Phenolic Acids Analysis in *Ligusticum Chuanxiong* Using HPLC

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Abstract

A reversed-phase high-performance liquid chromatographic method with diode array UV detection is developed for the determination of five kinds of phenolic acids common in herbal medicines. Based on this method, ferulic acid and caffeic acid are found to be two main phenolic acids in *Chuanxiong* (one of the important crude drugs in traditional Chinese medicine). More important, ferulic acid is found to exist in free form, and caffeic acid—a previously unreported component—is found to exist in esterified or insoluble-bound form.

Introduction

The dried rhizome of *Ligusticum Chuanxiong* is one of the important crude drugs in traditional Chinese medicine and has been used for headache, anaemia, and irregular menstrual cycle treatments (1). Among its active compounds, the phthalides, tetramethylpyrazine, and ferulic acid have attracted attention from scientists (2–7). Ferulic acid is a phenolic acid reported to have multiple pharmacological effects as an antioxidant, anti-inflammatory, platelet aggregation inhibitors, and antimicrobial (8). But little has been reported about other phenolic acids in *Chuanxiong*. Korean ginsengs contain phenolic acids in free, esterified, and insoluble-bound forms (9). Some kinds of phenolic acids could be detected only after the sample was hydrolyzed. This may suggest the existence of other forms of phenolic acids in *Chuanxiong*. Our research objective was to investigate the kinds and forms of phenolic acids present in *Chuanxiong* dry rhizomes. To date, several qualitative and quantitative analysis methods for phenolic acids have been set up for a variety of samples, such as wine (10), fruit (11), rice, etc. (12). Mun Yhung Jung used gas chromatography–mass spectrometry (MS) to determine the individual contents of these compounds in white and red Korean ginsengs (9), but the sample work-up was comparatively complicated. High-performance liquid chromatography (HPLC) is a simple way to analyze these kinds of components, and some analytical methods have been successfully established. For instance, a new HPLC stationary phase has been applied by Schieber to the analysis of phenolic acids and flavonoids with diode array UV and mass spectrometric detection. The separation of 26 standard compounds was achieved within 1 h (8). Thus, reversed-phase HPLC can be easily used to analyze phenolic acids. Five kinds of phenolic acids: gallic, protochatechuic, chlorogenic, caffeic, and ferulic acids are commonly encountered in herbal medicine and were investigated in this study.

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Experimental

Materials

Gallic, protochatechuic, chlorogenic, caffeic, and ferulic acids were purchased from China National Institute for the Control of Pharmaceutical and Biological Products. *Ligusticum Chuanxiong* was obtained from Chengdu (Si Chuan Province, China). Methanol was of HPLC grade. Other chemicals were of analytical grade.

Extraction of free phenolic acids

Twenty grams of *Chuanxiong* powders were soaked in 200 mL of 75% ethanol for 2 h at room temperature. The solid was filtrated and extracted respectively with 75% ethanol (2 × 200 mL, 2 h each) and deionized water (2 × 200 mL, 2 h each) at 90°C. The previous step and each extract were pooled. Enough ethanol was added to the mixture to precipitate protein. The mixture was then centrifuged at 4000 rpm for 10 min, and the supernatant was saved. The same centrifugation procedure was repeated once and the supernatants were combined. This extract was concentrated to 150 mL under reduced pressure. The concentrated extract was then partitioned four times, each with 40 mL of diethyl ether. The combined diethyl ether phase was then partitioned four times, each with 2% Na_2CO_3 solution to extract the acidic components. Then the pH of the combined aqueous layer was adjusted to 3 with 4 mol/L hydrochloric

acid. This acid solution was partitioned four times with diethyl ether again. These combined diethyl ether layers were evaporated to dryness at vacuum. Before analysis, the solid extract was dissolved in methanol and filtered through a 0.45-µm membrane filter.

Extraction of total phenolic acids

Five grams of *Chuanxiong* powder were hydrolyzed with 50 mL of 4 mol/L NaOH for 4 h under nitrogen and at room temperature. Hydrolysates were acidified with 6 mol/L HCl to pH 3 and filtered to remove a cloudy precipitate. Then the solution was extracted four times with hexane to remove free fatty acids. The combined aqueous phases were extracted four times with diethyl ether. This combined diethyl ether layer was evaporated to dryness at vacuum. Before being subjected to analysis, the solid extract was dissolved in methanol and filtered through 0.45 µm membrane filter.

