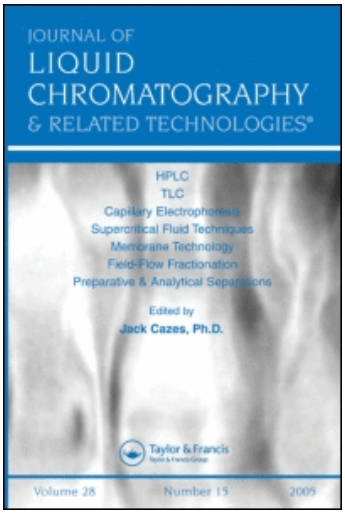

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Simultaneous Determination of Seven Bioactive Ingredients in a Chinese Herbal Preparation by HPLC

Lei Xu[a]; Qiao Wang[a]; De-Qiang Li[a]; Min Li[a]; Xiu-Juan Jing[a]; Lan-Tong Zhang[a]
[a] Department of Pharmaceutical Analysis, School of Pharmaceutical Science, Hebei Medical University, Shijiazhuang, Hebei, P. R. China

To cite this Article Xu, Lei , Wang, Qiao , Li, De-Qiang , Li, Min , Jing, Xiu-Juan and Zhang, Lan-Tong(2009) 'Simultaneous Determination of Seven Bioactive Ingredients in a Chinese Herbal Preparation by HPLC', Journal of Liquid Chromatography & Related Technologies, 32: 5, 732 — 745
To link to this Article: DOI: 10.1080/10826070802711246
URL: http://dx.doi.org/10.1080/10826070802711246

Journal of Liquid Chromatography & Related Technologies®, 32: 732–745, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802711246

Simultaneous Determination of Seven Bioactive Ingredients in a Chinese Herbal Preparation by HPLC

Lei Xu, Qiao Wang, De-Qiang Li, Min Li, Xiu-Juan Jing, and Lan-Tong Zhang

Department of Pharmaceutical Analysis, School of Pharmaceutical Science, Hebei Medical University, Shijiazhuang, Hebei, P. R. China

Abstract: A novel method based on high performance liquid chromatography with variable wavelength detection was developed for quantitative analysis of the bioactive ingredients in a Chinese herbal preparation called Kangnaoshuai capsule (KNS). Seven bioactive ingredients, namely puerarin, albiflorin, stilbene glucoside, salvianolic acid B, baicalin, baicalein, and wogonin were simultaneously determined. The separation was carried out on a RP-C_{18} column with acetonitrile and 0.1% phosphoric acid as mobile phase under gradient conditions. Five detection wavelengths, 248 nm for puerarin, 230 nm for albiflorin, 320 nm for stilbene glucoside, 275 nm for baicalin, baicalein and wogonin, and 286 nm for salvianolic acid B, were utilized during a single elution process. The validation data proved the developed method to be sensitive, precise, accurate, and selective, and was successfully applied for the determination of commercial KNS samples from different production batches and different manufacturers.

Keywords: Quantitative analysis, RP-HPLC, Simultaneous determination, Traditional Chinese medicine, Variable wavelength detection

Correspondence: Lan-Tong Zhang, Department of Pharmaceutical Analysis, School of Pharmaceutical Science, Hebei Medical University, Shijiazhuang, Hebei 050017, P. R. China. E-mail: zhanglantong@263.net

INTRODUCTION

Traditional Chinese medicine (TCM) has been used for thousands of years for the treatment of a wide variety of diseases, and it is still very prevalent in China nowadays in the form of modern herbal preparation. The Pharmacopoeia of the People's Republic of China has documented nearly six hundred Chinese herbal preparations, which are in extensive use at present.[1] Since TCM preparations are always complex mixtures consisting of multiple ingredients, their quality control by effective analysis tools are in urgent need for the modernization of TCM within modern pharmaceutical industry.

