric acid to pH 3) at ambient temperature. All mobile phase solutions were filtered and degassed before use. The gradient program was as follows: initial step 0–15 min linear change from 15:85 (v/v; A:B) to 20:80; 15–50 min linear change to 55:45; 50–55 min linear change to 90:10, which was maintained for 5 min; followed at 1 h by a return to the initial conditions. The flow-rate was kept constant at 1.0 mL/min. The injection volume was 20 μL. Under these conditions, the internal standard and the 12 bioactive standard compounds from Rhei rhizome, Coptidis rhizome and Scutellariae radix were separated satisfactorily within the run time stipulated.

Determination of 12 marker standards

Calibration curves. Methanol stock solutions containing the standards were prepared and diluted to an appropriate concentration range for the construction of calibration curves. The linearity of each calibration curve was determined in triplicate using six different concentrations. The concentration of the internal standard, indomethacin, was 30 μg/mL for all analyses. Calibration curves were analysed using the linear least-squares regression equation derived by plotting concentrations of each standard as a function of peak area ratio. The concentrations of these compounds in commercial products were calculated using the regression parameter obtained from the calibration curves.

Accuracy, precision and limits of detection. The relative standard deviation (RSD%) was taken as a measure of precision and the percentage difference (RE%) between the amount of analyte determined and the spiked amount was considered as a measure of accuracy in the developed assay. Three concentrations of standard samples spiked with the internal standard were also prepared similarly. The peak area ratio difference between test and control samples for each analyte was calculated, and the quantity of each analyte was subsequently obtained from the corresponding calibration curve.

The limit of detection (LOD) was determined by adding aliquots of standards to methanol in order to obtain appropriate concentrations. LOD was defined as the amount of analyte added such that the magnitude of the test peak signal was three times greater than the noise level.

Quantification of the marker components

In each crude herb. A 10 mg sample of pulverized Rhei rhizome, Coptidis rhizome or Scutellariae radix was extracted individually with 50 mL of 65% methanol by stirring at room temperature for 30 min. The suspension was centrifuged at 3000 rpm for 10 min, Hitachi Centrifuger 05 P-22 (Tokyo, Japan), internal standard added and the resultant mixture filtered and analysed by HPLC.

In commercial products of SHXXT. Dried commercial SHXXT samples (10 mg) were extracted with 50 mL of 65% methanol at ambient temperature for 1 h with stirring. The resultant mixture was centrifuged (12,000 rpm; 10 min) and aliquots were analysed by HPLC. The contents of the analytes were determined from the corresponding calibration curves.

RESULTS AND DISCUSSION

HPLC analysis

The HPLC assay described in the present study constituted an attempt to develop a simple chromatographic method capable of separating simultaneously the marker components in a crude herb mixture of Rhei rhizome, Coptidis rhizome and Scutellariae radix. Figure 2 depicts a representative chromatogram showing the separation of the 12 analytes under optimised

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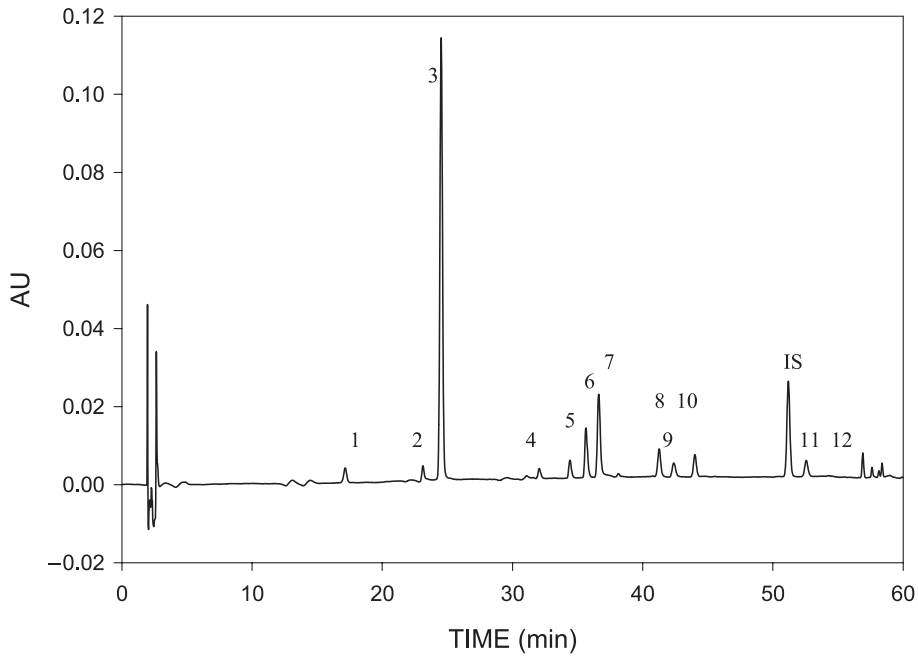

