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Pre-column derivatization and gas chromatographic determination of alkaloids in bulbs of *Fritillaria*

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Abstract

A method of precolumn derivatization GC with FID detection was developed for a simultaneous analysis of five major steroidal alkaloids of *Fritillaria* species, namely ebeiedine, ebeiedinone, verticine, verticinone and imperialine. Derivatization was carried out by trimethylsilylation of the hydroxyl-containing *Fritillaria* alkaloids to the corresponding trimethylsilylates with trimethylsilylimidazole. Reaction conditions were optimised and the alkaloids derivatives were characterised by on-line GC–MS. The validated GC method demonstrated a good linearity at the sampling ranges used. This analytical method is simple, convenient and reproducible. The developed assay was successfully applied to the determination of the major pharmacologically active alkaloids in three commonly used antitussive *Fritillaria* species: *F. cirrhosa, F. thunbergii* and *F. pallidiflora*.

Keywords: Derivatization, GC; Alkaloids; *Fritillaria* alkaloids

1. Introduction

Bulbus *Fritillariae* (Chinese name Beimu), derived from the bulbs of various species of the genus *Fritillaria* (*Liliaceae*), has been used as an antitussive and expectorant in traditional Chinese medicine for more than 2000 years [1]. It is well documented that the major constituents in Beimu are isosteroidal alkaloids, and ebeiedine, ebeiedinone, verticine, verticinone and imperialine (Fig. 1) are the major alkaloids in commonly used *Fritillaria* herbs [2–4]. Our recent in vivo pharmacological studies on guinea pigs suggested that ebeiedine, ebeiedinone, verticine and verticinone all possess antitussive activity [5,6]. Furthermore, the relaxation effects of ebeiedine, verticine, verticinone and imperialine on isolated rat tracheal and bronchial rings have also been demonstrated by our research team [7].

Many *Fritillaria* species are currently used as plant sources. However, the amounts and types of *Fritillaria* alkaloids vary in different species [8–11]. Therefore, quality control of Beimu is an important

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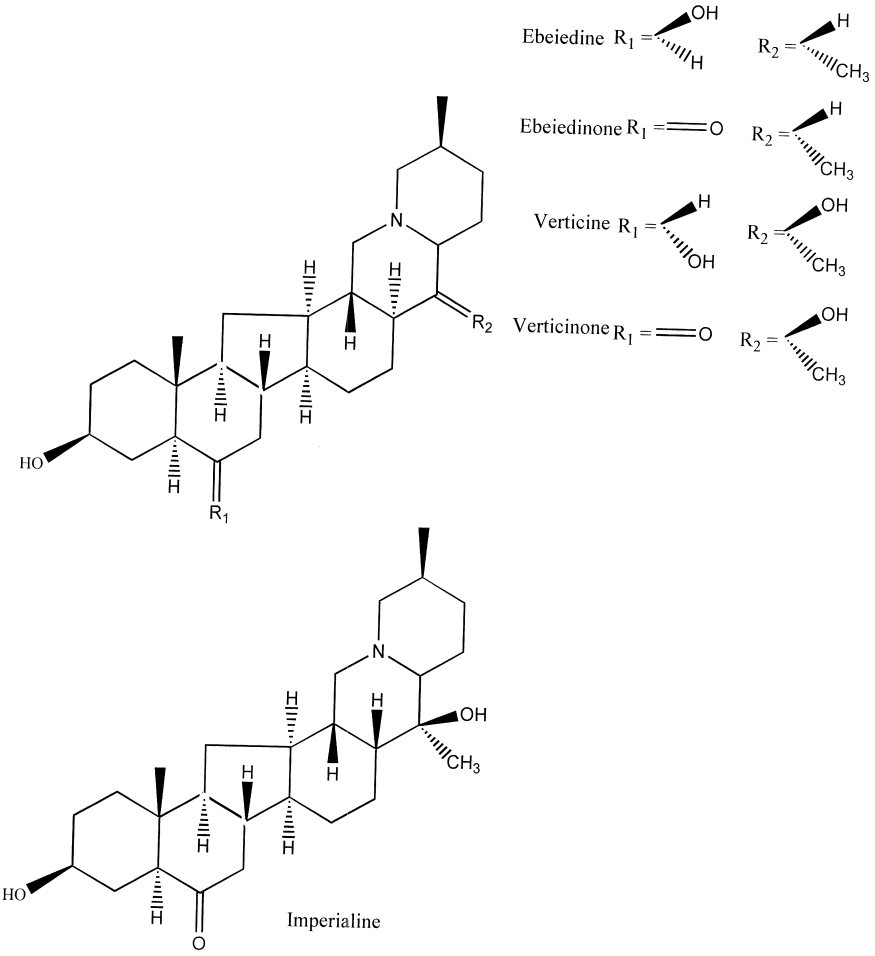

