Quantitative Determination of Five Glucosyloxybenzyl 2-Isobutylmalates in the Tubers of *Gymnadenia Conopsea* and *Coeloglossum Viride* var. bracteatum by HPLC

Min Li, Shun-Xing Guo*, Chun-Lan Wang, and **Pei-Gen Xiao**

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100094, China

Abstract

Glucosyloxybenzyl 2-isobutylmalates are one group of important active constituents in the tubers of *Gymnadenia conopsea* R. Br. and *Coeloglossum viride* (L.) Hartm. var. *bracteatum* (Willd.). For the purpose of quality evaluation of these two Chinese herbal medicines, it is necessary to use a rapid and reliable assay that is suitable for the determination of their active constituents. A high-performance liquid chromatography method is firstly developed for the simultaneous quantification of five glucosyloxybenzyl 2-isobutylmalates in the tubers. The analytes including dactylorhin B, dactylorhin E, loroglossin, dactylorhin A, and militarine are isolated from the tubers of *G. conopsea*. The compounds are separated on an Agilent Hydrosphere C_{18} (150 × 4.6 mm i.d., 5 µm) column using a mobile phase of acetonitrile-water including 0.3% acetic acid (adjusted with 36% acetic acid) with gradient elution at a flow rate of 1.0 mL/min. Detection is set at a UV wavelength of 221.5 nm. The recovery of the method is 97.7–101.0%, and linearity ($r > 0.9998$) is obtained for all the analytes. The assay is successfully applied to determine the contents of the analytes in the tubers of *G. conopsea* and *C. viride* var. *bracteatum* collected from different regions of China.

Introduction

Gymnadenia conopsea R. Br. and *Coeloglossum viride* (L.) *Hartm. var. bracteatum* (Willd.) are plants of the Orchidaceae family widely distributed in China (1–2). The tubers of these two plants have long been used as traditional Chinese medicines for the treatment of asthma, neurasthenia and chronic hepatitis, and as tonics especially in Mongolia and Tibet (3). Previous chemical investigations have indicated that glucosyloxybenzyl 2-isobutylmalates are one group of major constituents in the plants' tubers (4–6). Modern pharmacological studies have demonstrated that a special extract from the tubers of *C. viride var. bracteatum*, which is mainly composed of four glucosyloxybenzyl 2-isobutylmalates including dactylorhin B, loroglossin, dactylorhin A, and militarine, has important pharmacological effects such as resisting oxidation injury in subacute senescent model mice (7) and attenuating D-galactose and $NaNO_2$-induced memory impairment in mice (8). The use of *C. viride var. bracteatum* against dementia is protected by PCT patent [WO 2004/058244] (8). On the other hand, dactylorhin B has been reported to have the activity of reducing the toxic effects of *b*-amyloid fragment (25–35) on neuron cells and isolated rat brain mitochondria (9). Therefore, glucosyloxybenzyl 2-isobutylmalates are one group of major active constituents in the tubers of *G. conopsea* and *C. viride var. bracteatum*, and the quantitative determination of the active constituents would be helpful to evaluate the quality of these plants.

Currently, there are two reports comprised of analytical methods available for the determination of four glucosyloxybenzyl 2-isobutylmalates including dactylorhin B, loroglossin, dactylorhin A, and militarine by reversed-phase high-performance liquid chromatography (RP-HPLC) (8, 10). In the two reports, HPLC–diode array detection (DAD) and HPLC–DAD–tandem mass spectrometry (MS^n) methods were adopted in the quantitative and qualitative analyses of the analytes, respectively. One of them briefly reported the quantitative determination of the four compounds in the extract from the tubers of *C. viride var. bracteatum* by HPLC with UV–DAD as the main objective of the work was to investigate the pharmacological activities of CE (8). Another paper described the chromatographic fingerprint analysis of the tubers of *G. conopsea*, and seven main peaks in the fingerprint were identified as adenosine, 4-hydroxybenzyl alcohol, 4-hydroxybenzyl aldehyde, dactylorhin B, loroglossin, dactylorhin A, and militarine by using the HPLC–DAD–MS^n technique (10). But up to now, there have been not any reports on the quantitative determination of glucosyloxybenzyl 2-isobutylmalates in the tubers of *G. conopsea* and *C. viride var. bracteatum*.