Figure 2 HPLC chromatogram showing the separation of the 12 standard bioactive components of SHXXT under optimised conditions. Key to peak identities as in Fig. 1. (For chromatographic protocol see the Experimental section; UV detection at 280 nm.)

Table 1 Calibration curves, calibration ranges and detection limits for the 12 standard components of SHXXT

Compound	Retention time (min)	Regression equation[a]	Calibration range (μg/mL)	Limit of detection (ng/mL)
Sennoside B (1)	17.3	$y = (13.4 \pm 0.1)x + (0.0 \pm 0.0)$	0.3–6.4	50
Sennoside A (2)	23.3	$y = (19.9 \pm 0.2)x + (-0.0 \pm 0.0)$	0.3–6.4	75
Baicalin (3)	24.5	$y = (6.8 \pm 0.0)x + (-0.1 \pm 0.2)$	3.3–80	25
Coptisine (4)	32.4	$y = (9.1 \pm 0.1)x + (-0.0 \pm 0.0)$	0.1–2.4	25
Palmatine (5)	34.8	$y = (8.8 \pm 0.1)x + (-0.1 \pm 0.0)$	0.2–4	50
Berberine (6)	36.0	$y = (10.2 \pm 0.2)x + (-0.2 \pm 0.1)$	0.5–12	50
Baicalein (7)	36.9	$y = (7.3 \pm 0.1)x + (0.4 \pm 0.1)$	0.8–20	75
Aloe-emodin (8)	41.5	$y = (2.7 \pm 0.0)x + (0.0 \pm 0.0)$	0.1–2.4	50
Rhein (9)	42.4	$y = (13.4 \pm 0.1)x + (-0.0 \pm 0.0)$	0.3–6.4	75
Wogonin (10)	44.2	$y = (3.4 \pm 0.0)x + (0.0 \pm 0.0)$	0.1–2.4	25
Emodin (11)	52.7	$y = (4.4 \pm 0.0)x + (0.0 \pm 0.0)$	0.1–2.4	50
Chrysophanol (12)	57.0	$y = (5.4 \pm 0.0)x + (-0.0 \pm 0.0)$	0.1–2.4	25

[a] Equations in the form $y = (slope \pm SE)x + (intercept \pm SE)$; R^2 values for all calibration curves were >0.99.

conditions. The bioactive components were adequately separated within 1 h using the described HPLC method. Owing to the presence of highly polar compounds such as **1** and **2**, the composition of the solvent system was changed slowly during the initial step. Although the separation time could be reduced to 35 min using a faster gradient rate, the analysis of the commercial product SHXXT would be impaired by unknown components with short elution times. Based on the above considerations, we obtained an appropriate resolution for the crude herbs and the combinatorial formula of SHXXT.

Calibration curves were constructed by plotting the peak area ratio against concentration of each standard compound. Linear regression analysis was performed for each standard and acceptable linearity was obtained with respect to concentration and peak area ratio and the correlation coefficient (R^2) for each calibration curve was greater than 0.99. Table 1 lists the slopes, intercepts, correlation coefficients and calibration ranges obtained for the regression lines. The limits of detection at a signal-to-noise ratio of 3 for the analytes ranged from 25 to 75 ng/mL, as shown in Table 1.

