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Original Research Article

Quantitative and Qualitative Analysis of Alkaloids in *Coptis chinensis* (Coptidis Rhizoma) by LC-DAD and LC-ESI/MS

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Abstract - The quality control of natural products is principal key to guarantee the Good Manufacturing Practices (GMP) and Good Clinical Practices (GCP) for the functional food, pharmaceuticals and cosmeceuticals in the industry. In this study, we examined the quantitative analysis of berberine as marker substance of Coptidis Rhizoma by high performance liquid chromatography-photodiode array detector (HPLC-DAD). The HPLC method was validated and met all the requirements for the quality control analysis recommended by FDA and ICH. The berberine was separated on a Xterra C_{18} column (5 ㎛, 4.6 × 250 ㎜) using mobile phase consisting of distilled water and acetonitrile with KH_2PO_4 (3.4 g) and Na_2SO_4 (1.7 g). Calibration curve of berberine has been estimated (y = 42293.47x-41589 with the correlation coefficient 0.9999). The amount of berberine was calculated as 4.25%. And berberastine, palmatine, columbamine, jatrorrhizine, epiberberine, berberine and coptisine in the Coptidis Rhizoma were identified by high performance liquid chromatography - electrospray ionization-mass spectrometer (HPLC-ESI-MS) method.

Key words - Coptidis Rhizoma, 5 marker substances, LC-DAD, LC-ESI/MS

Introduction

Nowadays many researchers are endeavoring to control of quality on natural products since prescription of the functional foods, pharmaceuticals and cosmeceuticals is growing in the clinical field. Moreover, decoction, pill, powder, pellet, tablet and tincture which the government approved are produced by pharmaceutical company. And these products are recorded in Korean, Chinese and Japanese pharmacopoeia (Sim *et al.*, 2017; Yoo *et al.*, 2017).

In phytochemical analysis, almost analytical works on herbal medicines have been confined to single marker substance. The standardization of multiple components in the herbal medicine still remains to be settled, hence appropriate methods for quality control are needed. We are consistently studying on analysis of herbal formula such as Gami-Honghwa-Tang (Yu *et al*., 2005b; Yu *et al*., 2004) and Gami-Samhwang-San (Yu *et al*., 2005a) by HPLC. And we reported the analysis method of Sipjeondaebo-tang fomula (Shin *et al*., 2011) and Samul-tang (Yu *et al*., 2007a, b), herbal by HPLC-MS-MS.

The Coptidis rhizoma has been used for several thousand years in traditional Korean medicine. Coptidis Rhizoma has properties of bitter, cold and drying dampness in traditional Korean medicine. Because of its characteristics, it should be used with caution in those with cold of spleen and stomach. Coptidis Rhizoma enables it to cool heat and dry dampness. Hence it is best at draining heart and stomach heat (Moon *et al*., 2017). Coptidis Rhizoma has been reported to have the anti-inflammatory effect (Fujii *et al*., 2017), analgesic effect (Tjong *et al*., 2011), and inhibition of coronavirus replications (Kim *et al*., 2008). Also Coptidis Rhizoma are known as anti-herpes simplex virus effects (Chin *et al*., 2010), insulin resistance in 3T3-L1 adipocytes (Yuan *et al*., 2014), and anti-hyperglycemic effects (Wang *et al*., 2014). Coptidis Rhizoma contains a large amount of alkaloids such as berberine, palmatine, berberastine, columbamine, jatrorrhizine, epiberberine and coptisine which are known to have anti hypercholesterolemic effects (Wu *et al*., 2014) and cardio-vascular protective effects (Tan *et al*., 2016).

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Adverse effects of Coptidis rhizome reported transient diarrhea, abdominal distention, polyuria, loss of appetite, vomiting, nausea, and epigastric discomfort after oral ingestion (Chuang *et al*., 2006; Yi *et al*., 2013).

The research of herbal medicine that contain complicated chemical constituents is more difficult and complex than that of single chemicals. This study presents quantitative analysis of berberine using the high performance liquid chromatography-photodiode array detector (LC-DAD), and provides an identification of various alkaloids such as berberastine, columbamine, jatrorrhizine, epiberberine and coptisine using high performance liquid chromatography-electrospray ionization-mass spectrometer (LC-ESI/MS) methods in Coptidis Rhizoma.

Material and Method

Chemicals

Berberine and palmatine were purchased from Wako (Tokyo, Japan) and Sigma (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were purchased from Sigma (St. Louis, MO, USA). Ultra-pure distilled water with a resistivity greater than 18 mega ohm was used (Millipore, Bedford, MA, USA). All other chemicals were analytical grade and were purchased from Sigma.