In this work, five glucosyloxybenzyl 2-isobutylmalates including dactylorhin B, dactylorhin E, loroglossin, dactylorhin A, and militarine (Figure 1) were isolated from the tubers of G. conopsea and selected as the marker compounds, and a HPLC method was firstly developed to quantify the five markers simultaneously in the tubers of *G. conopsea* and *C. viride var. bracteatum*. Among the analytes, dactylorphin E, the same kind of chemical constituent in the same plant, was firstly determined in this paper because its content was higher in some

*Author to whom correspondence should be addressed: email sxguo2006@yahoo.com.cn.

tested samples. The aim of this study was to develop a sensitive HPLC method for the quality evaluation of these two Chinese herbal medicines.

Experiment

Chemicals and solvents

Five marker compounds including dactylorhin B, dactylorhin E, loroglossin, dactylorhin A, and militarine were isolated from the tubers of *G. conopsea* in our laboratory. The dried tubers of *G. conopsea* (4.8 kg) were extracted with 70% ethanol under reflux. The resulting extract (685 g) was suspended in water and partitioned successively with petroleum ether, ethyl acetate, and *n*-butanol. The n-butanol extract (70 g) was fractioned in a glass column using D101 macroporous resin as an adsorbent and eluted with water, 50% ethanol, and 95% ethanol to afford a 50% ethanol fraction (21 g), which was separated on a glass column with silica gel as an adsorbent and eluted with a gradient solvent of chloroform–methanol–water to give 13 fractions. Fraction 10, 11, and 12 were repeatedly purified over a reversed-phase silica gel column using a mixture of methanol–water with gradient elution to yield five compounds: dactylorhin B (0.9%), dactylorhin E (0.06%), loroglossin (0.008%), dactylorhin A (0.01%), and militarine (0.003%). These compounds were identified by directly comparing ^{1}H NMR, ^{13}C NMR, and mass spectral date with the literature (11). All of their purities confirmed by HPLC were greater than 99%.

HPLC-grade acetonitrile and methanol were purchased from Honeywell Burdick & Jackson Company (Muskegon, MI, USA). Analytical-grade ethanol used for sample preparation was obtained from Beijing Chemical Factory (Beijing, China). Analytical-grade 36% acetic acid was purchased from Tianjin Chemical Reagent No 1 Plant (Beijing, China). Water used for the preparation of mobile phase was prepared with a Milli-Q water purification system (Millipore, Billerica, MA).

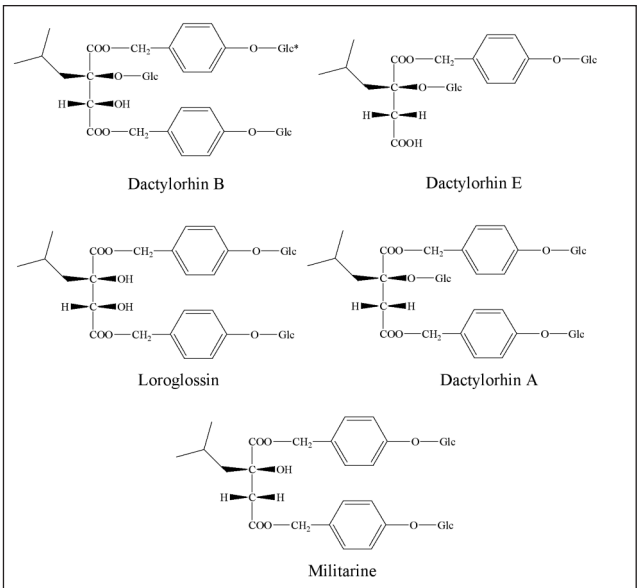

Figure 1. The chemical structures of five marker compounds.

Plant materials

The tested samples of tubers of *G. conopsea* were collected from Lijiang city, Yunnan province (sample I), Weixian city, Hebei province (sample II), Kangding city, Sichuan province (sample III), Tibet province (sample IV) and Xining city, Qinghai province (sample V), respectively, and tubers of C. *viride* var. *bracteatum* were collected from Lanzhou city, Gansu province (sample VI). Sample I was used for isolating the marker compounds and investigating the analytic method. All of these materials were authenticated by Professor Shunxing Guo (Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College).