The precision and accuracy of the method for each analyte expressed as the RSD and RE were calculated for each standard concentration and complied with the requirements of assay validation. All data showed the

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Table 2 Recovery and precision of the method at different spiked concentrations for each analyte

Compound	Spiked concentration (μg/mL)	Determined concentration (μg/mL)	RSD (%)	RE (%)
Sennoside B (**1**)	4.3	4.3 ± 0.1	3.3	0.7
	2.1	2.1 ± 0.1	2.4	0.0
	0.5	0.5 ± 0.1	9.4	0.0
Sennoside A (**2**)	4.3	4.5 ± 0.3	5.8	4.7
	2.1	2.3 ± 0.2	8.7	7.0
	0.5	0.5 ± 0.0	4.1	–8.2
Baicalin (**3**)	53.3	54.8 ± 0.8	1.4	2.6
	26.7	26.6 ± 0.6	2.3	–0.1
	6.7	6.6 ± 0.3	3.9	–0.7
Coptisine (**4**)	1.6	1.6 ± 0.1	4.9	1.2
	0.8	0.9 ± 0.1	6.7	11.1
	0.2	0.2 ± 0.0	10.0	0.0
Palmatine (**5**)	2.7	2.8 ± 0.1	2.2	3.3
	1.3	1.3 ± 0.0	0.8	0.0
	0.3	0.4 ± 0.0	8.1	10.8
Berberine (**6**)	8.0	8.9 ± 0.2	2.5	10.2
	4.0	4.4 ± 0.2	3.6	9.9
	1.0	1.1 ± 0.1	10.0	9.1
Baicalein (**7**)	13.3	13.7 ± 0.2	1.7	2.5
	6.7	6.5 ± 0.3	4.8	–2.8
	1.7	1.8 ± 0.1	5.6	7.2
Aloe-emodin (**8**)	1.6	1.7 ± 0.0	1.2	3.0
	0.8	0.8 ± 0.0	3.9	–3.9
	0.2	0.2 ± 0.0	10.5	–5.3
Rhein (**9**)	4.3	4.4 ± 0.1	2.9	3.8
	2.1	2.1 ± 0.0	1.5	–2.9
	0.5	0.5 ± 0.0	4.0	–6.0
Wogonin (**10**)	1.6	1.6 ± 0.0	1.2	1.2
	0.8	0.8 ± 0.1	7.4	1.2
	0.2	0.2 ± 0.0	4.8	4.8
Emodin (**11**)	1.6	1.6 ± 0.0	1.9	1.2
	0.8	0.8 ± 0.0	1.2	1.2
	0.2	0.2 ± 0.0	5.0	0.0
Chrysophanol (**12**)	1.6	1.6 ± 0.0	2.5	1.8
	0.8	0.8 ± 0.0	2.5	0.0
	0.2	0.2 ± 0.0	1.0	0.6

excellent repeatability of the sample analysis. Table 2 presents the RSD and RE at low, medium and high concentration levels of each standard compound, respectively. The RSD values ranged from 1.0 to 10.5% at low level, 0.8 to 8.7% at medium level, and 1.2 to 5.8% at high level. Thus, the HPLC method had the advantages of high resolution and efficiency.

Determination of 12 bioactive compounds in each crude herb and in commercial products of SHXXT

The validated HPLC method was subsequently applied to identify the components in the extracts of each crude herb and in the commercial products of SHXXT. Figure 3 shows typical chromatograms for Rhei rhizome (A), Coptidis rhizome (B), and Scutellariae radix (C) obtained using the optimised HPLC method. Six marker compounds were found in Rhei rhizome, and three marker compounds were individually found in both Coptidis rhizome and Scutellariae radix. The tentatively identified peaks of the marker compounds in the chromatograms were confirmed from the UV profiles of the pure individual components.