Sample Preparation for Chromagography

Crude drugs were purchased from their major producers (Suncheon, Chonnam province) and the market, respectively. A voucher specimens are deposited at Dept. of Herbal Pharmacology (KyungHee University, Seoul, Korea). According to the Korean Pharmacopoeial Requirements, quantitative analysis of berberine in Coptidis Rhizoma was carried out by HPLC. Coptidis Rhizoma was pulverized and was accurately weighed as 0.5 g. The powder was poured to the 30 ㎖ methanol mixture with dilute hydrochloric acid (100:1) and then the mixture was boiled for 30 min at circulating extractor for the first decocting, and a 50 ㎖ of methanol mixture was boiled for 30 min for the second decocting. After filtration, while hot, the two decoctions were mixed and were combined and mass up to 100 ㎖. Twenty ㎕ aliquots of samples were injected for analysis.

Standard Curve Preparation

To prepare a standard marker substance solution, berberine was accurately weighed and dissolved in 70% methanol to give various concentrations within the range 2-200 ㎍/㎖, respectively. The whole volume of standard solution mixture is 1 ㎖. Linearity of the responses was determined for five concentrations with three injections for each level. Calibration graphs were plotted after linear regression analysis of the peak-area ratios with concentration. The contents of these constituents in the test samples were calculated using the regression parameters obtained from the standard curve.

Recovery and Purity Angle test

An appropriate amount of the standard decoction was divided in to five portions (one as a control group), each portion (except the control) was spiked with different concentrations of standard solution to add various concentrations of berberine. All samples were injected for HPLC analysis to calculate the recovery. Purity Angle is a new technology used in Waters HPLC software, which can test the purity of every peak of a compound. When Purity Angle < Purity Threshold is computed it shows the data of the peak and is accurate for quantitative determination.

Library Match Test

Library matching test was allowed to identify peaks by comparing spectra from unknown peaks to spectra from standards. We created a library of known spectra from existing standards and compared unknown spectra to spectra in the library. If the Purity Angle is less than the Purity Threshold, the peak is spectrally homogeneous.

HPLC Analysis

The HPLC system consisted of a multi-solvent delivery pump (Waters 2690, USA), and a diode-array UV/Vis multi-wavelength detector (Waters 996, USA). The signals from the detector were collected and analyzed with a computer equipped with a software of 2010 system (Millenium 4.0). Separations were carried out on X-terra reversed-phase column (particle size 5 ㎛, 4.6x250 ㎜ i.d., Waters, USA). The mobile phase was composed of distilled water and acetonitrile (50:50, v/v, KH_2PO_4 (3.4 g) and Na_2SO_4 (1.7 g)

per 1,000 ㎖) with isocratic elution. The solvents were filtered through a 0.45 ㎜ Millipore filter and degassed prior to use. The flow rate was 1 ㎖/min with DAD scanning at 345 ㎚. The operating temperature was maintained at 40℃.

LC-MS analysis

LC-ESI/MS was performed using a Waters Alliance 2695 series liquid chromatograph interfaced to a ZQ Mass spectrometer equipped with an atmospheric pressure interface electrospray chamber. Waters MassLynx 4.0 was used for data collection and manipulation. For HPLC, a 150 x 2.1, 5 ㎛, Xterra Ms C_{18} column (Waters, MA, USA) was used at a flow rate of 0.2 ㎖/min. The HPLC gradient was solvent B (B= 0.05% TFA in CH_3CN) in solvent A (A=0.05% TFA in water): 10%, 0 min.; 30%, 30 min.-35 min.; 90% 50 min.-55 min., 10%, 60 min. and finally returned to initial concentration from 65 to 70 min, with diode array detection set at 190-600 ㎚. For optimum MS analysis, Conditions for ESI-MS analysis of HPLC peaks in both negative - and positive ion mode included a capillary voltage of 3.2k V, a cone voltage of 20, 40 and 60 V, a extractor of 5.0 V, a RF lens of 0.5V, a cone gas flow of 50 L/hr., a desolvation gas flow of 250 L/hr., a source temperature of 120℃ and a desolvation temperature of 170℃.