Preparation of standard stock solutions

Individual standard stock solutions of dactylorhin B (4.40 mg/mL), dactylorhin E (1.07 mg/mL), loroglossin (0.94 mg/mL), dactylorhin A (2.18 mg/mL), and militarine (0.95 mg/mL) were prepared in methanol and stored in the refrigerator at 5°C.

Preparation of sample solutions

Dry-powdered samples I–VI were passed through a 40 mesh sieve, and then accurately weighed with the results of 0.70, 0.70, 0.70, 0.35, 0.35, and 0.18 g, respectively. Each sample was refluxed with 35 mL of 70% ethanol for 1 h. The extracted solution was accurately weighed after cooling down, the weight lost in the extraction procedure was compensated for by adding 70% ethanol to the solution. Accurately measured 25 mL filtrate and evaporated it to dryness, and the residue was dissolved in methanol and transferred to a 5-mL volumetric flask.

All solutions were filtrated through 0.45-µm nylon filters (Whatman, Maidstone, Kent, UK) before being injected into the HPLC system.

Instruments and chromatographic conditions

All analyses were performed on a Waters HPLC system (Waters, Milford, MA), consisting of a Waters 600 pump, a Waters 600 system controller, a 2996 UV–DAD detector, and a model 7725 injector equipped with 20-mL sample loop. The chromatographic data was recorded and processed with a Waters empower workstation.

The samples were separated on an Agilent YMC Hydrosphere C_{18} column (150 × 4.6 mm i.d., 5 mm, Agilent, Santa Clara, CA) at 25°C. Mobile phase A was acetonitrile and mobile phase B was acetonitrile–water (5:95, v/v), both of which contained 0.3% acetic acid (v/v, adjusted with 36% acetic acid). The elution program was well optimized as follows: the elution composition of the mobile phase was 10:90 (mobile phase A: mobile phase B, v/v) during the first 27 min, changed to 12:88 in the next 3 min, and then changed to 26:74 in the following 25 min. The total analysis time was 55 min. Mobile phase was delivered at a flow rate of 1 mL/min. UV–DAD detection was performed at 221.5 nm, and the injection volume was 20 mL.

Method validation

The method was validated for linearity, sensitivity, precision, and accuracy. The working solutions of six different concen-

trations were prepared by accurately transferring 6, 12, 24, 48, 96, and 120 mL of the standard stock solutions into six 1-mL volumetric flasks, respectively, and making up to the final volume with methanol. The calibration curves were constructed by injecting the six working solutions within its linearity range. After determining the peak area, calibration lines of peak area versus the quantity of the standards were plotted.

The stock solutions were diluted step by step for the determination of the limit of detection (LOD) and limit of quantitation (LOQ). LOD was defined as the quantity for a signal-to-noise ratio (S/N) of 3 was obtained, and a S/N of 10 for LOQ.

Precision was determined by performing three concentration levels (low, middle, and high) of marker compounds, and each concentration was analyzed in five replicates. The intra-day and inter-day precisions were determined by analyzing the samples within a single day and over 5 days, respectively. Precision was evaluated by the relative standard deviation (RSD, %) values of the peak area of the analytes.

The accuracy of the method was confirmed by the determination of recovery. Six repetitive samples from the same batch of the tubers of G. conopsea were spiked with a known amount of the marker compounds before extraction. The mixtures were extracted and analyzed under the previously mentioned conditions. The percent recovery of the added markers was calculated as: % Recovery = $[(C - A)/B] \times 100$, where C is the measured value (µg), which is the total amount measured after adding the marker compounds; A is the computed value (µg), which equals the sample weight (g) multiplied by the average content of each analyte (µg/g) in the sample from the quantitative determination of six repetitive samples from the same batch of the tubers of G. conopsea; and B is the added amount (µg) of the marker compound in each sample.

