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Quantitative Determination of Protoberebrines from the Roots of *Coptis chinensis*

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Abstract – A simple reversed phase HPLC method was developed for extracting pharmacologically active compounds coptisine, palmatine, berberine, and epiberberine from the roots of *Coptis chinensis* using a binary gradient of acetonitrile : 10 mM hexanesulfonic acid-Na monohydrate with UV detection at 254 nm. The coptisine (**1**), palmatine (**2**), berberine (**3**), and epiberberine (**4**) contents of the roots of *C. chinensis* collected from sixteen district markets in Korea and China were 6.79 ~ 24.63 μg/g, 5.40 ~ 20.75 μg/g, 21.40 ~ 81.21 μg/g, and 3.45 ~ 12.04 μg/g, respectively.
Keywords – *Coptis chinensis*, HPLC, coptisine, palmatine, berberine, epiberberine

Introduction

The rhizome of *Coptis chinensis* Franch. (Ranunculaceae) is a traditional Chinese medicine with anti-inflammatory, anti-bacterial, analgesic and stomachic activities (Park *et al.*, 2005), and has been used to treat anxiety, antibacterial, antihypertensive and CNS depressant (Cho *et al.*, 2000; Min *et al.*, 2006). In the previous studies, the rhizomes of this plant are composed mainly of alkaloids, phenolic compounds, lignans, coumarinolignans and sesquilignan (Yahara *et al.*, 1985; Cho *et al.*, 2000; Min *et al.*, 2006). The MeOH extract of this plant exhibited inhibitory activity of the morphine-induced conditioned place preference through the regulation of *c*-fos expression in the mouse brain (Lee *et al.*, 2003). Protoberebrine alkaloids isolated from the rhizome of *C. japonica* inhibit the catecholamine biosynthesis in PC12 cells (Lee and Kim, 1996). The berberine have been reported to have antifungal activity tested against *Candida tropicalis* (resistant to nyastin, miconazole, and econazole) (Slobodnikova *et al.*, 2004), anti-inflammatory activity (Ivanovska and Philipov, 1996), anti-diabetic effect related to the property of stimulating insulin secretion and modulating lipids (Yang and Wang, 2003), and hepatoprotective activity (Tsai and Tsai, 2004; Wang *et al.*, 2004). It was previously reported that the 8-oxocoptisine showed significant P-gp multidrug resistance inhibitory activity (Min *et al.*, 2006) and coptisine inhibited MAO-A activity in the mouse whole brain (Ro *et al.*, 2001). The biological effect of neolignan woorenosides have been demonstrated *in vitro* anti-inflammatory activity (Cho *et al.*, 2000). *C. chinensis* contains numberous protoberberine alkaloids such as berberine, magnoflorine, coptisine, palmatine, worenine and epiberberine (Min *et al.*, 2006). This study quantified the levels of coptisine, palmatine, berberine and epiberberine from the roots of *C. chinensis* collected from the district markets in Korea and China.

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Experimental

General – The chromatographic system for quantitative analysis consisted of a 306 pump (Gilson, USA), 811C dynamic mixer (Gilson, USA), UV/VIS-156 detector (Gilson, USA), 231 XL sample injector (Gilson, USA), and GILSON UniPoint data processor (Gilson, USA). Separation was performed using an Agilent Eclipse XD8-C18 (Agilent Technologies, USA; 5 μm, 4.6 × 150 mm). Methanol (Burdick & Jackson, USA) and acetonitrile (Burdick & Jackson, USA) used in this work were of HPLC grade and other reagents were of analytical grade. Milli-Q (Millipore, MA, USA) treated water (with resistivity more than 17.5 MΩ cm) was used throughout the experiments. Hexansulfonic acid-Na monohydrate was purchased from Sigma Chemicals (St. Louis, MO, USA).