Kangnaoshuai capsule (KNS) is an over the counter herbal preparation mainly consisting of Radix Puerariae, Radix Paeoniae Alba, Radix Polygoni Multiflori, Radix Salviae Miltiorrhiae, and Radix Scutellariae. KNS is used to treat insomnia, amnesia, and neurasthenia. It has also been reported to be beneficial in the treatments for age related dementia, brain injuries caused by stroke, cerebral trauma, and encephalitis.[2–5] The modern pharmacology studies have revealed that puerarin from Radix Puerariae, albiflorin from Radix Paeoniae Alba, stilbene glucoside from Radix Polygoni Multiflori, salvianolic acid B from Radix Salviae Miltiorrhiae, baicalin, baicalein, and wogonin from Radix Scutellariae are the bioactive ingredients in these herbs,[6–12] therefore simultaneous determination of these seven ingredients is in urgent need to improve the quality control of KNS.

There has already been one HPLC method reported for quality control of KNS,[13] however, only puerarin, paeoniflorin, and baicalin have been determined. Although the methods for determination of these chemical components in herbs, such as Radix Puerariae, Radix Paeoniae Alba, Radix Polygoni Multiflori, Radix Salviae Miltiorrhiae, and Radix Scutellariae have been well reported,[14–17] as far as we know, the method for simultaneous separation and determination of the seven of them is not available in literatures.

High performance liquid chromatography with gradient elution, as one of the most powerful analytical technologies, is widely used in the routine analysis of complex mixtures because of its simplicity and suitability. The fact that the ingredients in complex mixtures usually possess different UV spectra and thus, different maximum absorption wavelengths, makes the single wavelength detection less selective and sensitive. A variable wavelength detector (VWD) can be programmed for determination at each component's maximum absorption wavelength by varying the detection wavelength during a single elution process. In this respect, VWD is more powerful than a single wavelength detector and more simple and sensitive than a photodiode array detector.

In this study, a novel method based on high performance liquid chromatography with variable wavelength detection was developed for

quantitative analysis of the seven bioactive ingredients in KNS. Five detection wavelengths were employed during a single elution process. The validation data proved the developed method to be sensitive, precise, accurate, and selective, and the method was also successfully applied to quantitative analysis of KNS capsules from different production batches and different manufacturers.

EXPERIMENTAL

Reagents, Chemicals, and Materials

Acetonitrile and methanol (TEDIA, Fairfield, USA) were of HPLC grade. Ultrapure water obtained from Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. Analytical grade phosphoric acid (Yongda, Tianjin, China) was used for preparation of the mobile phase.

The Reference chemical standards for quantitative analysis were puerarin, albiflorin, 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (stilbene glucoside), salvianolic acid B, baicalin, baicalein, and wogonin purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their purities were more than 98% by HPLC analysis. The chemical structures are shown in Figure 1.

KNS samples of different production batches and different manufacturers of the constituent herbs of KNS were provided by a pharmaceutical manufacturer in China.

Equipment and Chromatographic Conditions

The quantitative analysis was carried out on a Waters 1525 binary pump with a Waters 2487 UV detector (Waters, USA), a column apartment, and a 20 μL injection loop controlled by the workstation of Empower software (Waters). The mobile phase consisted of acetonitrile (A) and 0.1% aqueous phosphoric acid (v/v, B). The gradient program for quantitative analysis was: 0–15 min, 12–20% A; 15–30 min, 20–45% A; 30–40 min, 45–75% A; 40–50 min, 75–100% A; 50–55 min, 100–12% A. The detection wavelength was programmed as: 0.00–6.10 min, 320 nm; 6.10–8.50 min, 248 nm; 8.50–15.00 min, 230 nm; 15.00–25.00 min, 320 nm; 25.00–27.10 min, 275 nm; 27.10–32.50 min, 286 nm; 32.50–50.00 min, 275 nm. The sample injection volume was 20 μL. The seperation was carried out on a Waters Sunfire^{TM}-C18 column (150 × 4.6 mm I.D., 5 μm). The column was equilibrated with the initial composition of the mobile phase for 10 min after each run. The column temperature was maintained at 30°C and the flow rate was 1 mL/min.