The method was also employed to determine the content of the 12 markers in three different commercial products of SHXXT, which combined Rhei rhizome, Coptidis rhizome and Scutellariae radix. Figure 4 shows the chromatograms of the extracts of SHXXT obtained using 65% methanol as the extraction solvent. The marker compounds could be determined individually in each sample of SHXXT, and their identities were

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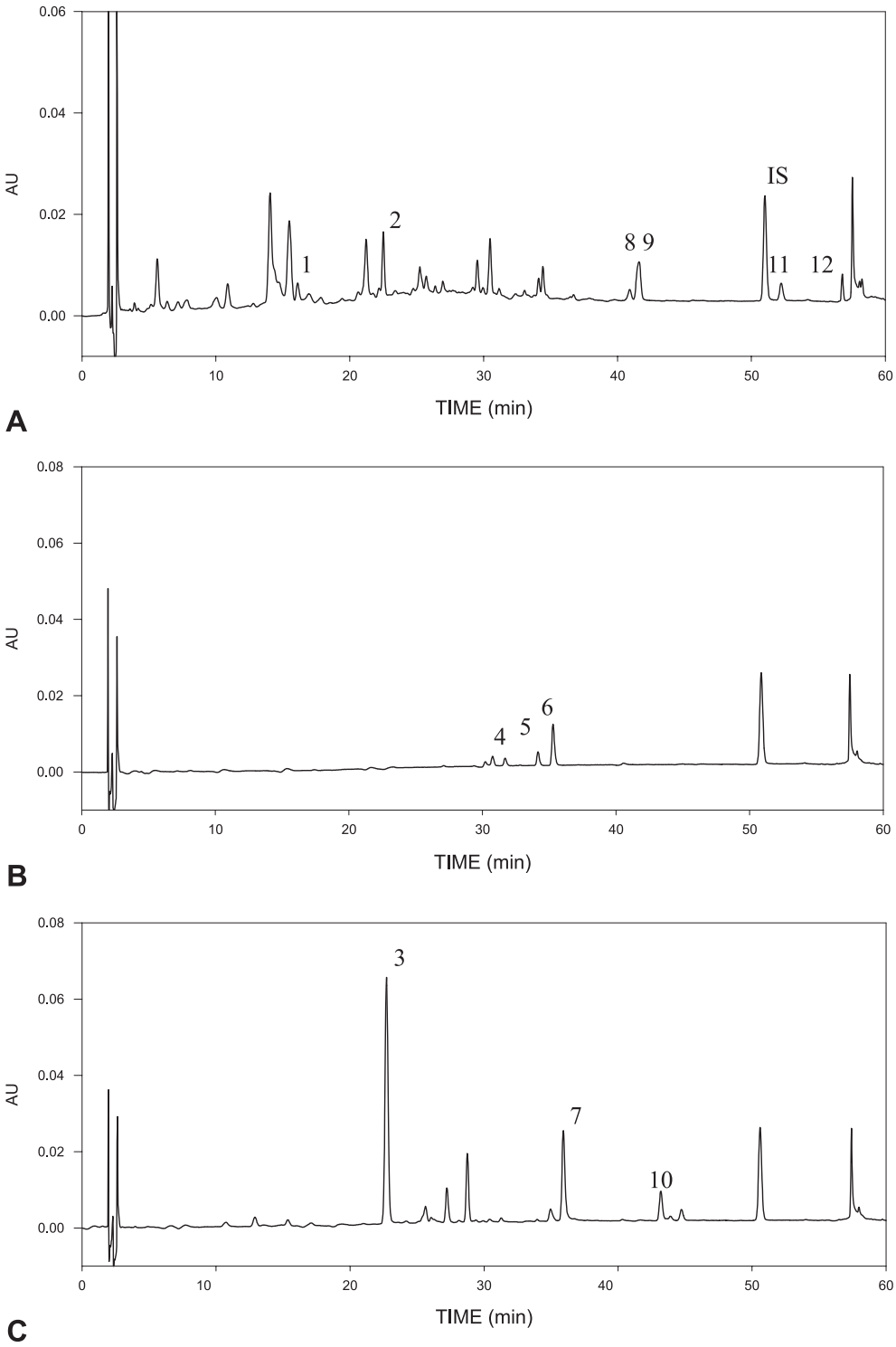

