SHORT COMMUNICATION

Simultaneous determination of five diterpenoid alkaloids in *Herba Delphinii* by HPLC/ELSD

Chao-Zhan Lin\(^a\), Si-Min Xie\(^a\), Chen-Chen Zhu\(^{a,*}\), Zeren-Dawa Bairu\(^b\), Suolang-Qimei Kangsa\(^b\), Dun Zhu\(^b\)

\(^a\)Institute of Clinical Pharmacology, Guangzhou University of Chinese Medicine, Guangzhou 510405, PR China
\(^b\)Tibet College of Tibetan Medicines, Lasa 850000, PR China

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**Abstract** A HPLC–ELSD method was developed and validated for simultaneous determination of five Hetisane-type diterpenoid alkaloids in a Tibetan traditional herbal medicine, “Gebu Dili” (*Herba Delphinii*), using a Kromasil C\(_{18}\) column (250 mm x 4.6 mm, 5 \(\mu\)m) with the mobile phase consisting of acetonitrile and 0.1% triethylamine in gradient (detected by evaporative light scattering detector). The linear ranges of five compounds were determined and method validation was evaluated completely. The established method is rapid and accurate with high repeatability, and can be applied for the quality control of *Herba Delphinii*.

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1. **Introduction**

The aerial parts of *Delphinium trichophorum* Franch (*Herba Delphinii, Ranunculaceae* Family), named “Gebu Dili”, is the manna for clearing away lung-heat and widely used for the treatment of cough or flu due to lung-heat in Tibetan medicine for a long time [1,2]. But the effective method for quality control of this drug has been absent till now.

Diterpenoid alkaloids are the main constituents in Genus *Delphinium* with over 230 identified structures [3]. These kinds of alkaloids possess not only pharmacological importance due to their analgesic [4], anti-epileptic [5–7] and anti-tumorigenic activities [8,9], but also toxic actions [10] as a neurotoxin and a cardiac toxin. Quality evaluation based on these kinds of constituents for herbal medicines originated from such species seems to be more practicable.

As a series of our studies on the phytochemistry and quality control of *Herba Delphinii*, this paper reports a method for simultaneous determination of five hetisane-type diterpenoid alkaloids in this crude drug by high-performance liquid chromatography with evaporative
light scattering detection (HPLC/ELSD). The results are expected to provide scientific evidence for quality control of this traditional Tibetan herbal medicine.

2. Experimental

2.1. Chemicals and reagents

Five reference compounds as trichodelphinineA-D and 2α, 11α, 13α-triacetyl-hetisane (Fig. 1) were isolated from D. trichoformum and their structures were elucidated according to their spectral data (UV, IR, MS, 1H NMR and 13C NMR). The purity of these compounds was determined to be more than 98% by normalization of the peak areas detected by HPLC–ELSD.

Acetonitrile (HPLC grade) was of Honeywell Company Inc. (USA). Super purified water was prepared with a Milli-Q system (Millipore, USA). The other solvents were all of Analytical Reagent (AR) grade and purchased from Guanghua Chemical Company Inc. (Shantou, China).

2.2. Samples preparation

All tested samples were collected from Tibet Autonomous Region, PR China, and authenticated by Professor Suolang-Qimei Kangsa and Professor Chao-Mei Pan (Botanical Teaching and Researching Section, School of Materia Medica, Guangzhou University of CM). The voucher specimens were deposited at Herbarium of Guangzhou University of CM, Guangzhou, PR China.

Fourteen patches of tested samples were dried at 50 °C until constant weight was attained. Approximately 1.0 g dried powder of each sample was accurately weighed, moistened with 0.2 mL ammonia water for 15 min and then extracted with 25 mL of 4:1 (v/v) CHCl3:MeOH for 45 min under Ultrasonic vibration. The supernatants were filtered through 0.45 μm microporous membranes into HPLC vials for future test. Twenty microliters was injected for analysis.

2.3. Chromatographic conditions

Analyses were performed using a Waters Alliance 2695-2998 HPLC system (Waters, MA, USA). The HPLC detector was an Alltech 3300 ELSD (evaporative light scattering detector, Alltech Associates, USA) whose signal was integrated to a 35900E interface. The detector was warmed up for 30 min prior to each run, and then operated at 75 °C with the optimum flow rate of the nebulizing gas (nitrogen, 99.99%) at 1.0 L/min. A Kromasil C18 (250 mm × 4.6 mm × 5 μm) column was combined with a guard column of the same stationary phase (10 mm × 4.6 mm × 5 μm). Column temperature was maintained at room temperature. The mobile phase consisting of acetonitrile (A) and 0.1% triethylamine was in gradient as follows: 30% A (0–21 min), 30% A–45% A (21–30 min), 45% A–55% A (30–55 min) at a flow rate of 1.0 mL/min. The injection volume was 20 μL. Data was collected and processed using Empower 2 software on a Dell computer.