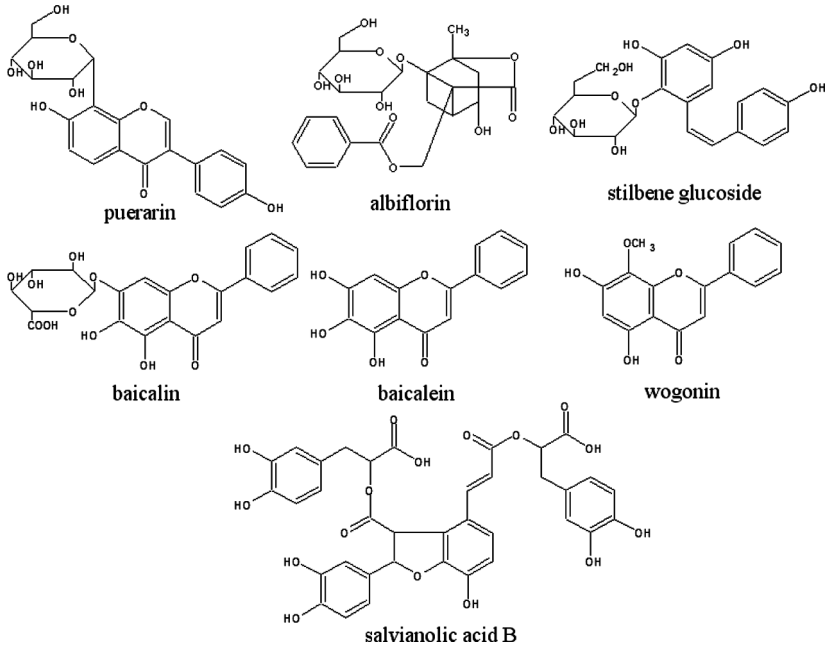

Figure 1. Chemical structures of the seven bioactive ingredients for quantitative analysis.

Standard Solution Preparation

The stock standard solution of puerarin (0.306 mg/mL), albiflorin (0.041 mg/mL), stilbene glucoside (0.166 mg/mL), salvianolic acid B (0.124 mg/mL), baicalin (0.266 mg/mL), baicalein (0.048 mg/mL), and wogonin (0.006 mg/mL) were prepared with 50% methanol and stored away from light at 4°C. Then, a series of seven calibration solutions were prepared by diluting the stock standard solutions of 0.1, 0.2, 0.5, 1.0, 2.0, 4.0, 8.0 mL, to 10 mL volumetric flask by 50% methanol for construction of the regression equations. All solutions were filtered through 0.45 μm membrane filter before injection.

Sample Preparation

An accurately weighted dried powder (100 mg) of the contents of KNS capsule was extracted with 25.0 mL 50% methanol (v/v) by a sonifier (25 KHz, 100 W) at room temperature for 30 min. The negative control samples with the absence of Radix Puerariae, Radix Paeoniae Alba,

Radix Polygoni Multiflori, Radix Salviae Miltiorrhiae, and Radix Scutellariae were prepared, respectively, with the same method by mixing the extracts of the other herbs.

Method Validation

The method validation parameters of this analytical method included linearity, precision, accuracy, and selectivity.

RESULTS AND DISCUSSION

Optimization of Extraction Method

Extraction solvents of different solvent compositions of water, 25%, 50%, 75%, and 100% methanol were tested for optimization of the extraction efficacy of KNS, and 50% methanol was employed because the chemical components were better extracted compared with the others as shown in Figure 2. During the tests, it was also found that the use of 50% methanol could avoid peak distortion caused by injecting the sample with a high concentration of methanol into the column equilibrated by the initial composition of the mobile phase with a low concentration of acetonitrile.

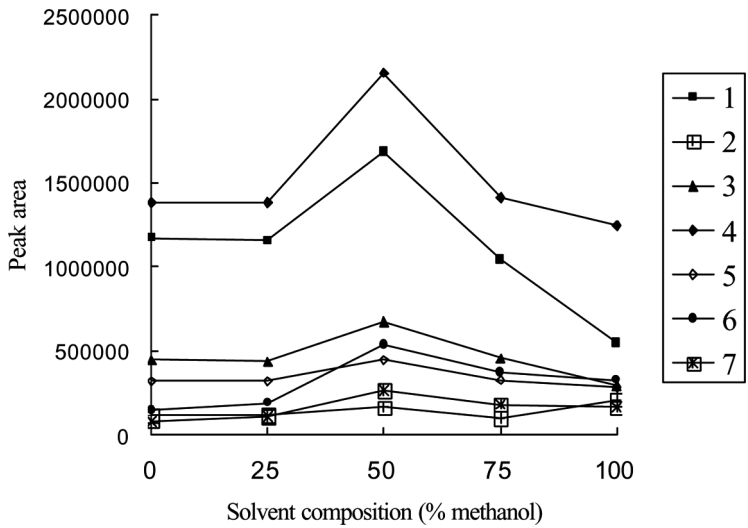

Figure 2. Comparison of different extraction solvents for optimization of the extraction efficacy of KNS. The numbers of the ingredients from 1–7 are puerarin, albiflorin, stilbene glucoside, baicalin, salvianolic acid B, baicalein, and wogonin.