Fig. 1. Structures of ebeiedine, ebeiedinone, verticine, verticinone and imperialine.

issue for the effective and safe use of these herbs. So far only a few methods have been reported on the analysis of *Fritillaria* alkaloids [12]. Since they lack ultraviolet (UV) absorption, most of these alkaloids are difficult to determine by conventional HPLC–UV analysis [12]. No direct GC assay was developed as these alkaloids are highly polar and can not be eluted from conventional GC columns. Recently we have established a HPLC method for the determination of *Fritillaria* alkaloids by precolumn derivatization of the hydroxyl functional groups in the alkaloids with 1-naphthoxyl chloride [13]. In the present study, a newly developed GC method with precolumn derivatization for the determination of five major *Fritillaria* alkaloids, namely ebeiedine, ebeiedinone, verticine, verticinone and imperialine, in Beimu is described.

2. Experimental

2.1. Materials

Fritillaria thunbergii was collected from Zhangshu, Zhejiang province, *F. pallidiflora* from Yili, Xinjiang autonomous region, and *F. cirrhosa* from Zhongdian, Yunnan province, P.R. China. Plants were authenticated by pharmacognosists in China Pharmaceutical University, and the voucher samples were deposited at the Herbarium of China

Pharmaceutical University. Pure samples of ebeiedine, ebeiedinone, verticine, verticinone and imperialine were isolated in our laboratories [3,11,14–19]. The purity and identity of each isolated alkaloid were determined by IR, ^{1}H- and ^{13}C-NMR, and MS analyses. *n*-Tricontone (GC-grade, as internal standard), *N*-methyl-*N*-(trimethylsilyl)trifluroacetamide (MSTFA), trimethylsilylimidazole (TMSI), trimethylchlorosilane (TMCS), bis-(trimethylsilyl)acetamide (BSA), bis(trimethylsilyl)-trifluroacetamide (BSTFA) and trifluroacetamide (TFAA) were all purchased from Sigma Chemical Co. (St. Louis MO, USA). Dichloromethane and all other solvents were of analytical reagent grade from Sigma Chemical Co.

2.2. Instrumentation

2.2.1. GC-analysis

GC analysis was performed on GC-R1A gas chromatography (Shimadzu) equipped with OPGU-500S hydrogen generator and flame ionization detector (FID). Data were recorded and analyzed by RPR-G1 GC- processor (Shimadzu). An OV-1 (HP, 12 m×0.53 mm, 0.33 μm) capillary column was used. The injector and detector temperatures were set at 260°C. Column temperature was programmed from 210 to 245°C at 1°C/min and held at 210°C for 5 min. Hydrogen was used as carrier gas with a flow-rate of 35 ml/min.

2.2.2. GC–MS analysis

Online GC–MS analysis was carried out on a Finnigan Mat GCQ GC–MS system. Helium was used as a carrier gas with a flow-rate of 40 ml/min. Both electron impact (EI, 70 eV) and chemical ionisation (CI, with methane as reactant gas) interfaces were utilised. Mass spectra were recorded with a scanning range of m/z 50–700.

2.3. Derivatization

Known amounts of each of the alkaloids or Beimu sample were transferred into vials, an aliquot of *n*-tricontone, the internal standard, solution (20 μl of 1.0 mg/ml in dichloromethane) was added to each sample, and the solvent was evaporated under a stream of nitrogen at 40°C. The sample was treated with 50 μl of TMSI in the vials sealed with screw-caps and then heated at 40°C for 20 min. Aliquot (0.3 μl) of the resultant sample was injected into GC or GC–MS system.

2.4. Optimal conditions of derivatization

Optimal derivatization conditions were obtained by testing for verticine and verticinone with the following variations: different derivatizing reagents, concentrations of derivatizing reagent (analyte/derivatizing reagent (w/v): 1:1.0, 1:1.5, 1:2.0, 1:2.5, 1:3.5 and 1:4.0), reaction temperatures (28, 40, 70 and 80 °C), and reaction time (10, 20, 40, 50 and 60 min).