HPLC method

HPLC and HPLC–MS Instrumentation and Conditions

As the final optimized HPLC method, the separation was carried out using a Zorbax SB-C18 (250- × 4.6-mm i.d., 5 µm) (Agilent Technologies, Palo Alto, CA). A mobile phase consisting of water with 0.1% (v/v) acetic acid (eluent A) and of methanol (eluent B) was delivered with a flow rate of 1.0 mL/min by a quaternary gradient pump (G1311A QuatPump, Agilent). The gradient program was as follows: eluent B from 5% to 100% and eluent A from 95% to 0 within 63 min. The injection volume was 20 µL. The UV spectra were recorded from 190 to 400 nm by a diode array detector (G1315A DAD, Agilent). The detection wavelength was set at 290 nm (band width 16 nm) to get the chromatogram, and the reference wavelength was 360 nm (band width 100 nm). The column temperature was ambient.

The HPLC–MS system used consisted of a binary solvent delivery system (Series 200 LC pumps) and a 785A UV–vis detector (PerkinElmer, Norwalk, CT) in tandem with a Sciex API 3000 triple quadrupole MS (Applied Biosystems, Beijing, China). The column, mobile phase and injection volume used were the same as those noted previously.

MS

HPLC–MS was carried out on an API 3000 with the interface of electrospray ionization (negative and positive ion mode) and mass range 100–500 (*m/z*).

Results and Discussion

Extraction of free phenolic acids

Methanol or ethanol aqueous solution is usually used to extract chemical components in plants (12,13). For simple species, such as rice, soluble phenolic acids could be extracted with 70% ethanol and subjected to analysis without further isolation. But for complex substances, such as Chinese traditional medicine (13), extraction with alcohol aqueous solution will make acid, neutral, and base components with different polarities come out together. The components that are not of importance to this study will interfere with the subsequent analysis severely. The main components of *Chuanxiong* are phthalide derivatives (14), which have wide polarity and are less polar than phenolic acids. When extracting with aqueous alcohol solution, almost all kinds of phthalide derivatives and more polar components were extracted out simultaneously. The diethyl ether (with moderate polarity) was then used to separate those ingredients more polar than phthalides derivatives. Lastly, alkaline solution was applied to extract acid components.

Extraction of total phenolic acids

Phenolic acids are reportedly present as three different forms of free, esterified, and insoluble-bound phenolic acid in plants (9). The latter two forms are usually called the conjugated form. Those conjugated phenolic acids need to be liberated by hydrolysis. The phenolic acid, particularly gallic acid, was unstable in alkaline conditions under air, and it was necessary to hydrolyze the sample under argon or nitrogen (11).

Identification of phenolic acids

Many of the more than 40 components present in *Chuanxiong* are phthalides derivatives (15). The HPLC–DAD–MS method was developed to simultaneously analyze 17 phthalide derivatives and 2 phenolic constituents. Ferulic acid was the only phenolic acid present in this material (14). As far as is known, there are no published data about other phenolic acids in conjugated form existing in *Chuanxiong*. For a reliable analysis of unknown components, the HPLC method employed should be coupled with other analysis instruments, such as MS. Currently, reversed-phase HPLC has been more readily used than HPLC–MS.

In order to produce an optimum separation, a C18 column and a series of aqueous mobile phases, in combination with dif-

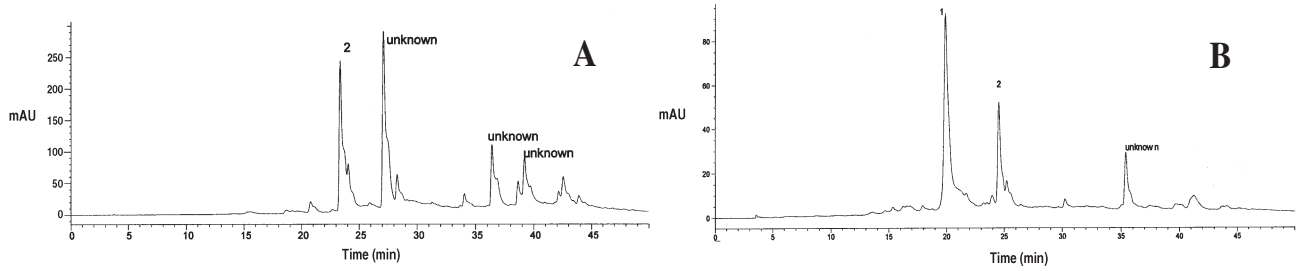

Figure 1. Chromatograms of free phenolic acids (A) and total phenolic acid (B) in *Chuanxiong*. The peaks correspond to caffeic acid (1) and ferulic acid (2).

ferent moderators including acetonitrile and methanol with different volume fractions, were tested. Methanol showed an acceptable result. Among several flow rates tested (0.5–1.5 mL/min), 1.0 mL/min was the best with respect to peak location and resolution. As a phenyl structure exists in the phenolic acid, the UV detector was applied.