Results and Discussion

Optimization of chromatographic conditions

The chromatographic conditions, especially the analytical column, the composition of mobile phase and gradient elution condition, were optimized through several trials. Four different C_{18} reversed-phase columns, Agilent YMC Hydrosphere C_{18} (150 × 4.6 mm i.d., 5 µm, Agilent, Santa Clara, CA), Agilent Zorbax SB- C_{18} (250 × 4.6 mm i.d., 5 µm, Agilent, Santa Clara, CA), LUNA Phenomenex C_{18} (250 × 4.6 mm i.d., 10 µm, Phenomenex, Torrance, CA) and Waters Symmetry C_{18} (150 × 3.9 mm i.d., 5 µm, Waters, Milford, MA) were evaluated. As a result, Agilent YMC Hydrosphere C_{18} (150 × 4.6 mm i.d., 5 µm, Agilent, Santa Clara, CA) was selected for its satisfactory separation performance.

Because of the wide range of polarity of the five glucosyloxybenzyl 2-isobutylmalates, a complete separation of each under isocratic conditions was not possible, and a gradient elution system was developed. Different mobile phase conditions (acid percentage, organic solvent) were also tested. In this study, different acid percentages such as 0.3%, 0.5%, and 0.8% (v/v, adjusted with 36% acetic acid) were compared with each other. When the proportion of 36% acetic acid was invariable, binary and ternary solvent (acetonitrile, methanol, water) mixtures were investigated. A mobile phase of acetonitrile–water including 0.3% of 36% acetic acid with gradient elution resulted

Table I. Contents of Marker Compounds (mg/g, *n* = 3*) in the Tubers of *G. conopsea*†

Solvent	Dactylorhin B	Dactylorhin E	Loroglossin	Dactylorhin A	Militarine	Total
Methanol	546.02	31.94	180.14	291.70	85.82	1135.62
	(2.56) ‡	(4.04)	(3.81)	(2.69)	(2.28	
Ethanol (50%)	1321.69	71.52	232.27	591.74	86.52	2303.74
	(2.27)	(3.12)	(3.41)	(2.02)	(2.46)	
Ethanol (70%)	1602.17	100.66	291.78	715.62	105.89	2816.12
	(2.52)	(2.68)	(1.79)	(1.21)	(1.64)	
Ethanol (95%)	404.98	24.72	148.03	221.69	74.16	873.58
	(3.22)	(6.18)	(3.56)	(2.34)	(3.79)	

* Number of determination (*n* = 3); † Samples are extracted with different solvents; ‡ Number in parentheses indicates RSD (%).

Table II. Contents of Marker Compounds (mg/g, *n* = 3*) in the Tubers of *G. conopsea*†

Method and time	Dactylorhin B	Dactylorhin E	Loroglossin	Dactylorhin A	Militarine	Total
Reflux	1595.58	97.60	272.17	712.40	97.79	2775.54
(0.5 h)	(1.11)‡	(2.43)	(2.14)	(1.48)	(1.21)	
Reflux	1611.05	103.24	290.10	717.65	101.90	2823.94
(1 h)	(2.54)	(2.02)	(2.08)	(1.11)	(2.32)	
Reflux	1622.83	102.51	287.13	702.61	102.07	2817.15
(2 h)	(0.98)	(3.01)	(1.96)	(2.45)	(3.86)	
Reflux	1643.34	97.15	275.37	723.93	99.10	2838.89
(4 h)	(1.76)	(3.45)	(1.56)	(1.79)	(1.04)	
MA	1320.80	81.5	256.00	607.03	94.71	2360.09
(10 min)	(3.21)5	(3.45)	(4.21)	(2.76)	(5.28)	
MA	1417.81	87.08	263.34	653.06	97.04	2518.33
(20 min)	(2.43)	(4.87)	(3.76)	(2.99)	(4.09)	
MA	1564.13	100.10	266.05	642.91	93.98	2667.17
(30 min)	(4.86)	(5.96)	(2.04)	(4.15)	(4.18)	
MA	1557.57	100.55	266.49	664.79	97.81	2687.21
(40 min)	(4.64)	(6.34)	(3.69)	(4.80)	(6.63)	
UA	1103.24	45.57	183.68	509.29	76.55	1918.33
(0.5 h)	(2.15)	(5.19)	(2.88)	(3.09)	(2.37)	
UA	1277.70	76.82	247.48	578.78)	94.26	2275.04
(1 h)	(1.98)	(4.06)	(2.76)	(2.11)	(1.21)	

* Number of determination (*n* = 3); † Samples are extracted with different methods and time
‡ Number in parentheses indicates RSD (%); § MA = microwave-assisted; ** UA = ultrasound-assisted

in the best peak shapes and separation effects for all the analytes. The addition of acetic acid in the mobile phase played a key role in enhancing peak symmetry and separation capacity.