2.5. Characterisation of the derivatives

The characterisation of the derivatives of five alkaloids was performed by GC–MS. The sample mixture obtained after optimal derivatization was directly subjected to the on-line GC–MS system with either EI or CI interface using a positive ion mode.

2.6. Stability of the derivatives

The stability of the derivatives of verticine and verticinone was determined by analyzing the sample at 0, 40, 80, 240, 540 and 1200 min, respectively, after derivatization.

2.7. Standard curves

A stock solution containing ebeiedine, ebeiedinone, verticine, verticinone and imperialine at a concentration of about 1.0 mg/ml in dichloromethane was prepared. Known amounts of each component (e.g. 5, 10, 15, 20, 25 and 30 μg) were transferred into vials by taking 5, 10, 15, 20, 25 and 30 μl of the stock solution. Samples were derivatized as described in Section 2.3. Aliquots (0.3 μl) of the samples were then injected into GC system. Samples for each concentration were analysed in triplicate. The standard curves were constructed by plotting peak area ratio (analyte/internal standard) versus concentration of the analyte.

2.8. Accuracy and precision

The measurements of intra- and inter-day variability were utilised to determine the precision and accuracy of the developed method. The assays were carried out by adding a known amount of ebeiedine, ebeiedinone, verticine and verticinone into the sample of *F. cirrhosa,* and imperialine into the sample of *F. pallidiflora*. The amounts of the alkaloids in both *F. cirrhosa* and *F. pallidiflora* were determined prior to standard addition. The spiked samples were then extracted and analyzed as described in Section 2.9. The relative standard variation (RSD) was taken as a measure of precision and the percentage difference between amounts determined and spiked was considered as a measure of accuracy. Each sample was analyzed in triplicate to obtain intra-day variability, and the inter-day variability was determined by analyzing samples in three separate days.

2.9. Analysis of Beimu sample from plant materials

Powder of *Fritillaria* bulbs (0.3 g for *F. cirrhosa* and 0.1 g for all other *F.* species) was extracted with 5.0 ml of diethyl ether-ammonium hydroxide (25:0.5, v/v) by vortex for 2.0 h. After centrifugation (1500 r/min, 10 min), the supernatant (1.0 ml) was transferred into vials, and then treated as described in Section 2.3. The reaction mixtures were directly subjected to GC analysis. Each sample was analysed in triplicate.

3. Results and discussion

3.1. Derivatization

Conventional GC methods can not be directly utilised to analyze *Fritillaria* alkaloids owing to the high polarity and low volatility of *Fritillaria* alkaloids. However, these alkaloids can be resolved well in conventional GC columns by trimethylsilylation and/or acetylation of the hydroxyl groups in the alkaloids. Several derivatizing reagents including MSTFA, TMSI, TMCS, BSA, BSTFA and TFAA were tested for the derivatization. It was found that using TMSI, all five alkaloids tested were rapidly and completely derivatized. The resultant TMS-derivatives were successfully separated under the developed GC conditions (Fig. 2). Thus, TMSI was chosen as the derivatizing reagent.

3.2. Optimal derivatization conditions

Verticine and verticinone were selected as the testing alkaloids for optimizing derivatization conditions, as they represent the two major types of *Fritillaria* alkaloids, with hydroxyl or ketone groups, respectively. Variations of the quantities of derivatizing reagent, reaction temperatures and reaction duration were examined systematically. As shown in Fig. 3, the ratio of analyte to derivatizing reagent, reaction temperature and reaction duration did not significantly affect the formation of derivatives of the two alkaloids examined. Therefore, in order to ensure a quantitative derivatization and a convenient determination, the optimal derivatization condition was chosen in that the analytes were heated with 2.5 fold excess of derivatizing reagent at 40°C for 20 min. These derivatizing conditions were much milder compared with that in HPLC assay previously developed by our research team [13].

3.3. GC-analysis

A variety of packed columns: Dexsil 300 (0.5 m×2 mm I.D.), OV-7 (2 m×2 mm I.D.), OV-1 2 m×2 mm I.D.), and capillary columns: OV-17 (15 m×0.53 mm I.D.), SE-54 (15 m×0.53 mm I.D.), and OV-1 (HP, 12 m×0.53 mm I.D.), have been tested with either H_2 or N_2 as carrier gas. The results demonstrated that capillary column OV-1 (HP) was the best column tested for the separation of the derivatives of *Fritillaria* alkaloids and all five alkaloids tested and the internal standard were separated well on this column using hydrogen as a carrier gas. Thus the developed GC assay for the determination of the major *Fritillaria* alkaloids in different Beimu species was performed on OV-1 capillary column. The representative chromatograms of various Beimu herbs are shown in Fig. 2.

