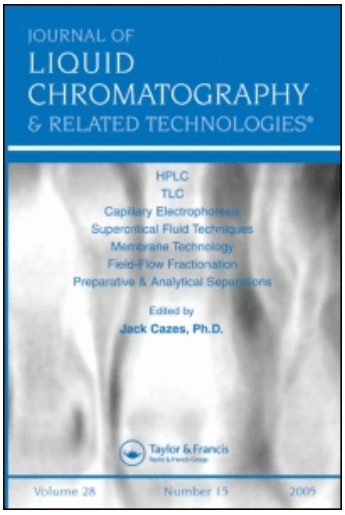


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

DETERMINATION OF ARISTOLOCHIC ACID IN TRADITIONAL CHINESE MEDICINAL PRESCRIPTIONS, CONTAINING RADIX ARISTOLOCHIAE FANGCHI, BY HPLC

Yuan-Shiun Chang[a]; Jeng-Shyan Deng[a]; Yoe-Ray Ku[b]
[a] Institute of Chinese Pharmaceutical Sciences, China Medical College, Taichung, Taiwan [b] Department of Health, National Laboratories of Foods and Drugs, Nankang, Taipei, Taiwan

Online publication date: 05 September 2002

To cite this Article Chang, Yuan-Shiun , Deng, Jeng-Shyan and Ku, Yoe-Ray(2002) 'DETERMINATION OF ARISTOLOCHIC ACID IN TRADITIONAL CHINESE MEDICINAL PRESCRIPTIONS, CONTAINING RADIX ARISTOLOCHIAE FANGCHI, BY HPLC', Journal of Liquid Chromatography & Related Technologies, 25: 6, 961 — 975
To link to this Article: DOI: 10.1081/JLC-120003273
URL: http://dx.doi.org/10.1081/JLC-120003273

J. LIQ. CHROM. & REL. TECHNOL., 25(6), 961–975 (2002)

DETERMINATION OF ARISTOLOCHIC ACID IN TRADITIONAL CHINESE MEDICINAL PRESCRIPTIONS, CONTAINING RADIX ARISTOLOCHIAE FANGCHI, BY HPLC

Yuan-Shiun Chang,[1,*] Jeng-Shyan Deng,[1] and Yoe-Ray Ku[2]

[1]Institute of Chinese Pharmaceutical Sciences, China Medical College, 91, Hsueh-Shih Road, Taichung 404, Taiwan
[2]National Laboratories of Foods and Drugs, Department of Health, Executive Yuan, 161-2, Kuen-Yang Street, Nankang, Taipei 115, Taiwan

ABSTRACT

A high performance liquid chromatographic method for determination of aristolochic acid, a nephrotoxicant in traditional Chinese medicinal prescriptions containing Radix Aristolochiae Fangchi, was established. (The prescriptions included Mu-Fang-Ji-Tang, Fang-Ji-Huang-Qi-Tang, Shu-Jing-Huo-Xue-Tang, Xiao-Xu-Ming-Tang, Shang-Zhong-Xia-Tong-Yong-Tong-Feng-Fang, and Fang-Ji-Fu-Ling-Tang.) The samples were analyzed by HPLC on a LiChrospher 100 RP-18e column and detected at

*Corresponding author. E-mail: yschang@mail.cmc.edu.tw

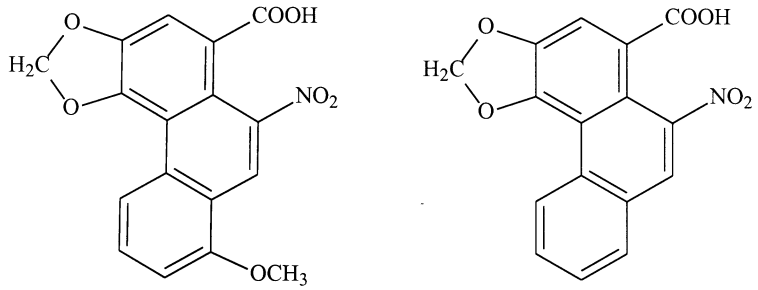
395 nm with acetonitrile water and 2% (v/v) acetic acid (45 : 55, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The regression equations of aristolochic acid I was $Y = 2.85e + 004X - 5.20e + 003$ ($r = 0.9999$). The intraday and interday relative standard deviations of aristolochic acid I were at the levels of 0.31–2.01% and 0.09–1.85%, respectively. The recoveries of aristolochic acid I added in the six kinds of blank prescriptions were between 93.0 and 102.0%. Thirty four samples of commercial concentrated preparation containing Fang-Ji were analyzed and the results showed that fourteen samples contained aristolochic acid I. The contents of aristolochic acid I in samples were between 0.30 to 1.84 mg/g.