The UV–DAD detector was set at the wavelength of 221.5 nm, which was the maximal absorbance wavelength (λmax) of the analytes.

Optimization of extraction conditions

Several factors, such as extraction solvent, extraction method, extraction time, solvent volume, and infusion time, were investigated in order to obtain the optimal extraction conditions. For the optimization of solvent, samples were refluxed with 35 mL of methanol, and 50%, 70%, and 95% ethanol for 4 h, respectively, after 12 h infusion. The samples were treated and assayed as described previously. It was found that 70% ethanol could extract the analytes in high yields (Table I). For optimization of the method and time, samples were refluxed for 0.5, 1, 2, and 4 h, ultrasound-assisted extracted for 0.5 and 1 h, and microwave-assisted extracted (300 W) for 10, 20, 30, and 40 min with 35 mL of 70% ethanol, respectively, after 12 h infusion. As a result, reflux (1 h) was proven to give a more efficient extraction than ultrasound-assisted extraction (1 h) and microwave-assisted extraction (40 min), which followed a repeated procedure of one-minute heating and then cooling, and reflux for 0.5 h was close to giving the highest yields (Table II). For the optimization of solvent volume and infusion time, samples were extracted with 20, 35, and 70 mL solvent, and infused for 0 and 12 h, respectively, both of which were followed by insignificant different effects on the final results (data not shown). In sum, direct reflux with 70% ethanol for 1 h was the optimal extraction conditions in this study.

Table III. Analytical Characteristics of the Calibration Graphs

Compound	$y = a + bx$*	Range (µg)	r	LOD (µg)	LOQ (µg)
Dactylorhin B	$y = -57568 + 979837x$	0.53–10	0.9999	0.048	0.19
Dactylorhin E	$y = 17982 + 927576x$	0.13–2.6	0.9999	0.032	0.13
Loroglossin	$y = -47069 + 1860748x$	0.11–2.2	0.9999	0.039	0.10
Dactylorhin A	$y = -24568 + 1311926x$	0.26–5.2	0.9998	0.010	0.039
Militarine	$y = 4551 + 1741200x$	0.11–2.2	0.9999	0.0066	0.019

* a = intercept on the ordinate; b = slope

Table IV. Inter-day and Intra-day Precisions of the method (n = 5)*

Compound	Concentration (µg/mL)	RSD (%)	
		Inter-day	Intra-day
Dactylorhin B	26.4	0.21	0.96
	105.6	0.12	0.56
	422.4	0.10	0.38
Dactylorhin E	6.4	0.88	1.11
	25.6	0.86	0.94
	102.4	0.25	1.21
Loroglossin	5.6	0.88	1.22
	22.4	0.64	0.97
	89.6	0.91	1.49
Dactylorhin A	13.1	0.52	1.03
	52.4	0.52	1.53
	209.6	0.43	1.02
Militarine	5.7	0.73	0.74
	22.8	0.49	0.57
	91.2	0.48	1.11

* Number of determination (n = 5).

Table V. Recovery of the method (n = 6)*

Compound	Added (µg)	Found (µg)	Recovery (%)	RSD (%)
Dactylorhin B	541.84	534.25	98.6	1.31
Dactylorhin E	29.38	28.70	97.7	1.91
Loroglossin	100.80	100.70	99.9	1.82
Dactylorhin A	253.76	256.30	101.0	2.70
Militarine	34.56	33.83	97.9	1.66

* Number of determination (n = 6).

Method validation

The calibration curves showed a good correlation (r > 0.9998) between peak areas (y) and the quantity of the standards (x, µg). The LOD ranged from 0.0066 µg to 0.048 µg while LOQ ranged from 0.019 µg to 0.19 µg for all the analytes. The linearity, linear range, correlation coefficient (r), LOD, and LOQ are shown in Table III.

As the dynamic range of the calibration curves was narrow, a preliminary experiment of the quantitative determination of the analytes in the sample was necessary to ensure that the real injected amount of the analytes on column was within the dynamic range. According to the result, the original amount of weighed sample was increased or decreased, or the sample solution was further diluted before being injected into the HPLC system.