2.4. Calibration curves

Methanol stock solution of mixture of the above standards was prepared containing 1256.0 μg/mL of trichodelphinine A, 648.4 μg/mL of trichodelphinine B, 370.0 μg/mL of trichodelphinine C, 794.4 μg/mL of trichodelphinine D and 1380.8 μg/mL of 2α, 11α, 13α-triacetyl-hetisane, and diluted 1, 2, 4, 8, 16 and 32 times to six different concentrations. Each concentration was injected three times. Calibration curves are constructed by plotting the logarithm of peak areas versus the logarithm of concentration of each analyte, and the results are shown in Table 1.

2.5. Quantification

Each sample solution was injected in triplet and the peak areas of the five analytes were recorded. Quantification was based on the two points external standard method using calibration curves fitted by linear regression analysis.

2.6. Method validation

Method validation including precision, repeatability, stability and sample recovery was evaluated. Precision and repeatability were assessed by the successive analysis of five injections of the same sample solution and five replicates of the same patch of sample, respectively. The analysis of six time period within a day (0, 2, 4, 8, 16, 24 h) was used to evaluate the stability of sample solution in 24 h. Recovery was determined by adding known quantities of standards to the tested samples, and calculated by comparing the added and obtained quantities using external standard linear regression.

3. Results and discussion

3.1. Optimization of sample pre-treatment

As for the low polarity and weak alkalinity of the analytes, multiple related extraction conditions were designed and evaluated, which involved the following factors and corresponding levels: extraction method (ultrasonication or reflux), extraction repetitions (1 or 2 times), extraction solvents (CHCl3:MeOH 4:1, 2:1, 1:1, 1:2, 1:4 and 1:5 v/v), solvent volume (25, 50, or 100 mL), extraction time (15, 30, 45 or
(60 min) and volume of ammonia water (0.1, 0.2, 0.3, 0.4 or 0.5 mL). By comparison of the contents of five analytes determined by the above experiments, the final optimized pre-treatment method for D. trichoforum was selected as follows: The accurately weighed sample powder was moistened with 0.2 mL ammonia water for 15 min and then extracted with 25 mL of 4:1 (v/v) CHCl₃:MeOH for 45 min under Ultrasonic vibration.

### 3.2. Optimization of HPLC conditions

Several HPLC parameters affecting the results of separation and content precision of the content determination were optimized, including mobile phase (acetonitrile–water, acetonitrile–0.1% triethylamine, or methanol–0.1% triethylamine), column temperature (20, 25, 30, 35, 40 or 50 °C), drift tube temperature (70, 75, 80, 85, 90, 95 or 100 °C) and flow rate of the nebulizing gas (0.6 L/min, 0.9 L/min, 1.2 L/min, 1.5 L/min, 1.8 L/min or 2.1 L/min). The final optimal HPLC condition was defined as adopting acetonitrile (A) and 0.1% triethylamine as the mobile phase in gradient as 30% A (0–21 min), 30% A–45% A (21–30 min), 45% A–55% A (30–55 min) at a flow rate of 1.0 mL/min. Column temperature was maintained at room temperature (20–35 °C). The detector was warmed up for 30 min prior to each run, and then operated at 75 °C with the optimum flow rate of the nebulizing gas at 1.0 L/min.

### 3.3. Validation of methodology

The values of relative standard deviation (RSD) of peak area (PA) of analytes for replicated injections of the same sample were in the range of 1.56–2.28%. Meanwhile, the RSD values for method

| Standards               | Linear range (μg) | Regression equation | Correlation factor (γ) | LOQ (μg) | LOD (μg) |
|-------------------------|-------------------|---------------------|------------------------|----------|----------|
| Trichodelphinine A      | 0.78–25.12        | Y = 1.2663X + 6.9481 | 0.9987                 | 0.40     | 0.04     |
| Trichodelphinine B      | 0.41–12.97        | Y = 1.3268X + 6.4905 | 0.9990                 | 0.21     | 0.02     |
| Trichodelphinine C      | 0.23–7.40         | Y = 1.2851X + 6.9061 | 0.9980                 | 0.12     | 0.06     |
| Trichodelphinine D      | 0.50–15.89        | Y = 1.3382X + 6.2680 | 0.9959                 | 0.25     | 0.06     |
| 2α,11α,13α-triacetyl-hetisane | 0.86–27.62   | Y = 1.2333X + 7.2241 | 0.9983                 | 0.44     | 0.04     |