INTRODUCTION

Radix Aristolochiae Fangchi (RAF) is the dried root of *Aristolochia fangchi* (Aristolochiaceae), which promotes diuresis and relieves edema, dispells wind and dampness, and relieves pain. The Fang-Ji available in Chinese medicine, includes not only *A. fangchi* (Aristolochiaceae), but also *Stephania tetrandra, Cocculus trilobus* and *Sinomenium acutumn* (Menispermaceae) (1).

Aristolochic acid (AA, mixture of aristolochic acid I and II, chemical structures are shown in Figure 1) is the major component of *Aristolochia* sp. Some of the species in *Aristolochia* are commonly used in traditional Chinese medicine, such as *A. fangchi*, *A. manshuriensis*, *A. mollissima*, *A. contorta*, and *A. debilis* (2).

A new cause of progressive interstitial fibrosis of the kidney was identified in Belgium and was related to a slimming regimen, which included Chinese



Aristolochic acid I Aristolochic acid II

Figure 1. Chemical structures of aristolochic acid I and aristolochic acid II.

medicine. In February 1993, Vanherweghem reported that many young women patients, after taking a slimming regimen that included Chinese herbs, experienced renal failure (3). The nephropathy that was characterized by a rapid deterioration in renal function and biopsy, displayed extensive interstitial fibrosis without glomerular lesions, as well as atrophy and loss of tubules. The nephrotic symptoms are called CHN (Chinese Herbs Nephropathy). Possible causal factors of this nephropathy are fungal and plant nephrotoxins such as ochratoxin A and AA (3). Further investigations indicated that DNA adducts, which were formed by AA metabolites were detected in kidney tissues obtained from CHN patients (4), but failed to demonstrate the presence of AA in the capsules taken by the patients (5). Therefore, an easy and suitable method for the detection and assay of AA in traditional Chinese medicinal prescriptions was developed.

Our previous studies have established HPLC methods for analysis of markers in prescriptions containing Radix Scrophulariae, Rhizoma Gastrodiae, and Folium Theae (6–8). In this study, we selected six kinds of traditional Chinese medicinal prescriptions: Mu-Fang-Ji-Tang (hereafter abbreviated as P1), Fang-Ji-Huang-Qi-Tang (P2), Shu-Jing-Huo-Xue-Tang (P3), Xiao-Xu-Ming-Tang (P4), Shang-Zhong-Xia-Tong-Yong-Tong-Feng-Fang (P5), and Fang-Ji-Fu-Ling-Tang (P6), all of which contain RAF. This paper deals with the HPLC method for determining the content of AA in each prescription.

EXPERIMENTAL

Materials

The materials used to prepare the traditional Chinese medicine prescriptions were as follows (9):

Mu-Fang-Ji-Tang (P1)

Radix Aristolochiae Fangchi (12.0 g), Ramulus Cinnamomi, Radix Codonopsis Pilosulae (10.0 g each), Gypsum Fibrosum (30.0 g).

Fang-Ji-Huang-Qi-Tang (P2)

Radix Aristolochiae Fangchi (4.0 g), Radix Astragali (4.4 g), Rhizoma Atractylodis Macrocephalae (3.0 g), Fructus Ziziphi Jujubae, Rhizoma Zingiberis Recens (1.0 g each), Radix Glycyrrhizae Praeparata (2.0 g).