The RSD (%) values for the intra-day and inter-day precisions were better than 0.91% and 1.53%, respectively (Table IV), so the precision of the method was considered to be satisfactory. The average recovery results ranged from 97.1% to 101.0%, with RSD values of 1.31% to 2.70% (Table V).

Sample analysis

Figures 2 and 3 display the chromatograms of standard mixture solution and sample solution of G. conopsea, respectively. Peak identification of the analytes was performed by comparing the retention time and absorption spectra with those of the marker compounds and also by spiking the sample extracts with the markers when necessary. The peak purity was determined by comparing the values of the purity angle and purity threshold with Waters empower software. The resolution was calculated as, Rs = 1.18 $(t_2 - t_1)/(W_{0.5,1} + W_{0.5,2})$, where t_1 and t_2 represent retention time, and $W_{0.5,1}$ and $W_{0.5,2}$ stand for half bandwidths of two peaks. All of the results of Rs were over 2.5.

Table VI. Contents of Marker Compounds (mg/g, *n* = 3*) in the Tubers of *G. conopsea* and *C. viride* var. *bracteatum*

S. no.	Dactylorhin B	Dactylorhin E	Loroglossin	Dactylorhin A	Militarine
I	1638.28 (1.11)#	100.19 (2.43)	279.91 (2.14)	717.22 (1.48)	101.78 (1.21)
II	665.82 (1.26)	92.12 (2.79)	248.15 (2.57)	507.65 (1.72)	138.57 (3.35)
III	707.84 (4.39)	85.64 (0.72)	322.63 (3.24)	705.70 (3.06)	137.30 (0.25)
IV	2207.30 (2.40)	204.46 (1.74)	604.81 (1.44)	2213.39 (3.38)	228.04 (3.32)
V	4073.89 (2.06)	156.81 (2.13)	3060.63 (2.48)	2330.67 (3.91)	563.56 (1.25)
VI	6561.90 (1.64)	1231.81 (0.62)	1832.15 (2.22)	3517.10 (1.93)	843.12 (1.25)

* Number of determination (*n* = 3) except sample I, which is *n* = 6.
# Number in parentheses indicates RSD (%).

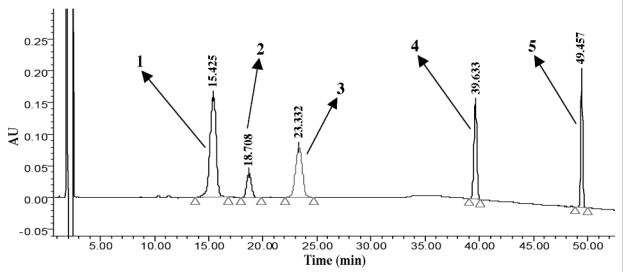

Figure 2. HPLC–UV (DAD) chromatogram (221.5 nm) of mixture of five marker compounds. Peak numbers are: 1, 524.51; 2, 807.81; 3, 233.32; 4, 336.93; 5, 754.94.

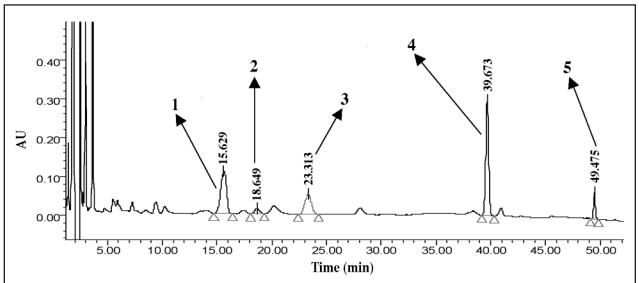

Figure 3. HPLC–UV (DAD) chromatogram (221.5 nm) of extract of sample IV. Peak numbers are: 1, 6.51; 2, 946.81; 3, 313.32; 4, 376.93; 5, 574.94.