*Y: the logarithm of peak areas; X: the logarithm of concentration.

| Analytes                | Sample amount (g) | Amount in sample (mg) | Added amount (mg) | Detected amount (mg) | Recovery (%) | Average recovery (%) | RSD (%) |
|-------------------------|-------------------|-----------------------|-------------------|----------------------|--------------|----------------------|---------|
| Trichodelphinine A      | 0.5001            | 1.10                  | 1.11              | 2.20                 | 98.4         | 99.7                 | 2.08    |
|                         | 0.5003            | 1.10                  | 1.11              | 2.18                 | 97.2         |                      |         |
|                         | 0.5001            | 1.10                  | 1.11              | 2.25                 | 103.6        |                      |         |
|                         | 0.5002            | 1.10                  | 1.11              | 2.22                 | 100.9        |                      |         |
|                         | 0.5000            | 1.10                  | 1.11              | 2.20                 | 99.1         |                      |         |
|                         | 0.5001            | 1.10                  | 1.11              | 2.20                 | 99.1         |                      |         |
| Trichodelphinine B      | 0.5001            | 0.64                  | 0.63              | 1.27                 | 100.0        | 99.7                 | 2.23    |
|                         | 0.5003            | 0.64                  | 0.63              | 1.27                 | 100.0        |                      |         |
|                         | 0.5001            | 0.64                  | 0.63              | 1.27                 | 100.0        |                      |         |
|                         | 0.5002            | 0.64                  | 0.63              | 1.29                 | 103.2        |                      |         |
|                         | 0.5000            | 0.64                  | 0.63              | 1.26                 | 98.4         |                      |         |
|                         | 0.5001            | 0.64                  | 0.63              | 1.25                 | 96.8         |                      |         |
| Trichodelphinine C      | 0.5001            | 0.24                  | 0.24              | 0.49                 | 103.0        | 101.2                | 2.09    |
|                         | 0.5003            | 0.24                  | 0.24              | 0.48                 | 98.6         |                      |         |
|                         | 0.5001            | 0.24                  | 0.24              | 0.49                 | 102.9        |                      |         |
|                         | 0.5002            | 0.24                  | 0.24              | 0.48                 | 98.0         |                      |         |
|                         | 0.5000            | 0.24                  | 0.24              | 0.49                 | 102.3        |                      |         |
|                         | 0.5001            | 0.24                  | 0.24              | 0.49                 | 102.7        |                      |         |
| Trichodelphinine D      | 0.5001            | 0.85                  | 0.85              | 1.69                 | 98.0         | 100.4                | 2.84    |
|                         | 0.5003            | 0.85                  | 0.85              | 1.68                 | 97.5         |                      |         |
|                         | 0.5001            | 0.85                  | 0.85              | 1.74                 | 104.9        |                      |         |
|                         | 0.5002            | 0.85                  | 0.85              | 1.71                 | 101.0        |                      |         |
|                         | 0.5000            | 0.85                  | 0.85              | 1.72                 | 102.3        |                      |         |
|                         | 0.5001            | 0.85                  | 0.85              | 1.69                 | 98.9         |                      |         |
| 2α,11α,13α-triacetyl-hetisane | 0.5001     | 1.11                  | 1.00              | 2.01                 | 99.7         | 101.1                | 1.91    |
|                         | 0.5003            | 1.11                  | 1.00              | 2.00                 | 98.9         |                      |         |
|                         | 0.5001            | 1.11                  | 1.00              | 2.05                 | 104.3        |                      |         |
|                         | 0.5002            | 1.11                  | 1.00              | 2.02                 | 100.6        |                      |         |
|                         | 0.5000            | 1.11                  | 1.00              | 2.03                 | 102.2        |                      |         |
|                         | 0.5001            | 1.11                  | 1.00              | 2.02                 | 100.7        |                      |         |
repeatability were from 2.18% to 2.53%. Moreover, the RSD values for sample stability test were less than 2.56%. The results of sample recovery indicated that the average recovery of the analytes was from 99.7% to 101.2% and the RSD values were less than 3.0% (see Table 2). All these results indicated that the established method of determination was valid and feasible.

3.4. Analysis of diterpenoid alkaloids in D. trichoforum

The five analytes were identified in 14 samples by comparing their retention time (tR) with standards and quantified by the two points external standard method using calibration curves fitted by linear regression analysis. The contents of all tested samples are listed in Table 3 and the chromatograms of representative samples are shown in Fig. 2. T-test was used to analyze the variance of contents of the five analytes and total alkaloids of samples from different collection locations and times.