Shu-Jing-Huo-Xue-Tang (P3)

Radix Paeoniae Albae (2.5 g), Radix Angelcae Sinensis, Rhizoma Ligustici Chuanxiong, Radix Rehmanniae Praeparata, Rhizoma Atractylodis Macrocephalae, Semen Persicae, Poria (2.0 g each), Radix Achyranthis Bidentatae, Radix Clematidis, Radix Aristolochiae Fangchi, Rhizoma seu Radix Notopterygii, Radix Ledebouriellae, Radix Gentianae, Radix Angelicae Dahuricae, Exocarpium Citri Grandis, Rhizoma Zingiberis Recens (1.5 g each), Radix Glycyrrhizae Praeparata (1.0 g).

Xiao-Xu-Ming-Tang (P4)

Radix Aconiti Praeparata (1.2 g), Radix Ledebouriellae (2.5 g), Radix Paeoniae Albae, Radix Aristolochiae Fangchi, Herba Ephedrae, Rhizoma Ligustici Chuanxiong, Radix Scutellariae, Ramulus Cinnamomi (1.8 g each), Rhizoma Zingiberis Recens, Semen Armeniacae Amarum, Radix Glycyrrhizae Praeparata, Frucutus Ziziphi Jujubae (1.5 g each), Radix Codonopsis Pilosulae (1.8 g).

Shang-Zhong-Xia-Tong-Yong-Tong-Feng-Fang (P5)

Cortex Phellodendri, Rhizoma Atractylodis, Rhizoma Arisaematis (3.0 g each), Massa Fermentata Medicinalis, Rhizoma Ligustici Chuanxiong, Semen Persicae, Radix Gentianae, Radix Aristolochiae Fangchi, Radix Angelicae Dahuricae (1.5 g each), Rhizoma seu Radix Notopterygii, Radix Clematidis, Ramulus Cinnamomi, Flos Carthami (0.5 g each).

Fang-Ji-Fu-Ling-Tang (P6)

Radix Aristolochiae Fangchi (4.0 g), Radix Astragali (4.4 g), Ramulus Cinnamomi, Poria, Radix Glycyrrhizae Praeparata (2.0 g each).

All materials were obtained from Chinese Crude Drug Pharmacy, China Medical College Hospital, and all crude drugs were cut into pieces and homogenized. Five prescriptions (P1–P5) of commercial concentrated preparations were purchased from eight pharmaceutical companies, and a total of thirty-four samples were collected.

Chemicals and Reagents

AA standard was purchased from SIGMA (St. Louis, USA) (mixture of AA, predominantly I and II; with 43% of AAI and 54% of AAII), methanol and acetonitrile (HPLC grade) were purchased from Mallinckrodt (Kentucky, USA), glacial acetic acid (HPLC grade) was purchased from ALPS (Taipei, Taiwan), Ultrapure distilled water with a resistance greater than 18 MΩ was used.

Instruments

The analysis was performed using a Waters 2690 separations module pump with a Waters 996 photodiode array detector and a Waters 717 autosampler. Merck column LiChrospher 100 RP-18e (5 μm, 4.0 I.D × 250 mm) was used. Peak area was calculated using Millennium 3.2 edition software in the computer integrator. The mobile phase was the mixtures of acetonitrile water and 2.0% (v/v) acetic acid (45 : 55 v/v). The flow rate was 1.0 mL/min and the detective wavelength was 395 nm.

Extraction Condition

In order to obtain better extraction of AAI from crude drugs or prescriptions, different times of extraction with methanol were tested. Five groups of 1.0 g RAF were accurately weighed and extracted with 50 mL methanol for 30 min by ultrasonication, respectively. The extraction of these five group samples were performed once, twice, three-, four-, or fivefold, respectively. After sedimentation of undissolved material, the clear suspension was filtered. The residue and the container were then washed with 10 mL methanol. The extracts were combined and filtered. The combined filtrates were concentrated at 40°C, using a rotary evaporator under vacuum to remove organic solvents. The residue was quantitatively transferred into 10 mL volumetric flasks and diluted to volume with methanol. These solutions were filtered through 0.45 μm Millipore filters before use. AAI in the samples of these five groups that were extracted with methanol at different times was quantified, respectively. Besides, Fang-Ji-Huang-Qi Tang (P2) 1.54 g (one tenth per daily dose) was also treated by the same method.