The elution order of the five compounds in the chromatogram was dactylorhin B, dactylorhin E, loroglossin, dactylorhin A, and militarine. The difference in polarity between the separated compounds was the main factor that influenced the elution order, but the position and spatial configuration of the substituent groups also played an important role because they would affect the formation of intermolecular forces between the substituent groups and the mobile phase. Among these substituent groups, the C_3-hydroxyl group was a particularly advantageous group that could form stronger intermolecular forces with the mobile phase, and so the compounds with C_3-hydroxyl group were eluted more easily by the mobile phase. Therefore, loroglossin showed a lower retention value than dactylorhin A. It was a little difficult to explain clearly the retention order of dactylorhin E between dactylorhin B or loroglossin because there were larger differences between their structures, but the polarity and the spatial configuration of their substituent groups were also the important factors that influenced the elution order. The only difference between the structures of dactylorhin B and loroglossin as well as dactylorhin A and militarine was 2-substituted group. Therefore, it was easy to explain the elution order because glucose groups had stronger polarity than hydrogen groups.

The newly established method was successfully applied to determine the contents of the analytes in the tubers of *G. conopsea* and *C. viride* var. *bracteatum*. The contents and RSD (%) values are listed in Table VI, and the results showed a high level of variation of the five secondary metabolites among the tubers of *G. conopsea* collected from five different regions of China.

Conclusion

An HPLC method was firstly developed for the simultaneous quantization of five glucosyloxybenzyl 2-isobutylmalates in the tubers of *G. conopsea* and *C. viride var. bracteatum*. The assay, which was validated in terms of linearity, precision, sensitivity, and accuracy, had been successfully applied to determine the contents of the analytes in the tubers of the two plants collected from different regions of China.

Acknowledgements

The work was supported by a grant from National Science Fund for Distinguished Young Scholars (No. 30325047).

References

1. Flora of China. Vol 17, Science Press, Beijing, China, 1999, pp. 388–394.
2. Flora of China. Vol 17, Science Press, Beijing, China, 1999, pp. 328–329.
3. Dictionary of Chinese Materia Medica. Shanghai Scientific and Technical Publishers, Shanghai, China, 1975, pp. 436–437.
4. T. Morikawa, H.H. Xie, H. Matsuda, and M.Yoshikawa. Glucosyloxybenzyl 2-isobutylmalates from the tubers of Gymnadenia conopse. *J. Nat. Prod.* **69(6):** 881–886 (2006).
5. T. Morikawa, H.H. Xie, H. Matsuda, T. Wang, and M. Yoshikawa. Bioactive constituents from chinese natural medicines. XVII. Constituents with radical scavenging effect and new glucosyloxybenzyl 2-isobutylmalates from *Gymnadenia conopsea*. *Chem. Pharm. Bull.* **54(4):** 506–513 (2006).
6. S.Y. Huang, G.Q. Li, J.G. Shi, S.Y. MO, S.J. Wang, and Y.C. Yang. Chemical constituents of the rhizomes of Coeloglossum viride var. bracteatum. *J. Asian Nat. Prod. Res.* **6(1):** 49–61 (2004).
7. D. Zhang and J.J. Zhang. Effect of Coeloglossum. viride var. bracteatum Extract on oxidation injury in sub-acute senescent model mice. *Acta. Aca. Med. Sin.* **27(6):** 729–733 (2005).
8. D. Zhang, G.T. Liu, J.G. Shi, and J.J. Zhang. Coeloglossum viride var. bracteatum extract attenuates D-galactose and NaNO2 induced memory impairment in mice. *J. Ethno-Pharmacol.* **104:** 250–256 (2006).
9. D. Zhang, Y. Zhang, G.T. Liu, and J.J. Zhang. Dactylorhin B reduces toxic effects of b-amyloid fragment (25-35) on neuron cells and isolated rat brain mitochondria. *Nau. Sch. Arch. Pharmacol.* **374(2):** 117–125 (2006).
10. M. Cai, Y. Zhou, S.L. Gesang, C. Bianba, and L.S. Ding. Chemical fingerprint analysis of rhizomes of Gymnadenia conopsea by HPLC–DAD–MS. *J. Chromatogr. B* **844:** 301–307 (2006).
11. H. Kizu, E, Kaneko, and T. Tomimori. Studies on Nepalese crude drugs. XXVI. Chemical constituents of panch aunle, the roots of Dactylorhiza hatagirea D. *Don. Chem. Pharm. Bull.* **47(11):** 1618–1625 (1999).

Manuscript received March 22, 2007;
Revision received February 17, 2008.