Figure 3 HPLC chromatograms of Rhei rhizome (A), Coptidis rhizome (B) and Scutellariae radix (C) obtained under optimised chromatographic conditions. Key to peak identities as in Fig. 1. (For chromatographic protocol see the Experimental section; UV detection at 280 nm.)

confirmed by comparing both retention times and UV spectra with those of the standards. In the extract of commercial products of SHXXT, **6** and **7** were present in the highest amounts with percentages up to 4.0 and 3.3% (sample C), respectively (Table 3). Compounds **1**, **8**, **11** and **12** were present at less than 0.1% in SHXXT.

The composition of the traditional Chinese medicinal preparation is quite complex and some components may alter during the manufacturing process. Hence,

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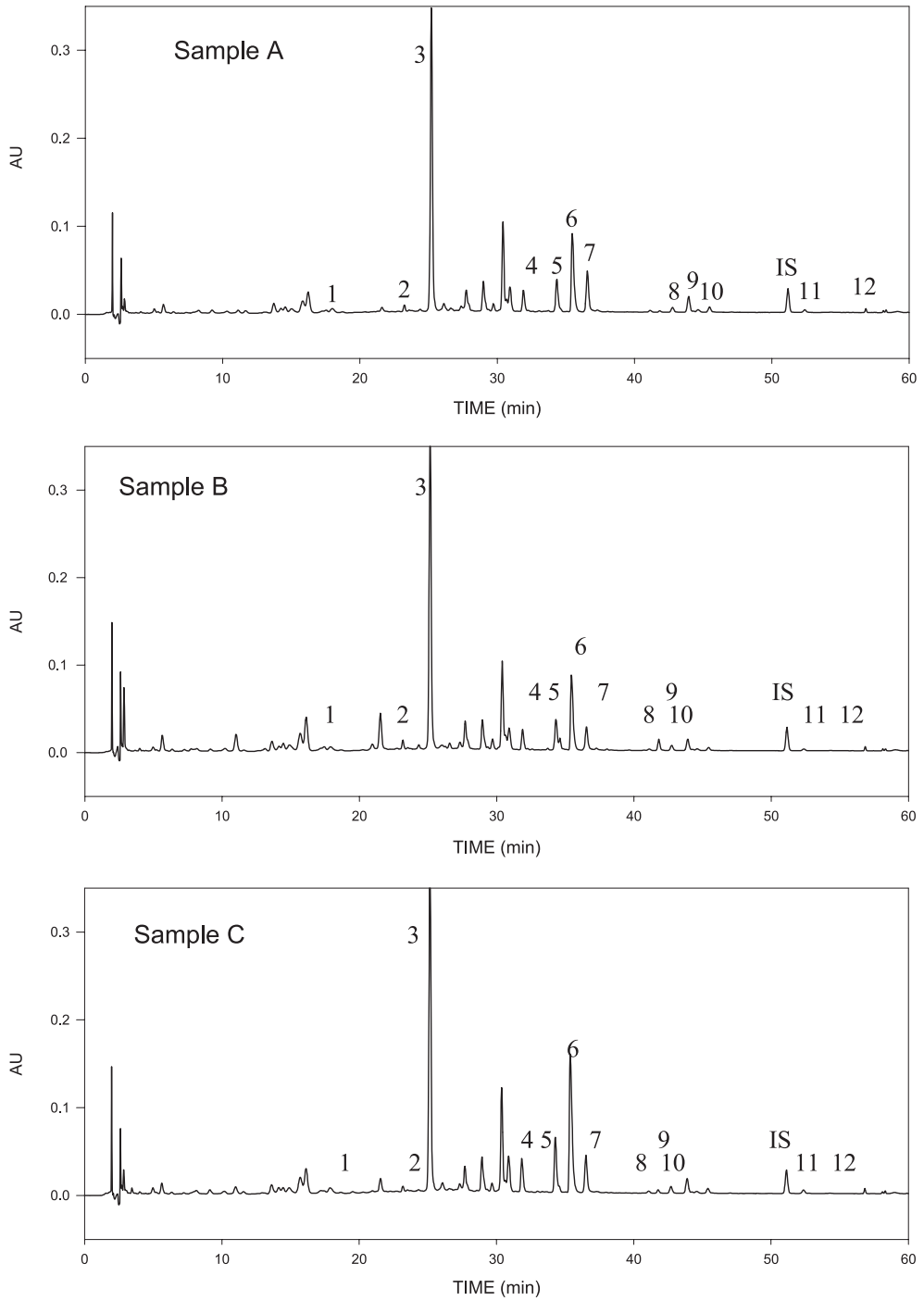