Preparation of Standard Solution

AA standard (11.6 mg) was accurately weighed into 50 mL volumetric flasks (AAI 5.0 mg), and dissolved in methanol to volume (AAI 100 μg/mL). The

stock solution was serially diluted with methanol to obtain concentrations ranging from 0.63–100.00 μg/mL of AAI. These solutions were filtered through 0.45 μm Millipore filters before use. 20 μL of each dilution was injected into HPLC. Calibration curves were plotted, based on linear regression analysis of peak-areas versus concentrations.

Preparation of Sample Solution

Reconstituted Prescriptions Decoction

The accurately weighed amount representing one tenth per daily dose according to traditional Chinese medical prescriptions, was extracted three times with 50 mL methanol for 30 min by ultrasonication. After sedimentation of undissolved material, the clear suspension was filtered. The residue and the container were then washed with 10 mL methanol. The extracts were combined and filtered. The combined filtrates were concentrated at 40°C using a rotary evaporator under vacuum to remove organic solvents. The residue was quantitatively transferred into 10 mL volumetric flasks and diluted to volume with methanol. These solutions were filtered through 0.45 μm Millipore filters before use.

Blank Prescriptions Decoction

RAF was omitted from traditional Chinese medical prescriptions and the blank prescriptions were treated according to the method described above for the preparation of the reconstituted prescriptions decoction.

Commercial Concentrated Preparation Decoction

0.6 g of concentrated preparation (one tenth per daily dose) were accurately weighed, and the samples were treated according to the method described above for the preparation of the reconstituted prescriptions decoction.

System Suitability Test

To assess the linearity of these methods, four concentrations of AA (AAI 2.5, 10, 40, 100 μg/mL) were determined five times on the same day and in a five-day period. The intraday and interday variations were studied.

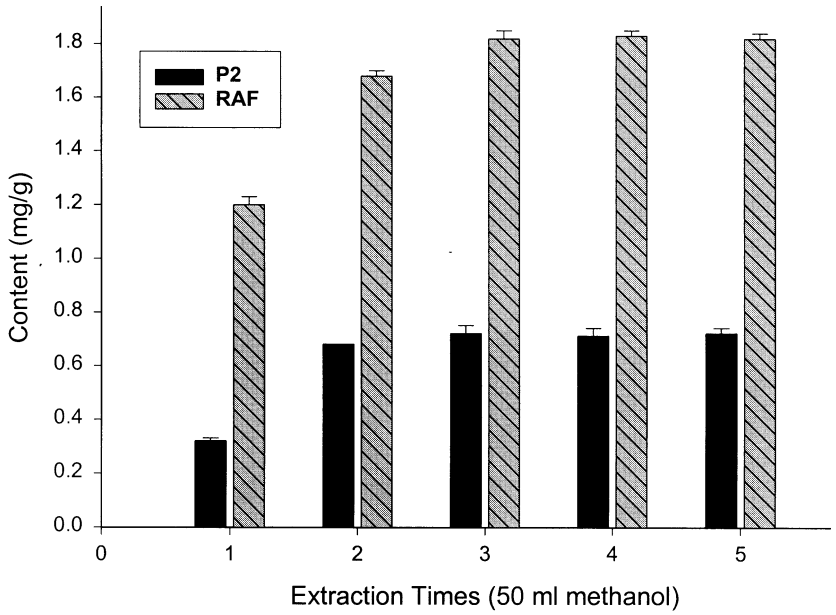


Figure 2. The exhaust test of aristolochic acid I in Radix Aristolochiae Fangchi (RAF) and Fang-Ji-Huang-Qi-Tang (P2).

Recovery Test

Three different concentrations of AAI (10.0, 20.0, and 40.0 μg/mL) were added to each blank prescription. These mixtures were treated according to the method described above for the preparation of the reconstituted prescription decoction. The sample solutions (20 μL) were separately injected into HPLC. Recoveries were calculated using the following formula:

$$\text{Recovery } (\%) = (\text{Amount measured}/\text{Amount added}) \times 100\%$$

RESULTS AND DISCUSSION

Chemical constituents of each Chinese herb are very complex. It is even more difficult to analyze the chemical components of traditional Chinese medical prescriptions, which are composed of numerous Chinese herbs. In this study, more than four crude drugs were combined in each prescription, some prescriptions contain up to 17 crude drugs. The kinds of composed crude

drugs and weight ratios of RAF were 4 and 19.4%, 6 and 26.0%, 17 and 5.2%, 13 and 8.1%, 13 and 7.5%, 5 and 27.8% in these six prescriptions (P1–P6), respectively. All possess very complex chemical compositions and the content of AA naturally occurring in RAF seldom exceeds 0.3% (10). Proper methods are required for the detection and assay of AAI in traditional Chinese medical prescriptions.