Optimization of Chromatographic Conditions

At the beginning of this study, a variety of analytical columns (all 250×4.6 mm I.D., 5 μm) were tested, but none of them could achieve the resolution requirement within 90 min; the analytical time was so long that shorter columns were employed. After comparing different columns such as Waters Sunfire^{TM}-C18, Agilent Zorbax SB-C18, Agilent Eclipse XDB-C18, (all 150×4.6 mm I.D., 5 μm), Waters Sunfire column was employed for its better performance of separation within 50 min.

Since the seven ingredients possess different maximum absorption wavelengths at 248 nm, 230 nm, 320 nm, 275 nm, and 286 nm, the single wavelength detection for quantitative analysis would be less efficient and sensitive. During the process of quantitative analysis, the UV detector was programmed to monitor the chemical components at each component's maximum absorption wavelength by varying the detection wavelength during a single elution process. The chromatograms of the varied wavelength detection and single wavelength detection of the standard solution are shown in Figure 3. As can be seen, the application of the varied wavelength detection method can provide more sensitive and selective quantitative information than single wavelength detection.

Methanol-water, acetonitrile-water, and acetonitrile-aqueous acid were tested for mobile phase systems. Acid was used to improve the peak shape and different acids were tested for optimization. Acetonitrile and phosphoric acid were employed for their lower background absorption compared with methanol and formic acid or acetic acid. The use of acetonitrile also had the advantage of better resolution and relatively short analysis time. Various gradient programs according to the initial composition of the mobile phase, the gradient time, and the gradient shape were tested in order to separate the seven ingredients with high resolution.

Different column temperatures of 25°C, 30°C, 35°C, and 40°C were tested and it was observed that increasing the column temperature had no obvious effect on the separation of the investigated ingredients, except the reduction of analysis time. The reduction time between 25°C and 40°C was not more than 0.82 min and it was not remarkable compared with the total analysis time, therefore, the column temperature was determined at 30°C.

Method Validation

Linearity

The determination of the seven ingredients was carried out using the external standard method. The seven peaks in the sample were identified by comparing their retention times to those of the standard solution, and