Results and Discussion

The characterization of structural information on the constituents of interest in complex mixtures requires sophisticated hyphenated techniques, which should provide good sensitivity and selectivity. The combination of the high separation efficiency of HPLC with UV-photodiode array detection (LC/DAD), LC/mass spectrometry (LC/MS)(Chen *et al*., 2015) and LC/nuclear magnetic resonance (LC/NMR) (Wolfender *et al*., 2001) has made possible the acquisition of on-line complementary spectroscopic data on an LC peak of interest within a complex mixture. As crude plant extracts represent very complex mixtures containing up to hundreds of constituents, these new-hyphenated techniques have been rapidly integrated for the study of crude plant extracts (Wolfender *et al*., 2015).

In this study, we employed a method, which can effectively determine the marker substances simultaneously. Validation of HPLC methods means evaluating the performance parameters of the method, which include the system suitability, accuracy, precision, specificity or selectivity, linearity, limit of quantification and limit of detection. Results of linearity test give assurance that the methods are valid for their intended use throughout the specified range. The prepared standard mixtures were injected in triplicate and the response factors were calculated. Results were inputted into a Microsoft excel spread sheet program so calibration curves could be plotted.

The regression equations of the graphs and the correlation coefficients for berberine are given y = 42293.47x-41589. Calibration graphs for berberine were obtained over the ranges 2-200 ㎍/㎖. R2 value is greater than 0.9999, indicating their acceptability, for each analyte. To check the precision of this method, we injected standard solutions of berberine at the concentrations of 1.22 ㎍/㎖, three times on the same day. On the basis of peak-area ratios for three replicate injections, the Inter-day relative standard deviation (RSD) was 0.97. The inter day RSD obtained for a 5-day period was 1.19, respectively. In all instances the accepted criteria of % RSD of less than 2% was met (Table 1). Accuracy of the method was studied by recovery investigation. For all the nine analytes in decoction at different concentration levels, the recovery values were found to meet the accecptance criteria of 99-102%. For appropriate analysis method, the values mentioned above indicated acceptable precision and accuracy (Table 2). Fig. 1 shows representative chromatograms (345 ㎚) berberine standard and Coptidis Rhizoma extracts acquired the HPLC-DAD system. The amount of berberine was calculated as 4.25% through the

Table 1. Inter-day and intra-day relative standard deviations of berberine (n=3)

Marker substance	Concentration (㎍/㎖)	Peak area of marker substance		R.S.D (%)	
		Inter-day	Intra-day	Inter-day	Intra-day
Berberine	1.22	49674.12	49668.44	1.19	0.97

Table 2. Recovery of berberine (n=3)

Marker substance	Added (㎍/㎖)	Found (㎍)	Relative recovery (%)	Mean±S.D. (%)	R.S.D (%)
Berberine	12.2	12.42	101.81		
	24.4	24.11	98.80		
	36.6	37.03	101.17		
	48.8	49.15	100.72	100.63 ± 1.30	1.29

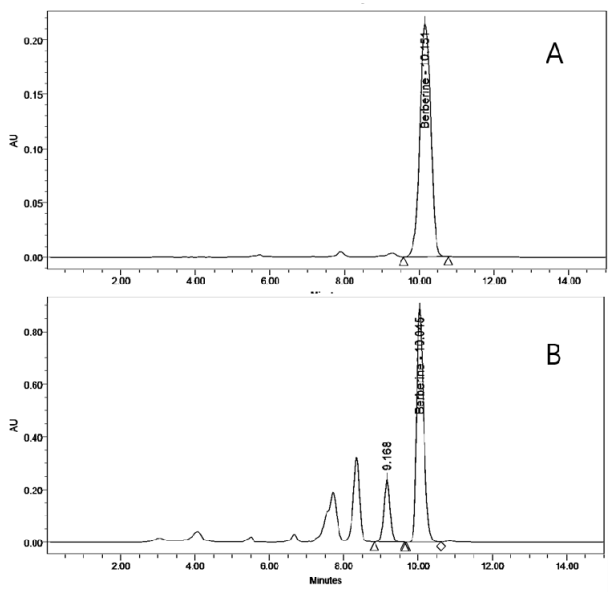

Fig. 1. HPLC chromatograms of Berberine standard (A) and Coptidis Rhizoma extracts (B).

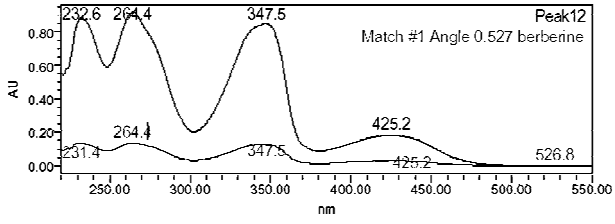

Fig. 2. Match plot compared with maker substance Coptidis Rhizoma and standard berberine in data library.