Plant material – The roots of *C. chinensis* were purchased from oriental medicinal markets, such as, CDU-1 (Hwaseung-sa, Daegu, Korea, cultured in China), CDU-2 (Hyunjin-sa, Daegu, Korea, cultured in China), CDU-3 (Kyungsan-mart, Daegu, Korea, cultured in

China), CDU-4 (Hobuk-mart, Hobuk-sung, China, cultured in China), CDU-5 (Kwangsu-mart, Kwangsu-sung, China, cultured in China), CDU-6 (Joongdo-hospital, Daejeon, Korea, cultured in China), CDU-7 (Baegje Co., Daejeon, Korea, cultured in China), CDU-8 (Kyungdong Co., Daejeon, Korea, cultured in China), CDU-9 (Kyungdong-mart, Seoul, Korea, cultured in China), CDU-10 (Byungin Co., Seoul, Korea, cultured in China), CDU-11 (Kyungshin Co., Yeongchun, Korea, cultured in China), CDU-12 (Yeongchun-mart1, Yeongchun, Korea, cultured in China), CDU-13 (Yeongchun-mart2, Yeongchun, Korea, cultured in China), CDU-14 (Sehwa-dang, Kwangju, Korea, cultured in China), CDU-15 (Kwangduk Co., Kwangju, Korea, cultured in China), and CDU-16 (Johwa Co., Kwangju, Korea, cultured in China). All of plant materials identified by Prof. KiHwan Bae, Chungnam National University, Korea and voucher specimens have been deposited at the Herbarium of College of Pharmacy, Catholic University of Daegu, Korea.

Isolation of standard compounds – The roots (10 kg) of *C. chinensis* were refluxed with MeOH for three hours (3 × 20 L). The total filtrate was concentrated to dryness in vacuum at 40 °C in order to render the MeOH extract (2.2 kg) and this extract was suspended in 10% MeOH and sequentially partitioned with CH_2Cl_2 (230 g) and BuOH (1100 g), and H_2O (840 g) in sequence. The BuOH-soluble fraction (1100 g) was chromatographed over Si gel, MCI-CHP20, and Sephadex LH20 columns using CH_2Cl_2-MeOH under gradient conditions to yield coptisine (**1**), palmatine (**2**) and berberine (**3**). The aqueous layer (840 g) was chromatographed on MCI-CHP20 and Sephadex LH-20 gel to isolate epiberberine (**4**).

Coptisine (1) – yellow amorphous powder; UV λ_{max} (MeOH): 226, 239, 264, 356; ^{1}H-NMR (400 MHz, CD_3OD) δ: 9.71 (1H, s, H-8), 8.71 (1H, s, H-13), 7.87 (1H, d, J = 8.3 Hz, H-11), 7.83 (1H, d, J = 8.0 Hz, H-12), 7.63 (1H, s, H-1), 6.84 (1H, s, H-4), 6.45 (2H, s, OCH_2O), 6.09 (2H, s, OCH_2O), 4.89 (2H, m, H-6), 3.23 (2H, m, H-5); ^{13}C-NMR (100 MHz, CD_3OD) δ: 151.0 (C-10), 148.8 (C-3), 148.1 (C-2), 144.6 (C-8), 144.1 (C-9), 137.8 (C-13a), 133.2 (C-12a), 130.6 (C-4a), 121.9 (C-12), 121.6 (C-13b), 120.7 (C-11), 112.5 (C-8a), 108.2 (C-4), 105.3 (C-1), 105.0 (OCH_2O), 102.5 (OCH_2O), 56.0 (C-6), 27.0 (C-5).