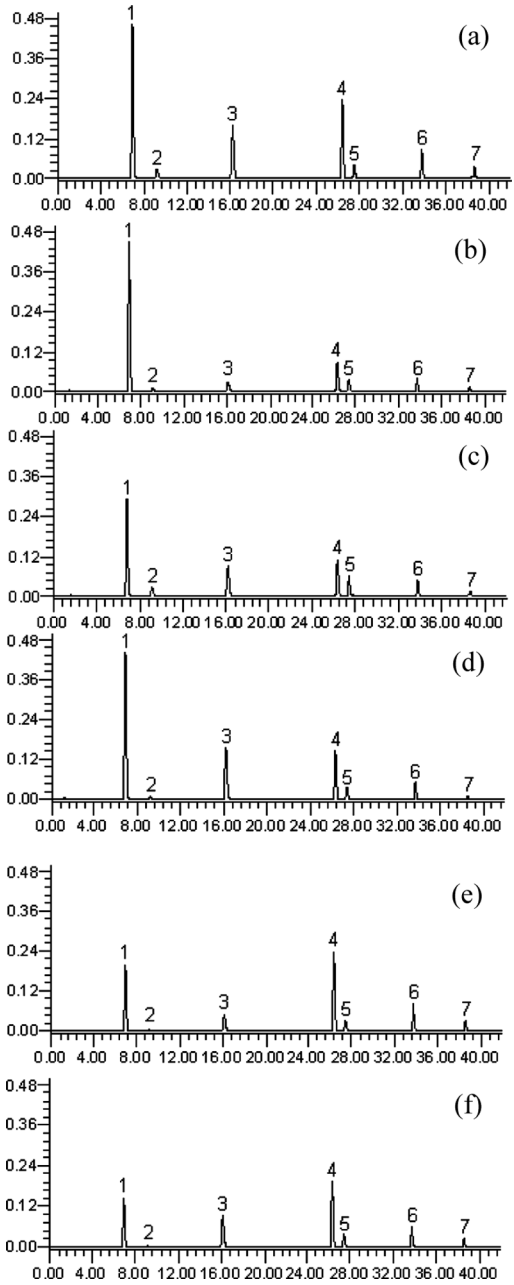

Figure 3. HPLC chromatograms of the standard solution by (a) varied wavelength detection and single-wavelength detection at (b) 248 nm; (c) 230 nm; (d) 320 nm; (e) 275 nm; (f) 286 nm. The numbers of the ingredients are the same as shown in Figure 2.

Table 1. Calibration curves, LODs and LOQs of the seven bioactive ingredients

Analytes	Regression Equation[a]	Test range (μg/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)
Puerarin	$y = 55380x - 118834$	3.06–244.80	0.9997	4.90	19.58
Albiflorin	$y = 41287x - 9970$	0.41–32.85	0.9998	16.40	65.68
Stilbene glucoside	$y = 75532x - 135070$	1.66–132.96	0.9996	6.65	13.30
Baicalin	$y = 51986x + 1635$	2.66–212.90	0.9999	4.26	17.03
Salvianolic acid B	$y = 21587x - 9038$	1.24–98.80	0.9999	1.98	7.92
Baicalein	$y = 126575x - 23912$	0.49–38.80	0.9999	3.88	7.76
Wogonin	$y = 270578x + 2530$	0.06–5.19	1	1.04	2.08

[a]y is the peak area and x is the concentration of the ingredient (μg/mL).

were further confirmed by spiking standards. The regression equation of each ingredient was calculated with seven different concentrations by plotting the peak areas versus the concentrations. All the seven ingredients showed good linearity within their test ranges. The limits of detection (LOD) and quantification (LOQ) were determined at a signal to noise ratio of about 3 and 10 by analyzing the diluted standard solution. The regression equations, correlation coefficients, test ranges, LODs, and LOQs are shown in Table 1.

Precision

The retention times and the peak areas of the seven ingredients were analyzed for evaluation of the precision. The injection precision was evaluated by analyzing the six repeated injections of the standard solution. Intra-day precision was evaluated by analyzing six replications prepared from the KNS sample within a day. Duplicate copies of the KNS sample were prepared within a day for the consecutive three days, and inter-day precision was evaluated by analyzing the six sample solutions of three days. As shown in Table 2, the RSDs of the retention times and the peak areas for injection, intra-day and inter-day precision tests were not more than 0.24% and 0.85%, 0.37% and 3.03%, 0.46% and 2.94%, respectively.

Accuracy

The recovery test was carried out to evaluate the accuracy of the developed method. Accurately weighted amounts of each reference compound at three levels were mixed with a fixed amount of the contents of KNS sample. Three replications at each level were extracted and analyzed. The results of the recovery test are shown in Table 3. The RSDs of the

Table 2. Precision of the seven ingredients for quantitative analysis

Analytes	Intra-day precision (RSD%, n = 6)		Inter-day precision (RSD%, n = 6)		Injection precision (RSD%, n = 6)	
	Retention time	Peak area	Retention time	Peak area	Retention time	Peak area
Puerarin	0.25	0.85	0.43	0.91	0.24	0.70
Albiflorin	0.18	2.01	0.32	1.77	0.15	0.57
Stilbene glucoside	0.19	1.04	0.46	1.00	0.09	0.85
Baicalin	0.25	0.69	0.17	1.14	0.09	0.52
Salvianolic acid B	0.37	2.48	0.23	2.94	0.13	0.44
Baicalein	0.11	3.03	0.24	1.39	0.02	0.39
Wogonin	0.04	2.22	0.05	0.65	0.02	0.53