Photodiode array detection was used in this experiment so that UV spectra of AAI could be compared with the reference standard.

The results of the extraction test indicated that extraction three times with 50 mL methanol for 30 min by ultrasonication and washing the residue with 10 mL methanol afforded the highest yield of AAI in the samples (Figure 2). This condition was used in the extraction protocol.

The linearity of the plot of peak-area (x) versus concentration (y, μg/mL) for AAI was investigated. The regression equations of AAI was $Y = 2.85e + 004X - 5.20e + 003$ (correlation coefficients = 0.9999). It showed a satisfactory linearity.

A signal three times higher than the peak noise height was regarded as the detection limit. The detection limit of AAI was 0.21 μg/mL. This result indicates that the developed method is sensitive enough to be an official method for monitoring AAI in traditional Chinese medical prescriptions.

The precision of the method was evaluated by measuring the reproducibility [relative standard deviation (R.S.D.)] and the accuracy was determined by recovery tests. The precision R.S.D.s of the proposed method of AAI, on the basis of peak-area for five replicate injections, were 0.31–2.01% for intraday, 0.09–1.85% for interday, respectively (Table 1). Precision of the method was high, with relative standard deviations below 2.5%. The results for the recoveries of AAI added in six kinds of blank prescriptions ranging from 93.0 to 102.0%. The R.S.D.s ranged from 1.34 to 3.34% (Table 2). Recoveries of AAI were excellent (> 90%) from the prescriptions by the simple extraction procedure.

Table 1. Intra-Day and Inter-Day Analytical Precisions of Four Concentrations (2.5–100.0 μg/mL) of Aristolochic Acid I

Chemical compound	Concentration (μg/mL)	Intra-day (R.S.D.,%)	Inter-day (R.S.D.,%)
Aristolochic acid I	2.5	1.12	1.59
	10.0	0.46	0.09
	40.0	2.01	1.85
	100.0	0.31	1.60

n = 5.

Table 2. Recoveries of Aristolochic Acid I Added in Blank Prescriptions

Blank	Amount added (μg/mL)	Amount measured (μg/mL)	Recovery (%)	Mean ± S.D (%)	R.S.D (%)
P1	10.0	9.8	98.0	96.8 ± 1.3	1.34
	20.0	19.1	95.5		
	40.0	38.7	96.8		
P2	10.0	10.1	101.0	99.4 ± 3.2	3.22
	20.0	20.3	101.5		
	40.0	38.3	95.8		
P3	10.0	10.2	102.0	98.7 ± 3.3	3.34
	20.0	19.7	98.5		
	40.0	38.2	95.5		
P4	10.0	9.6	96.0	94.8 ± 1.6	1.69
	20.0	18.6	93.0		
	40.0	38.1	95.3		
P5	10.0	9.8	98.5	98.8 ± 1.7	1.72
	20.0	20.1	100.7		
	40.0	38.9	97.3		
P6	10.0	10.2	102.0	100.4 ± 1.5	1.49
	20.0	19.8	99.0		
	40.0	40.1	100.3		

n = 3.

The wavelength at 254 nm was suitable to detect AAI in RAF (6), but it would be interfered by other constituents in the prescriptions. When the detective wavelength was changed to 395 nm, the interference of AAI can be avoided from others. Therefore, we selected UV 395 nm as a detective wavelength in this study.

There was only a small amount of AAII contained in RAF, and no matter which detector wavelength, 254 or 395 nm, was used, there would still be interference by other constituents in the prescriptions. Therefore, AAII was not chosen as marker in the prescriptions.