Figure 1 shows the chromatograms of free (A) and total (B) phenolic acids extracted from *Chuanxiong*. Figure 2 shows a standard chromatogram of the most common phenolic acids in plants. The identification of the phenolic acid peaks was carried out by comparing their HPLC retention times with those of the standard samples. Upon comparison, the retention times for caffeic and ferulic acids in Figures 1 and 2 were found to not be totally the same. As further confirmation, the UV and mass spectra of peak 1 and 2 in Figure 1 and peaks of caffeic and ferulic acids in Figure 2 were compared. The results revealed that the mass fragments and UV spectra of peaks 1 and 2 in Figure 1 and peaks of caffeic and ferulic acids in Figure 2 were identical. According to these, peaks 1 and 2 were confirmed to be caffeic acid and ferulic acid, respectively.

As shown in Figure 1B, there is a doublet in the peak 2. Interestingly, when the ferulic acid standard (trans form) was analyzed after being left in a refrigerator for approximately 1 week, the single peak became doublet. For this phenomenon, an intensive study was made (16). It is noted that the ferulic acid contains a double bond (Figure 3), and usually ferulic acid is referred to it trans-conformation. Under some conditions of heat and light, the trans-conformation could be gradually changed to cis-conformation. Furthermore, this coexistence of both cis- and trans-ferulic acids was detected in many plants and was also found in other herbal medicine (9). The HPLC–MS test of the molecular mass fragments and HPLC–DAD test of the online UV spectra of the split peak in Figure 2B show that both have similar mass fragments and UV spectra. On the basis of all previous considerations, this doublet could be confirmed as cis- and trans-ferulic acids, respectively.

Many unknown peaks were found to exist in the chromatograms of "free phenolic acids" and "total phenolic acids". In terms of the earlier paper, the unknown peaks in Figure 1A correspond to some phthalide derivatives (17). The unknown peak in Figure 1B could be tentatively deduced to be some hydrolysate of phthalide derivatives.

Phenolic acids contents in *Chuanxiong*

Method qualification tests

The reliability of quantitation by HPLC method first requires a qualification. Stock solutions of 1090 mg/L of gallic acid, 1090 mg/L of protochatechuic acid, 860 mg/L of chlorogenic acid, 1060 mg/L of caffeic acid, and 1010 mg/L of ferulic acid were prepared in methanol, and the other concentrations of these phenolic acids were prepared by diluting these stock solutions with the proper amount of methanol.

These prepared samples were injected directly into the chromatograph in three separate runs, and in each case, the linear regression analysis was carried out on the known concentrations of these five kinds of phenolic acids against the corresponding peak areas. The linear equations are shown in Table I.

Three samples with known concentrations used for the con-

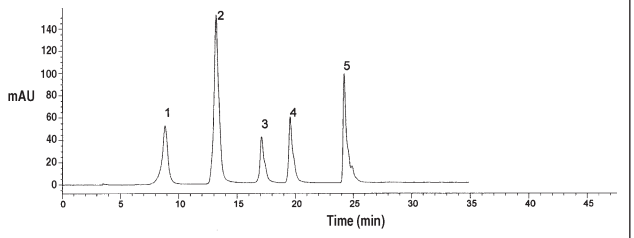

Figure 2. Chromatogram of standard phenolic acids found commonly in plants. The peaks correspond to: gallic acid (1), protochatechuic acid (2), chlorogenic acid (3), caffeic acid (4), and ferulic acid (5).

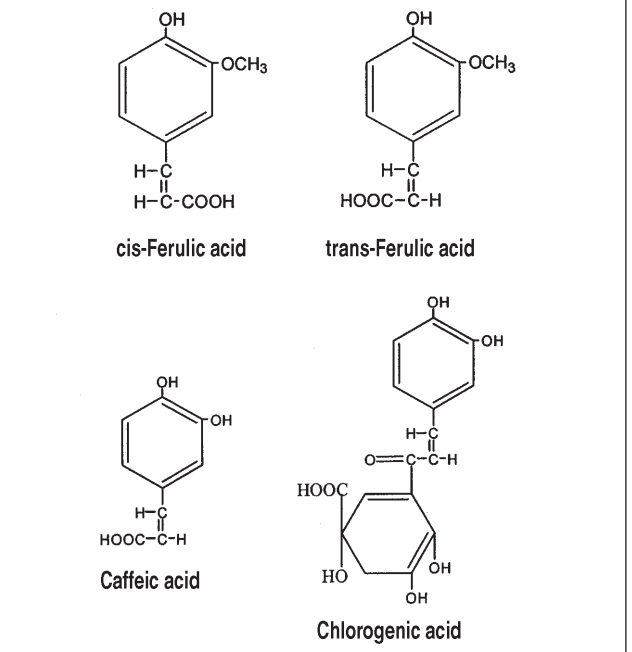

Figure 3. Structures of some phenolic acids.