Table 3. Recoveries of the seven ingredients for quantitative analysis

Analytes	Original (μg)	Spiked (μg)	Found (μg)	Recovery (%)	RSD (%)
Puerarin	35.48	17.93	55.54	104.9	1.2
		35.86	75.56	108.3	0.5
		44.83	83.84	105.1	0.6
Albiflorin	3.41	1.72	5.25	106.8	1.4
		3.45	7.03	104.8	1.0
		4.32	7.98	105.9	1.9
Stilbene glucoside	12.00	6.78	18.45	95.2	1.6
		13.56	25.06	96.3	0.6
		16.95	28.40	96.7	0.8
Baicalin	42.29	21.28	58.68	92.3	0.9
		42.58	82.07	96.7	1.2
		53.20	91.76	96.1	0.4
Salvianolic acid B	20.60	9.45	28.07	93.4	2.7
		18.82	35.87	91.0	3.8
		23.52	42.66	96.7	4.7
Baicalein	5.66	3.88	9.10	95.4	2.9
		7.76	12.08	90.0	1.2
		9.70	14.13	92.0	1.9
Wogonin	1.34	0.52	1.80	96.6	1.2
		1.04	2.15	90.2	2.3
		2.35	3.49	94.6	1.9

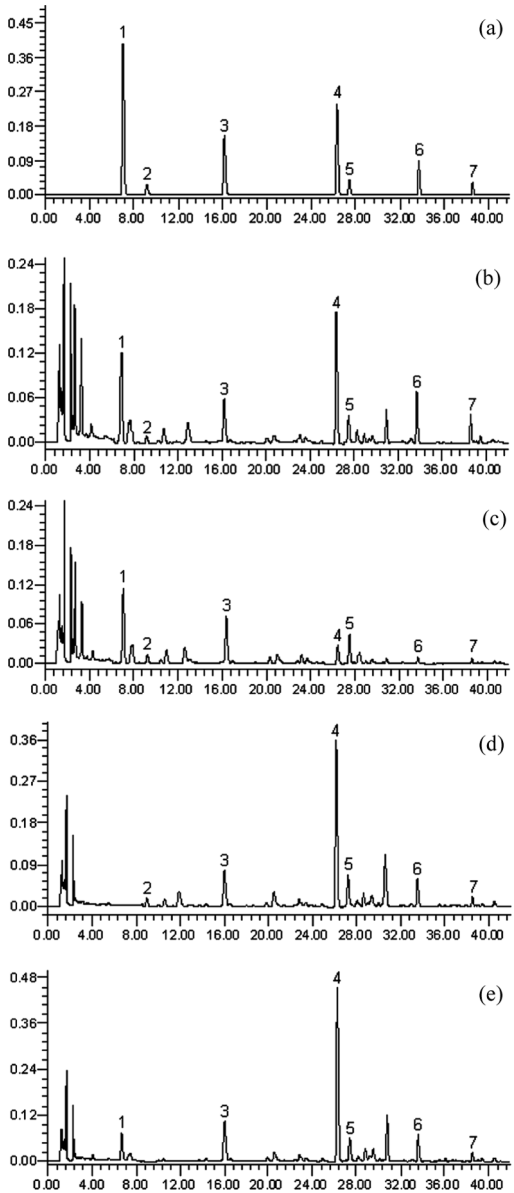

Figure 4. HPLC chromatograms of the quantitative analysis of (a) standard solution; (b) KNS sample (batch of 0843); (c) KNS sample (batch of 1250) and negative control samples of (d) Radix Puerariae; (e) Radix Paeoniae Alba; (f) Radix Polygoni Multiflori; (g) Radix Scutellariae; (h) Radix Salviae Miltiorrhiae. The numbers of the ingredients are the same as shown in Figure 2.

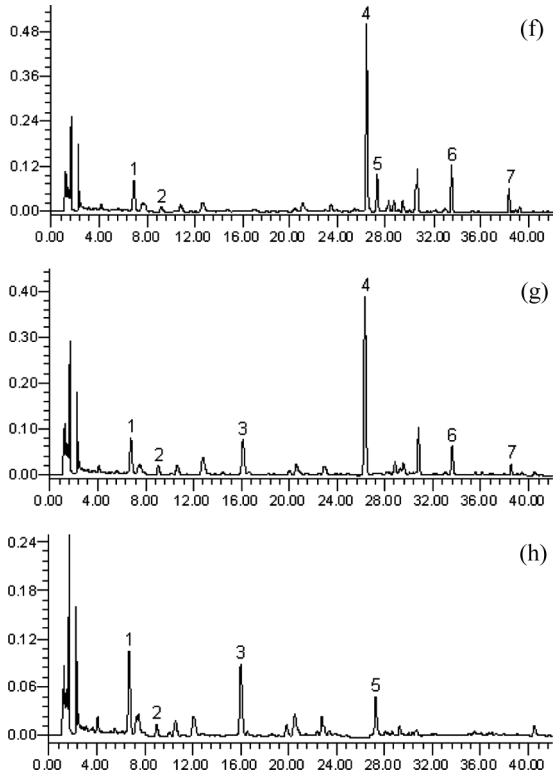

Figure 4. Continued.