Figure 4 HPLC chromatograms of the extract of commercial products A, B and C of SHXXT. Key to peak identities as in Fig. 1. (For chromatographic protocol see the Experimental section; UV detection at 280 nm.)

the analyte contents in the commercial products were different from those of the individual single herbs taken together. However, the HPLC method with UV detector could rapidly and efficiently analyse the constituents in the crude herb and in traditional Chinese medicinal preparations containing Rhei rhizome, Coptidis rhizome and Scutellariae radix. At the same time, the developed HPLC assay could be utilised as a quality control method for crude herbs or commercial products.

In conclusion, the 12 marker compounds could be completely separated within 1 h using a gradient elution system. The analytical method was successfully applied to determine the quantity of marker components extracted from the crude herbs Rhei rhizome, Coptidis rhizome and Scutellariae radix. Moreover,

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Table 3 The percentage of bioactive compounds in commercial products of SHXXT

	Sample A (n = 3)		Sample B (n = 3)		Sample C (n = 3)	
Compound	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)
Sennoside B (**1**)	0.0 ± 0.0	7.8	0.1 ± 0.0	7.7	0.0 ± 0.0	6.3
Sennoside A (**2**)	0.2 ± 0.0	6.2	0.4 ± 0.0	4.0	0.2 ± 0.0	9.7
Baicalin (**3**)	3.5 ± 0.1	2.2	3.8 ± 0.1	2.4	4.0 ± 0.0	0.7
Coptisine (**4**)	0.3 ± 0.1	16.5	0.3 ± 0.0	3.3	0.6 ± 0.0	1.0
Palmatine (**5**)	0.5 ± 0.0	8.2	0.6 ± 0.0	2.9	1.0 ± 0.0	4.4
Berberine (**6**)	1.8 ± 0.2	9.2	1.7 ± 0.0	2.5	3.3 ± 0.1	1.6
Baicalein (**7**)	0.7 ± 0.0	2.6	0.4 ± 0.0	3.1	0.6 ± 0.0	0.9
Aloe-emodin (**8**)	0.0 ± 0.0	17.0	0.0 ± 0.0	4.6	0.0 ± 0.0	7.5
Rhein (**9**)	0.2 ± 0.0	6.4	0.3 ± 0.0	2.4	0.2 ± 0.0	2.8
Wogonin (**10**)	0.1 ± 0.0	2.2	0.1 ± 0.0	5.5	0.1 ± 0.0	1.5
Emodin (**11**)	0.0 ± 0.0	1.4	0.0 ± 0.0	3.7	0.0 ± 0.0	4.9
Chrysophanol (**12**)	0.1 ± 0.0	2.9	0.1 ± 0.0	3.9	0.1 ± 0.0	4.2

commercial products of SHXXT, which combined the three herbs, could be determined using the HPLC method with a relatively simple extraction procedure.

Acknowledgements

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