The contents of five diterpenoid alkaloids in 10 aerial part samples of D. trichoforum are in the ranges of 1.12–2.37 mg/g (trichodelphinine A), 1.01–2.30 mg/g (trichodelphinine B), 0.30–0.72 mg/g (trichodelphinine C), 1.27–2.87 mg/g (trichodelphinine D) and 1.30–2.36 mg/g (2α, 11α, 13α-triacetyl-hetisane), and the sum amount of the above analytes is in the range 3.45–8.85 mg/g, e.g.; the average amount was 7.23 mg/g.

The contents of alkaloids in samples of various parts including roots, stems, leaves and flowers of D. trichoforum were also determined and the content of total alkaloids was the highest in the leaves, and too low in the stems.

For samples from different collection times (2009 and 2010), the T values of contents of trichodelphinine D (t1, 5.787), trichodelphinine B (t2, 5.302) and 2α, 11α, 13α-triacetyl-hetisane (t4, 3.162) were calculated.

Table 3  Contents of the five components in 14 tested samples.

| No. | Tested parts | Collection time | Collection location | Content (mg/g) | SUM  |
|-----|--------------|-----------------|---------------------|----------------|------|
|     |              |                 |                     | Trichodelphinine A | Trichodelphinine B | Trichodelphinine C | Trichodelphinine D | 2α, 11α, 13α-triacetyl-hetisane |  |
| 1   | Aerial       | 2009.08         | Changdu (wild)      | 1.51           | 1.95            | 0.48          | 2.47          | 1.92          | 8.33 |
| 2   | Aerial       | 2009.08         | Changdu (commercial)| 1.12           | 1.85            | 0.30          | 2.16          | 1.30          | 6.73 |
| 3   | Aerial       | 2009.08         | Linzhi (wild)       | 1.76           | 2.30            | 0.53          | 2.59          | 1.67          | 8.85 |
| 4   | Aerial       | 2009.08         | Linzhi (wild)       | 1.70           | 2.23            | 0.42          | 2.87          | 1.41          | 8.63 |
| 5   | Aerial       | 2009.08         | Linzhi (commercial) | 1.27           | 1.76            | 0.72          | 2.11          | 3.24          | 6.10 |
| 6   | Aerial       | 2010.07         | Changdu (commercial)| 1.93           | 1.07            | 0.45          | +            | +            | 3.45 |
| 7   | Aerial       | 2010.07         | Changdu (wild)      | 2.37           | 1.54            | 0.50          | 1.52          | +            | 5.93 |
| 8   | Aerial       | 2010.07         | Linzhi (commercial) | 1.75           | 1.01            | 0.43          | 1.27          | 1.84          | 6.30 |
| 9   | Aerial       | 2010.07         | Linzhi (wild)       | 2.37           | 1.38            | 0.58          | 1.48          | 2.36          | 8.17 |
| 10  | Aerial       | 2010.07         | Linzhi (wild)       | 2.21           | 1.57            | 0.49          | 1.71          | 2.22          | 7.90 |
| 11  | Leaf         | 2009.08         | Linzhi (wild)       | 3.01           | 1.76            | 0.72          | 2.10          | 3.61          | 11.20 |
| 12  | Stem         | 2009.08         | Linzhi (wild)       | +             | +               | +            | +            | +            | --d |
| 13  | Flower       | 2009.08         | Linzhi (wild)       | 4.03           | 1.20            | 1.13          | 1.38          | 2.89          | 10.63 |
| 14  | Root         | 2009.08         | Linzhi (wild)       | +             | +               | +            | +            | +            | --c |

*Each value is a mean value of three samples (n = 3).

bTrace.
cNot detected.
dNot calculated.

dNot calculated.

Fig. 2  The chromatogram of five reference compounds (A) and representative sample of D. trichoforum (B).
showed statistical significance compared with \( t_{0.05, \, 8} \) (2.306); meanwhile, trichodelphinine A \( (t_3, 0.776) \), trichodelphinine C \( (t_5, 0) \) and total alkaloids \( (t_{total}, 2.273) \) exhibited no difference. For those from two locations (Linzhi and Changdu), only the T value of trichodelphinine A \( (t_3, 2.956) \) displayed statistical significance, although, the sum content of five analytes of samples from Linzhi showed a little higher value than those from Changdu.

Considering about the quality evaluation of *Herba Delphinium*, the results of our research revealed that the contents of five diterpenoid alkaloids varied relatively greatly in 10 patches of herbal samples, which suggested that the content of any single analyte would not objectively affect the quality of this drug. Meanwhile, the sum contents of five analytes exhibited slight difference between samples from two different collecting locations to some extent. Therefore, simultaneous determination of five analytes and the sum contents of tested analytes would be necessary for comprehensive assessment of this drug.

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