The HPLC chromatograms of RAF and AA standards are shown in Figure 3. The contents of AAI in RAF and reconstituted prescription decoctions (P1–P6) ranged from 1.68 to 1.84 mg/g, and are shown in Table 3. The comparison of chromatograms of reconstituted prescription, blank prescription, and blank prescriptions with AA in the six prescriptions (P1–P6), are shown in Figure 4 and Figure 5. The contents of AAI in commercial concentrated preparations can also be analyzed with the same method. Thirty four samples of commercial concentrated preparations, containing Fang-Ji from eight pharmaceutical companies, were analyzed and the results showed that fourteen samples

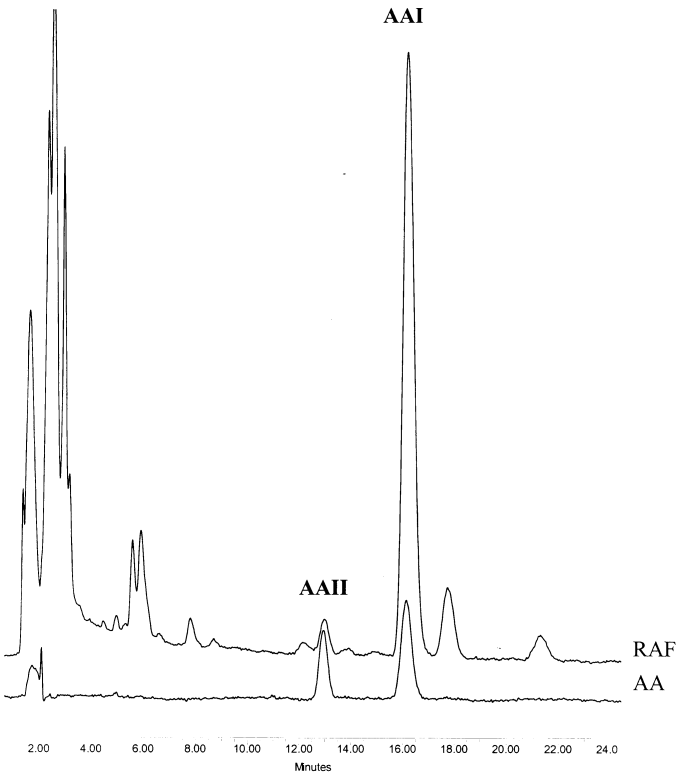


Figure 3. The HPLC chromatograms of Radix Aristolochiae Fangchi (RAF) and aristolochic acid (AA). AAI, aristolochic acid I; AAII, aristolochic acid II.

Table 3. The Contents of Aristolochic Acid I in Radix Aristolochiae Fangchi and Reconstituted Prescriptions Decoctions (P1–P6)

Sample	Content Mean ± S.D. (mg/g)
Radix Aristolochia Fangchi	1.83 ± 0.15
P1	1.81 ± 0.03
P2	1.72 ± 0.11
P3	1.84 ± 0.06
P4	1.76 ± 0.04
P5	1.68 ± 0.13
P6	1.84 ± 0.05

n = 3.