recovery were not more than 4.7% and the recovery was all within the range of 90.0–108.3%, which indicates the good accuracy of the developed method.

Selectivity

Since so many constituent herbs coexist, negative control samples were analyzed to evaluate the selectivity of the developed method. Negative control samples were prepared, respectively, with absence of the constituent herbs from which the seven ingredients were originated. The chromatograms of the negative control samples of Radix Puerariae, Radix Paeoniae Alba, Radix Polygoni Multiflori, Radix Salviae Miltiorrhiae, and Radix Scutellariae are shown in Figure 4. The results showed that the seven ingredients were determined without the interference of other chemical components which originated from the coexisting herbs. The results indicated that the developed method for quantitative analysis was sensitive, precise, accurate, and selective.

Table 4. Content (mg/g) of the seven ingredients of different production batches

Samples[a]	Puerarin	Albiflorin	Stilbene glucoside	Baicalin	Salvianolic acid B	Baicalein	Wogonin
0535	6.41[b]	0.73	2.11	11.86	5.47	1.16	0.24
0843	8.87	0.85	3.00	10.57	5.15	1.42	0.34
0844	8.74	0.82	2.96	10.58	4.92	1.39	0.34
0845	8.75	0.75	2.38	10.20	4.52	1.37	0.37
1249	7.81	0.75	3.46	9.64	4.44	1.40	0.33
1250	6.33	1.13	3.53	1.73	5.53	0.27	0.06
1251	5.86	1.11	3.57	10.98	5.47	1.13	0.25
1254	6.17	1.05	2.46	11.53	5.61	1.13	0.25
1255	7.92	0.96	2.61	10.29	5.29	1.12	0.24
1256	6.58	1.07	2.03	11.04	4.84	1.21	0.28

[a]The number of the production batch.
[b]The average data of three replications.

Application

The developed method was applied for determination of commercial KNS samples from different production batches and different manufacturers. The assay was calculated from the regression equation of each ingredient by the mean peak area of three replications. The results are shown in Table 4 and the variation of the content of the seven ingredients was prominent. The content of Baicalin, Baicalein, and Wogonin of the batch of 1250 is extremely lower than the mean value. The chromatograms of the batch of 0843 and 1250 for quantitative analysis are shown in Figure 4. The results confirmed the quality inconsistency among different production batches and different manufacturers.

CONCLUSION

In this study, a sensitive and simple analytical method for simultaneous determination of seven ingredients in a Chinese herbal medicine KNS was developed. High linearity, sensitivity, precision, accuracy, and selectivity were presented in the method validation procedure. Therefore, the proposed HPLC-UV method is effective to show the quality variation of KNS and, thus, is suitable to improve the quality control level of this Chinese herbal preparation.