Palmatine (2) – yellow amorphous powder; mp, 208 °C; UV λ_{max} (EtOH): 228, 240, 268, 276, 343, 350, 433; ^{1}H-NMR (400 MHz, CD_3OD) δ: 9.78 (1H, s, H-8), 8.88 (1H, s, H-13), 8.10 (1H, d, J = 8.0 Hz, H-11), 8.00 (1H, d, J = 8.0 Hz, H-12), 7.63 (1H, s, H-1), 7.03 (1H, s, H-4), 4.87 (2H, m, H-6), 4.19 (3H, s, 9-OCH_3), 4.08 (3H, s, 10-OCH_3), 3.97 (3H, s, 2-OCH_3), 3.92 (3H, s 3- OCH_3), 3.52 (2H, m, H-5); ^{13}C-NMR (100 MHz, CD_3OD) δ: 152.6 (C-3), 150.7 (C-10), 149.7 (C-2), 145.2 (C-8), 144.5 (C-9), 138.6 (C-13a), 134.0 (C-12a), 128.9 (C-4a), 126.8 (C-12), 123.3 (C-11), 122.1 (C-13b), 120.1 (C-13), 119.3 (C-8a), 111.0 (C-4), 108.7 (C-1), 61.3 (C-9, OCH_3), 56.4 (C-10, OCH_3), 56.1 (C-2, OCH_3), 55.8 (C-3, OCH_3), 55.4 (C-6), 26.6 (C-5); LC-ESI-MS/MS *m/z*: 352 $[M]^+$.

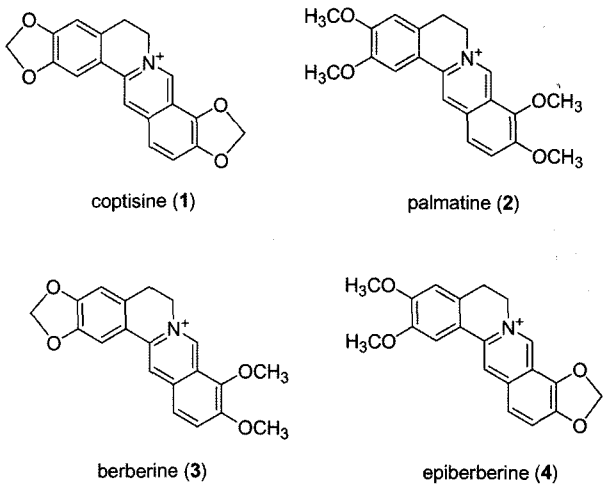

Fig. 1. Structure of coptisine (**1**), palmatine (**2**), berberine (**3**) and epiberberine (**4**) isolated from *C. chinensis*.

Berberine (3) – yellow amorphous powder; mp, 158-160 °C; UV λ_{max} (EtOH): 230, 266, 352, 432; ^{1}H-NMR (400 MHz, CD_3OD) δ: 9.75 (1H, s, H-8), 8.69 (1H, s, H-13), 8.10 (1H, d, J = 8.3 Hz, H-11), 7.98 (1H, d, J = 8.4 Hz, H-12), 7.64 (1H, s, H-1), 6.94 (1H, s, H-4), 6.09 (2H, s, OCH_2O), 4.91 (2H, t, J = 6.4 Hz, H-6), 4.18 (3H, s, 9-OCH_3), 4.09 (3H, s, 10-OCH_3), 3.24 (2H, t, J = 6.4 Hz, H-5); ^{13}C-NMR (100 MHz, CD_3OD) δ: 151.0 (C-10), 150.8 (C-3), 148.7 (C-2), 144.6 (C-9), 145.2 (C-8), 138.5 (C-13a), 134.0 (C-12a), 130.7 (C-4a), 126.8 (C-11), 123.3 (C-12), 122.1 (C-8a), 120.7 (C-13b), 120.3 (C-13), 108.2 (C-4), 105.3 (C-1), 102.5 (OCH_2O), 61.3 (C-9, OCH_3), 56.4 (C-10, OCH_3), 56.0 (C-6), 27.0 (C-5).