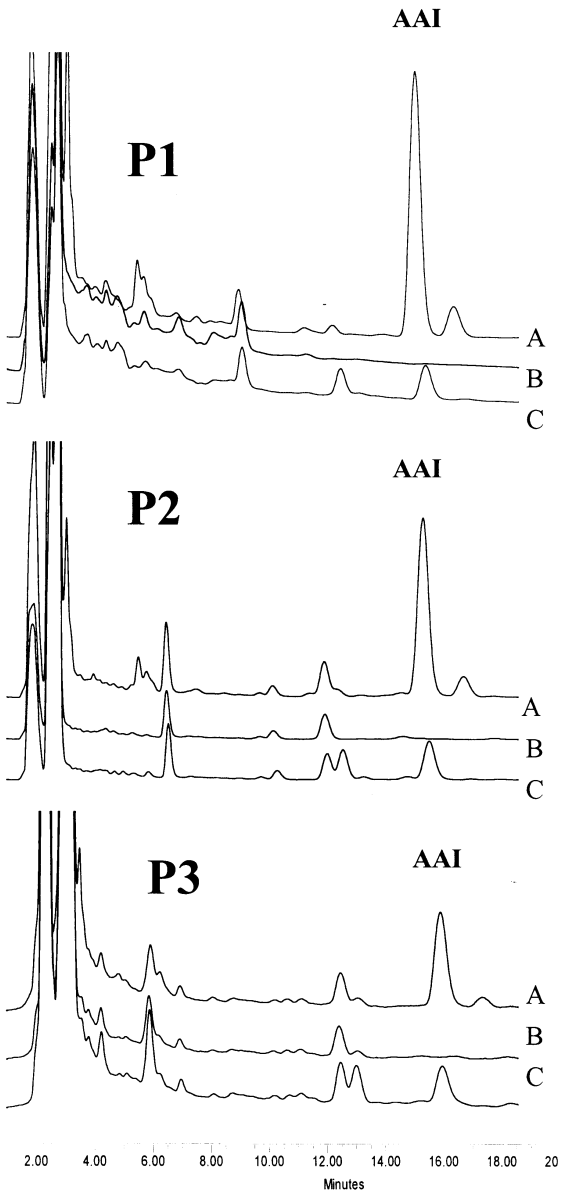


Figure 4. Comparison of HPLC chromatgrams of A, B and C in prescriptions (P1–P3). A, reconstituted prescription; B, blank (prescription without Radix Aristolochiae Fangchi); C, blank + aristolochic acid; AAI, aristolochic acid I.

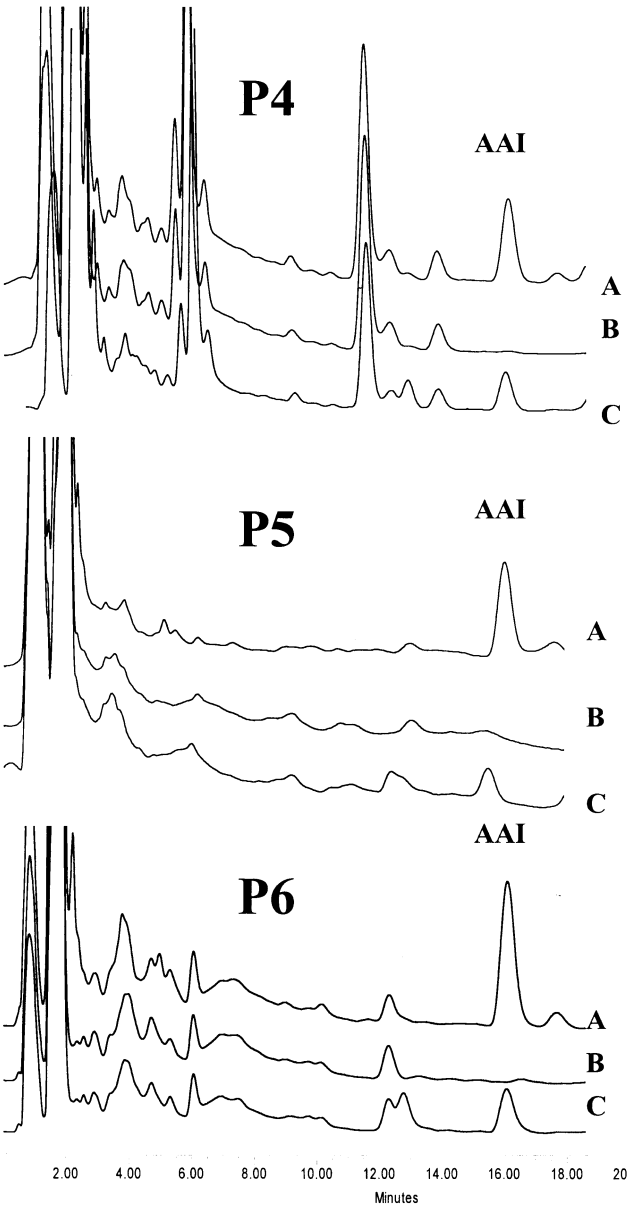


Figure 5. Comparison of HPLC chromatgrams of A, B, and C in prescriptions (P4–P6). A, reconstituted prescription; B, blank (prescription without Radix Aristolochiae Fangchi); C, blank + aristolochic acid; AAI, aristolochic acid I.