Table I. Regression Analysis of Calibration Curves and Detection Limits of Five Phenolic Acids*

Phenolic acid	Regression equation	*r*	Linear range (mg/L)	Detection limit (mg/L)
Gallic acid	$Y = 413.88575 + 44.98489X$	0.99943	2.02–1090	0.27
Protochatechuic acid	$Y = 349.26834 + 40.61787X$	0.99940	1.01–1090	0.27
Chlorogenic acid	$Y = -70.03911 + 17.49389X$	0.99962	4.30–860	0.86
Caffeic acid	$Y = 400.66054 + 42.09453X$	0.99511	1.06–530	0.13
Ferulic acid	$Y = 306.59591 + 39.29196X$	0.99654	1.01–505	0.13

* X = mass concentration, mg/L; Y = peak area.

struction of standard curve were added to three total phenolic acid samples, and the concentration of each phenolic acid in each sample was determined using the standard curve. Then, the percent ratios of measured concentration to known added concentration were calculated in each case. The mean absolute recovery values of the method for three selected concentrations within the linear range are shown in Table II. It's obvious that the method is adequately accurate and this ensures obtaining reliable results.

Three concentrations used for the construction of a standard curve were prepared as three replicates and analyzed by the HPLC method. Then, the coefficients of variation (%CV) of responses were calculated in each case. The variations of the method through three selected concentrations are shown in Table III. These data indicate an adequate reproducibility.

The limit of detection is commonly defined as the analyte concentration that gives an instrumental signal significantly different from a blank or background signal. Methanol as the blank solvent was analyzed 10 times to obtain the average area of various noise peaks at the situation corresponding to the peaks of various phenolic acids when various phenolic acids were injected. Then, the standard solutions were diluted successively and injected to obtain peak areas, which were almost three times the areas of the respective noise peaks. The detection limits in concentration unit for five phenolic acids are shown in Table I.

Table II. Absolute Recovery of the HPLC Method for Determination of Phenolic Acids*

Sample	Added (mg/L)	Found (mg/L)	Recovery (%)
Caffeic acid	0	29.3	
	5.3	34.3	94.3
	53.0	85.8	106.6
	265.0	336.4	115.9
Ferulic	0	16.82	
	5.05	21.47	92.1
	50.50	68.72	102.8
	252.50	290.90	108.6

* $n = 3$; the reference is the sample of total phenolic acids.

Table III. Reproducibility of the HPLC Method ($n = 3$)

Sample	Concentration (mg/L)	RSD (peak area) (%)	RSD (retention time) (%)
Gallic acid	5.45	5.81	2.18
	50.45	1.23	1.23
	272.50	1.33	1.21
Protochatechuic acid	5.45	5.97	1.73
	50.45	9.33	1.03
	272.50	1.09	1.01
Chlorogenic acid	43.00	7.70	0.75
	215.00	3.05	0.67
	430.00	2.45	1.02
Caffeic acid	5.30	7.08	1.37
	53.00	5.28	0.78
	265.00	1.96	0.71
Ferulic acid	5.05	2.59	1.02
	50.50	9.84	0.55
	252.50	1.67	0.52

Table IV. Phenolic Acids in *Chuanxiong* ($n = 3$)

Peak no.	Phenolic acid	Retention time (min)	Free form (mg/100 g)	Total (mg/100 g)
1	Caffeic acid	18.912	–	0.23 ± 0.02
2	Ferulic acid	23.608, 24.291	0.16 ± 0.01	0.13 ± 0.01

Determination of phenolic acid concentrations and forms

Two forms of phenolic acids prepared in the experiment were injected in the column. Table IV shows the contents of both free and total phenolic acids in *Chuanxiong*.

When the ester was hydrolyzed, more ferulic acid was let free. Thus, the total content of ferulic acid should be at least equal to, but not higher than that of free form. But from Table IV, the content of total ferulic acid was lower than that of the free form. It could be concluded that most ferulic acid in *Chuanxiong* exists in free form, which is the reason why most of the papers about acidic components in *Chuanxiong* were focused only on ferulic acid.

As for caffeic acid, there is no corresponding peak in Figure 1B. Thus, caffeic acid doesn't exist in free form but in esterified or insoluble-bound form. As is known, chlorogenic acid (Figure 3) is one usual form of esterfied caffeic acid, with biochemical and pharmacological activities widely present in plant (18). When it is hydrolyzed, caffeic, as a part of its structure, was released. But from Figures 1 and 2, chlorogenic acid was not found in *Chuanxiong*. So caffeic acid must exist in other esterified or insoluble-bound forms in *Chuanxiong*.

Conclusion

A reversed-phase HPLC method has been described for the analysis of five phenolic acids common in herbal medicine. According to the quantitative analysis, ferulic acid was found to exist in free form and caffeic acid in esterified or insoluble-bound form in *Chuanxiong*.

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