Epiberberine (4) – yellow amorphous powder; mp, 260 °C; UV λ_{max} (EtOH): 227, 245, 268, 361; ^{1}H-NMR (400 MHz, CD_3OD) δ: 9.70 (1H, s, H-8), 8.81 (1H, s, H-13), 7.88 (1H, d, J = 8.0 Hz, H-11), 7.83 (1H, d, J = 8.0 Hz, H-12), 7.63 (1H, s, H-1), 7.03 (1H, s, H-4), 6.45 (2H, s, OCH_2O), 4.88 (2H, m, H-6), 3.97 (3H, s, 2-OCH_3), 3.92 (3H, s, 3-OCH_3), 3.26 (2H, m, H-5); ^{13}C-NMR (100 MHz, CD_3OD) δ: 152.6 (C-3), 149.7 (C-2), 147.9 (C-10), 144.5 (C-9), 144.1 (C-8), 137.9 (C-13a), 133.3 (C-12a), 128.7 (C-4a), 121.8 (C-12), 121.1 (C-11), 121.0 (C-13), 119.3 (C-13b), 112.5 (C-8a), 111.0 (C-4), 108.5 (C-1),

104.9 (OCH_2O), 56.2 (C-6), 55.8 (C-2, OCH_3), 55.5 (C-3, OCH_3), 26.5 (C-5).

Preparation of test sample – Air-dried roots (500 mg) was finely powdered and refluxed with 40 mL 70% MeOH (+ 1% HCl) for 2 hours, and filtered using filter paper. The methanol extracts were evaporated to dryness in vacuum. The residue was dissolved in 90 mL 70% MeOH and 10 mL 0.002% butylparaben (70% methanol solution). The crude extract then filtered with a 0.45 μm pore size and a 10 μL sample subjected to HPLC analysis.

HPLC analysis – Method for protoberberine alkaloids analysis was modified from those previously described (Feng *et al.*, 2005) by using a reverse phase system (Hypersil C-18, 5 μm, 4.6 × 250 mm i.d.). Elution was initially with acetonitrile-10 mM hexanesulfonic acid-Na monohydrate (15 : 85), which was changed according to linear gradient over 40 min to acetonitrile-10 mM hexanesulfonic acid-Na monohydrate (80 : 20). The flow rate was 1 mL/min, and 10 μL aliquots of samples were injected for analysis and UV detection was carried out at 254 nm.

Calibration – Stock solutions (2 mg/mL) of coptisine (**1**), palmatine (**2**), berberine (**3**) and epiberberine (**4**) isolated from *C. chinensis* were prepared individually in methanol, and different concentrations (2, 5, 10, 25, 50, 100 μg/mL) of these were loaded onto an HPLC for the preparation of the calibration functions. The calibration function of coptisine, palmatine, berberine and epiberberine calculated with peak area (y), concentration (x, mg/mL), and mean values (n = 5) ± standard deviation.

Results and Discussion

The optimal mobile phase composition for the analysis of coptisine (**1**), palmatine (**2**), berberine (**3**) and epiberberine (**4**) from the 70% MeOH (+ 1% HCl) extracts of the roots of *C. chinensis* was selected by performing several HPLC runs with various concentrations of acetonitrile in 10 mM hexansulfonic acid-Na monohydrate as a mobile phase. A solution of the initial 15% acetonitrile in 10 mM hexansulfonic acid-Na monohydrate, which was changed gradually over 40 min to 80% acetonitrile, was selected as the mobile phase. The HPLC peaks of the coptisine (**1**), palmatine (**2**), berberine (**3**) and epiberberine (**4**) contained in each roots of *C. chinensis* were verified using the standard reference material. The chromatographic system used produced symmetrical peaks with a baseline resolution for coptisine, palmatine, berberine and epiberberine using a simple gradient profile (Lee *et al.*, 2004). Butylparaben was used as an internal

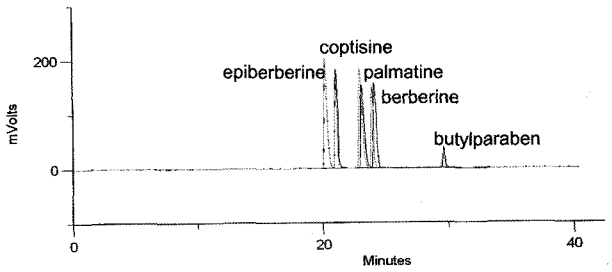

Fig. 2. HPLC chromatogram of coptisine (**1**), palmatine (**2**), berberine (**3**) and epiberberine (**4**) isolated from *C. chinensis*.