REFERENCES

1. Pharmacopoeia Commission of People's Republic of China. *Chinese Pharmacopoeia*, Vol. 1; Chemical Industry Press: Beijing, China, 2005.
2. Li, R.J.; Wang, R.C.; Ha, Z.Y.; Hu, F.Q.; Wang, R.D.; Han, Y. Clinical observation and therapeutic efficacy analysis of kangnaoshuai capsule in 291 clinical cases. Chin. Tradit. Pat. Med. **1987**, *8*, 20–23.
3. Cheng, R.Y. The beneficial effects of kangnaoshuai capsule for the treatments of brain injuries caused by cerebral trauma. Zhejiang Zhongxiyijiehe Zazhi **2002**, *9*, 547–548.
4. Tan, Y.; Zhu, Y.H.; Yin, M.; Yan, Y.; Li, Y. Clinical observation of kangnaoshuai capsule in 46 clinical cases of vascular related dementia. Yunnan Yiyao **1998**, *3*, 200.
5. Huang, Y.G. Therapeutic efficacy analysis of kangnaoshuai capsule in 100 clinical cases of insomnia. Shiyong Yixue Zazhi **2003**, *5*, 554–555.
6. Yeung, K.Y.; Leung, W.S.; Xu, Y.C.; Vanhoutte, M.; Man, Y.K. Puerarin, an isoflavonoid derived from Radix puerariae, potentiates endothelium-independent relaxation via the cyclic AMP pathway in porcine coronary artery. Euro J. Pharma. **2006**, *552*, 105–111.
7. Wu, S.H.; Chen, Y.W.; Yang, L.Y.; Li, S.L.; Li, Z.Y. Monoterpene glycosides from Paeonia delavayi. Fitoterapia. **2007**, *78*, 76–78.
8. Misiti, F.; Sampaolese, B.; Mezzogori, D.; Orsini, F.; Pezzotti, M.; Giardina, B.; Clementi, M.E. Protective effect of rhubarb derivatives on amyloid beta (1–42) peptide-induced apoptosis in IMR-32 cells: A case of nutrigenomic. Brain. Res. Bull. **2006**, *71*, 29–36.
9. Tian, L.L.; Wang, X.J.; Sun, Y.N.; Li, C.R.; Xing, Y.L.; Zhao, H.B.; Duan, M.; Zhou, Z.; Wang, S.Q. Salvianolic acid B, an antioxidant from Salvia miltiorrhiza, prevents 6-hydroxydopamine induced apoptosis in SH-SY5Y cells. Inter. J. Biochem. & Cell Bio. **2008**, *40*, 409–422.
10. Liu, L.L.; Gong, L.K.; Wang, H.; Xiao, Y.; Wu, X.F.; Zhang, Y.H.; Xue, X.; Qi, X.M.; Ren, J. Baicalin inhibits macrophage activation by lipopolysaccharide and protects mice from endotoxin shock. Biochem. Pharmaco. **2008**, *75*, 914–922.
11. Lapchak, P.A.; Maher, P.; Schubert, D.; Zivin, J.A. Baicalein, an antioxidant 12/15-lipoxygenase inhibitor improves clinical rating scores following multiple infarct embolic strokes. Neurosci. **2007**, *150*, 585–591.
12. Lu, N.; Gao, Y.; Ling, Y.; Chen, Y.; Yang, Y.; Gu, H.Y.; Qi, Q.; Liu, W.; Wang, X.T.; You, Q.D.; Guo, Q.L. Wogonin suppresses tumor growth in vivo and VEGF-induced angiogenesis through inhibiting tyrosine phosphorylation of VEGFR2. Life Sci. **2008**, *82*, 956–963.
13. Wang, F.L.; Han, S.Z.; Wang, Y.; Yuan, B.; Feng, L. Combination of quantitative analysis with fingerprint study used for quality evaluating of Kangnaoshuai Capsule. Chin. Tradit. Herb. Drugs. **2007**, *3*, 375–379.
14. Wen, K.C.; Huang, C.Y.; Lu, F.L. Determination of baicalin and puerarin in traditional Chinese medicinal preparations by high-performance liquid chromatography. J. Chromatogr. A. **1993**, *631*, 241–250.

15. Liao, H.M.; Wang, L.L.; Tan, L.L.; Hu, Z.H. Determination of stilbene glucoside for different origins of Radix Polygoni Multiflori. Chin. Trad. Herb. Drugs. **2006**, *37*, 603–604.
16. Jiang, L.H.; Xu, T.S.; Chen, X.X.; Liu, Q. A Study on the HPLC Method for Salvianolic Acid B in Radix Salviae Miltiorrhizae. Lishizhen Med. Materia Med. Res. **2007**, *18*, 1395–1396.
17. Zhang, H.; Tian, K.; Tang, J.H.; Qi, S.D.; Chen, H.L.; Chen, X.G.; Hu, Z.D. Analysis of baicalein, baicalin and wogonin in Scutellariae radix and its preparation by microemulsion electrokinetic chromatography with 1-butyl-3-methylimizolium tetrafluoborate ionic liquid as additive. J. Chromatogr. A. **2006**, *1129*, 304–307.

Received September 30, 2008
Accepted October 23, 2008
Manuscript 6414