Table 4. The Contents of Aristolochic Acid I in Commercial Concentrated Preparations from Eight Pharmaceutical Companies (A–H)

	Preparations				
Companies	P1 Mean ± S.D. (mg/g)	P2 Mean ± S.D. (mg/g)	P3 Mean ± S.D. (mg/g)	P4 Mean ± S.D. (mg/g)	P5 Mean ± S.D. (mg/g)
A		ND	ND	ND	ND
B	1.43 ± 0.02	0.69 ± 0.01	1.30 ± 0.04	0.47 ± 0.02	1.60 ± 0.04
C	1.82 ± 0.01	0.50 ± 0.03	1.84 ± 0.04	0.52 ± 0.01	1.23 ± 0.02
D		ND	ND	ND	ND
E		0.30 ± 0.01	1.66 ± 0.03	ND	ND
F		ND	ND	ND	ND
G		0.33 ± 0.01	ND	0.78 ± 0.03	ND
H		ND	ND	ND	ND

n = 3.
ND: not detected.

contained AAI. The amounts of AAI in samples were between 0.30 to 1.84 mg/g (Table 4). Unexpectedly, the scientific name of Fang-Ji, on the package directions of commercial concentrated preparations from eight pharmaceutical companies, is labeled as Radix Stephaniae Tetrandrae. Our data show that some of the concentrated preparations under the name of *S. tetrandra* contained AA and corresponded, most probably, to *Aristolochia* sp.

In conclusion, this study had shown that the HPLC method could be applied successfully to analyze AAI occurring in the six kinds of traditional Chinese medicinal prescriptions and their commercial concentrated preparations. Therefore, an easy and suitable method for the detection and assay of AAI in traditional Chinese medicines was established.

ACKNOWLEDGMENTS

We are grateful to China Medical College Hospital, Taichung, Taiwan, R.O.C, for the financial support of this work under contract DMR-88-051 and to Dr. Li-Kang Ho, National Yang-Ming University, for helpful discussions.

REFERENCES

1. Chen, H.-A.; Xu, G.-J.; Jing, L.-R.; Xu, L.-S. *Species Systematization and Quality Evaluation of Commonly Used Chinese Traditional Drugs*; Fujian Science Technology Publications: Fujian, China, 1994; 212–215.
2. Xie, Z.-W.; Fan, C.-S.; Shu, Z.-Y. *Editing the Collections of Chinese Herb Drugs in China*; People Sanitation Publications: Beijing, China, 1996; 24, 89, 331, 369, 510.
3. Vanherweghem, J.-L.; Depierreux, M.; Tielemans, C.; Abramowicz, D.; Dratwa, M.; Jadoul, M.; Richard, C.; Vandervelde, D.; Verbeelen, D.; Vanhaelen-Fastre, R. J. Lancet **1993**, *341*, 387–391.
4. Schmeiser, H.-H.; Bieler, C.-A.; Wiessler, M.; Van Ypersele de Strihou, C.; Cosyns, J.-P. Cancer Res. **1996**, *56*, 2025–2028.
5. Vanhaelen, M.; Vanhaelen-Fastre, R.; But, P.; Vanherweghem, J.L. J. Lancet **1994**, *343*, 174.
6. Ku, Y.R.; Lin, Y.-Z.; Wen, K.-C.; Lin, J.-H.; Liao, C.-H. J. Liq. Chrom. & Rel. Technol. **1996**, *19*, 3265.
7. Lin, J.-H.; Ku, Y.-R.; Huang, Y.-S.; Wen, K.-C.; Liao, C.-H. J. Liq. Chrom. & Rel. Technol. **1997**, *20*, 1617.
8. Ku, Y.-R.; Wen, K.-C.; Ho, L.-K.; Chang, Y.-S. J. Pharm. Biomed. Anal. **1999**, *20*, 351.

9. Ou, M. *Chinese-English Manual of Common-Used Prescriptions in Traditional Chinese Medecine*; Guangdong Science Technology Publications: Hong Kong, China, 1991; 97, 100, 124, 281.
10. Hsu, Y.-H.; Tseng, H.-H.; Wen, K.-C. Ann. Rept. NLFD **1997**, *15*, 136–142.

Received July 20, 2001
Accepted November 21, 2001
Manuscript 5616