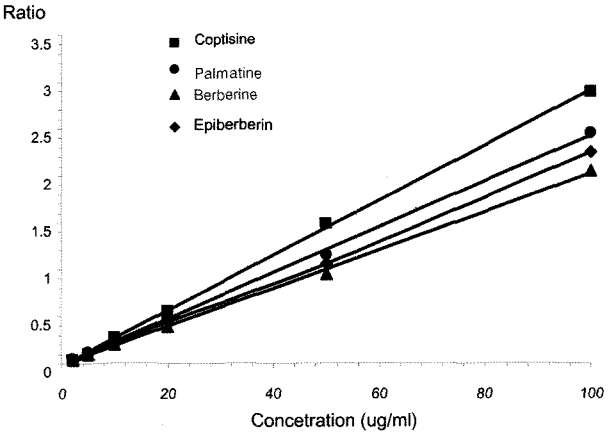

Fig. 3. Calibration curve of coptisine, palmatine, berberine and epiberberine.

standard. The retention time of coptisine (**1**), palmatine (**2**), berberine (**3**), epiberberine (**4**) and butylparaben were 20.17, 21.04, 23.10, 24.08 and 29.62 min, respectively (Fig. 2). The correlation coefficients of each calibration curve of coptisine (**1**), palmatine (**2**), berberine (**3**) and epiberberine (**4**) were 0.9994, 0.9992, 0.9987 and 0.9994, respectively (Table 1, Fig. 3). Under the above HPLC condition the detection limit of compounds were 0.25 μg/mL (UV_{254} nm).

The performance of the expressed method was tested by applying it to a simultaneous assay of coptisine (**1**), palmatine (**2**), berberine (**3**) and epiberberine (**4**) in the roots of *C. chinensis* obtained from the sixteen of oriental medicinal markets in Korea and China. The test samples were prepared as described previously and injected in duplicate (Li and Wang, 2004). The results are summarized in Table 2. It was found that the protoberberine alkaloid contents of the herbal samples were quite different. Berbeine (**3**) is the major compound in the roots of *C. chinensis*. Of these roots of *C. chinensis*, the sample from Hobuk-mart (CDU-4) in China had the highest alkaloids contents (coptisine, 24.63 ± 4.11 μg/g; palmatine, 20.75 ± 4.38 μg/g; berebrine, 81.16 ± 11.59 μg/g; epiberberine,

Table 1. Calibration graphs, linear ranges, LOD and LOQ of coptisine (**1**), palmatine (**2**), berberine (**3**) and epiberberine (**4**)

Compound	linear range (μg/mL)	Slope (a)	Intercept (b)	Correlation Coefficient (r^2)	LOD[a] (μg/mL)	LOQ[b] (μg/mL)
coptisine (**1**)	2 ~ 100	–0.0236	0.02938	0.9994	0.1	0.25
palmatine (**2**)	2 ~ 100	–0.001	0.02440	0.9992	0.1	0.25
berberine (**3**)	2 ~ 100	0.0151	0.02039	0.9987	0.1	0.25
epiberberine (**4**)	2 ~ 100	0.0015	0.02457	0.9994	0.1	0.25

[a]Limit of detection. [b]Limit of quality control

Table 2. Analytical results of coptisine (**1**), palmatine (**2**), berberine (**3**) and epiberberine (**4**) of *C. chinensis* purchased from markets in Korea and China