HPLC quantitative analysis. Also we established a library of known spectra from existing standards. And the berberine in the Coptidis Rhizoma was identified by comparing spectrum from the library. The berberine in the Coptidis Rhizoma extracts was identified from the HPLC retention time characteristics and UV-visible spectra of standards in a library (Fig. 2).

Although we conducted HPLC quantitative analysis, we

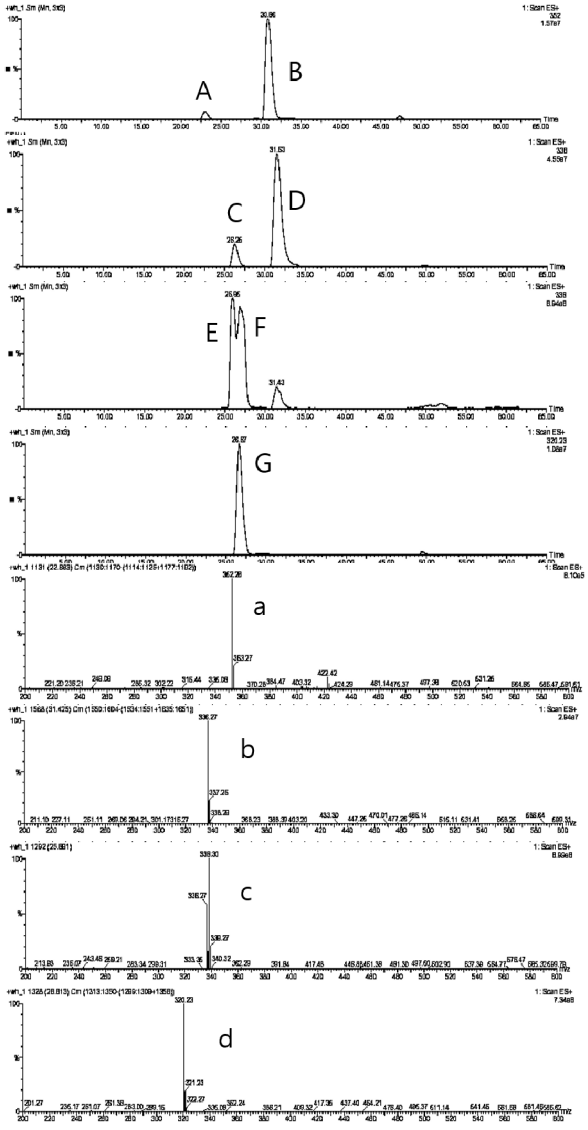

Fig. 3. EIC (Extract ion current) chromatogram and mass spectra of berberastatine (A, a), palmatine (B, a), epiberberine (C, b), berberine (D, b), columbamine (E, c), jatrorrhizine (F, c) and coptisine (G, d) in Coptidis Rhizoma.

could not assign peaks of the other alkaloids because marker substance could not isolate the plants. We need more certain

the chemical information of various compounds in Coptidis Rhizoma. For that reason, we were analyzed the Coptidis Rhizoma by LC-ESI-MS. The results showed that the all marker substances were always detected as the base peaks in the positive ion mode. ESI/MS spectra of compounds are presented in Fig. 3. The alkaloids of Coptidis Rhizoma showed a strong base peak $[M]^+$ in the positive detection mode to give the following as; berberastine (Rt 24.77, m/z 352 $[M]^+$), palmatine (Rt 30.86, m/z 352 $[M]^+$), columbamine (Rt 25.95, m/z 338 $[M]^+$), jatrorrhizine (Rt 26.87, m/z 338 $[M]^+$), epiberberine (Rt. 26.25, m/z 336 $[M]^+$), berberine (Rt. 31.53, m/z 336 $[M]^+$), and coptisine (Rt 26.67, m/z; 320 $[M]^+$). These results were agreed with fragment pattern and molecular weight of literature (Wolfender *et al*., 2015; Yang *et al*., 2016; Zi-Min *et al*., 2017).

According to the above results, HPLC-DAD was employed to determine the quantities and the qualities of berberine in Coptidis Rhizoma. HPLC-ESI-MS method permits assignment of tentative structures such as berberastine, columbamine, jatrorrhizine, epiberberine and coptisine in Coptidis Rhizoma (Fig. 3). We have been studied the standardization and identification of bioactive components from the traditional Korean medicine. This study on standardization of Coptidis Rhizoma would be provide the fundamental data increasing the scientific evidence of natural products.

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