Samle	Content (mg/g)			
	coptisine (**1**)	palmatine (**2**)	berberine (**3**)	epiberberine (**4**)
CUD-1[a]	9.66 ± 1.10	7.87 ± 1.00	33.28 ± 5.34	4.49 ± 0.39
CUD-2[b]	17.43 ± 3.80	15.43 ± 0.06	57.25 ± 11.00	7.77 ± 1.74
CUD-3[c]	9.48 ± 1.25	9.62 ± 0.83	30.76 ± 2.24	5.24 ± 0.54
CUD-4[d]	24.63 ± 4.11	20.75 ± 4.38	81.16 ± 11.59	12.04 ± 2.55
CUD-5[e]	6.79 ± 0.85	5.40 ± 0.27	21.40 ± 2.53	3.76 ± 0.21
CUD-6[f]	12.54 ± 0.55	10.00 ± 0.42	45.62 ± 0.10	5.97 ± 0.58
CUD-7[g]	13.32 ± 0.40	11.97 ± 0.52	48.16 ± 2.39	7.36 ± 1.09
CUD-8[h]	12.38 ± 0.59	9.90 ± 0.13	43.43 ± 0.61	7.02 ± 0.48
CUD-9[i]	9.53 ± 0.15	8.80 ± 0.82	33.87 ± 2.05	4.97 ± 0.31
CUD-10[j]	10.42 ± 0.79	8.46 ± 0.99	35.68 ± 3.30	5.39 ± 0.53
CUD-11[k]	8.50 ± 0.39	6.57 ± 0.06	27.61 ± 0.23	3.45 ± 0.02
CUD-12[l]	11.03 ± 1.49	8.49 ± 1.47	38.65 ± 4.34	4.60 ± 0.93
CUD-13[m]	13.65 ± 0.53	12.17 ± 0.80	41.34 ± 10.15	6.51 ± 0.50
CUD-14[n]	23.47 ± 2.87	17.06 ± 0.60	77.21 ± 5.36	10.28 ± 0.97
CUD-15[o]	22.77 ± 0.36	16.62 ± 2.09	81.21 ± 6.98	10.55 ± 0.18
CUD-16[p]	17.61 ± 0.68	13.00 ± 0.46	53.36 ± 2.64	9.32 ± 0.29

[a]CDU-1 (Hwaseung-sa, Daegu, Korea, cultured in China), [b]CDU-2 (Hyunjin-sa, Daegu, Korea, cultured in China), [c]CDU-3 (Kyungsan-mart, Daegu, Korea, cultured in China), [d]CDU-4 (Hobuk-mart, Hobuk-sung, China, cultured in China), [e]CDU-5 (Kwangsu-mart, Kwangsu-sung, China, cultured in China), [f]CDU-6 (Joongdo-hospital, Daejeon, Korea, cultured in China), [g]CDU-7 (Baegje Co., Daejeon, Korea, cultured in China), [h]CDU-8 (Kyungdong Co., Daejeon, Korea, cultured in China), [i]CDU-9 (Kyungdong-mart, Seoul, Korea, cultured in China), [j]CDU-10 (Byungin Co., Seoul, Korea, cultured in China), [k]CDU-11 (Kyungshin Co., Yeongchun, Korea, cultured in China), [l]CDU-12 (Yeongchun-mart1, Yeongchun, Korea, cultured in China), [m]CDU-13 (Yeongchun-mart2, Yeongchun, Korea, cultured in China), [n]CDU-14 (Sehwa-dang, Kwangju, Korea, cultured in China), [o]CDU-15 (Kwangduk Co., Kwangju, Korea, cultured in China), [p]CDU-16 (Johwa Co., Kwangju, Korea, cultured in China)

12.04 ± 2.55 μg/g). These results suggest that this method might be used more conveniently for the monitoring the quality of protoberberine alkaloids from the roots of *C. chinensis*.

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