3.4. Characterization of the derivatives

Characterization of the derivatives was performed

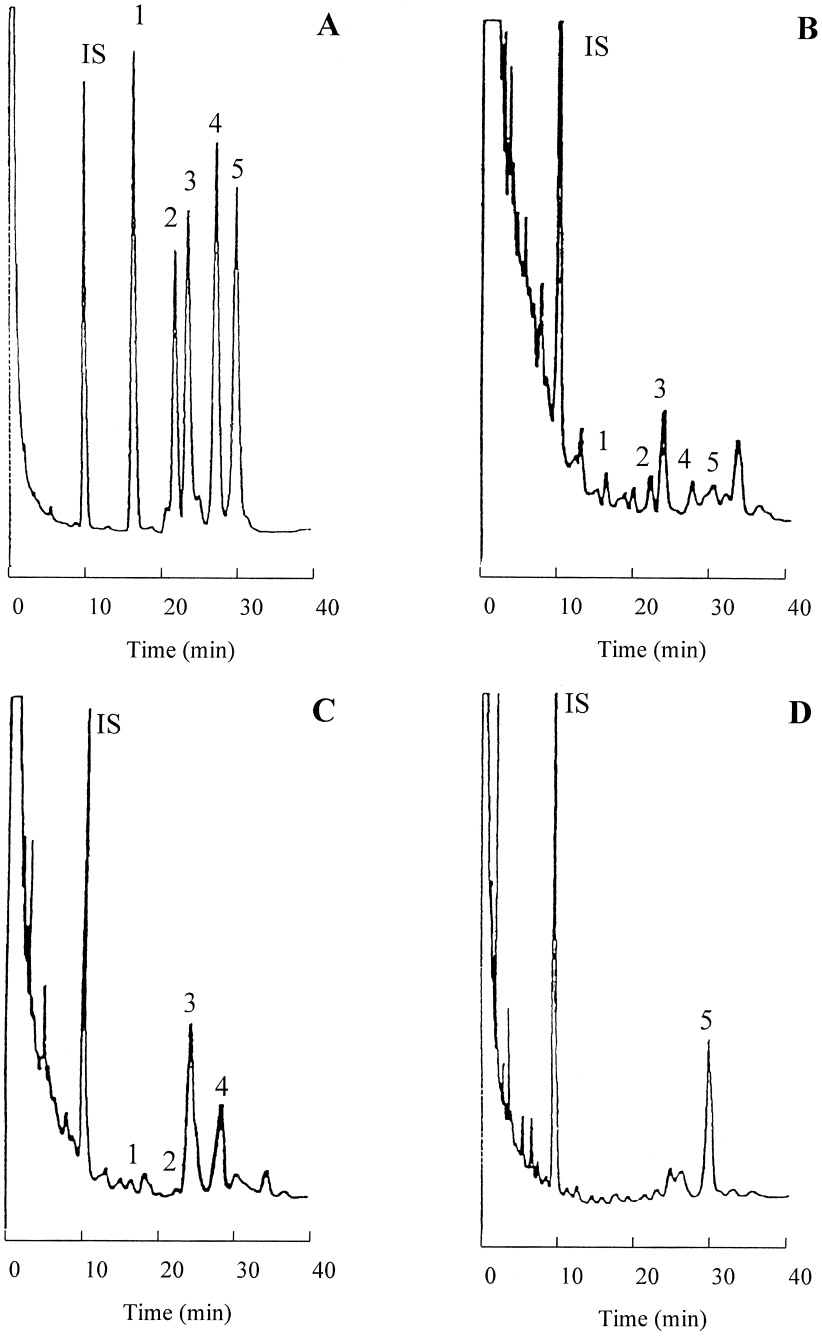

Fig. 2. GC chromatograms of derivatized mixtures of spiked ebeiedine, ebeiedinone, verticine, verticinone and imperialine (A), derivatized samples from *F. cirrhosa* (B), *F. thunbergii* (C) and *F. pallidiflora* (D). (IS) Internal Standard; (1) ebeiedine derivative; (2) ebeiedinone derivative; (3) verticine derivative; (4) verticinone derivative; (5) imperialine derivative.

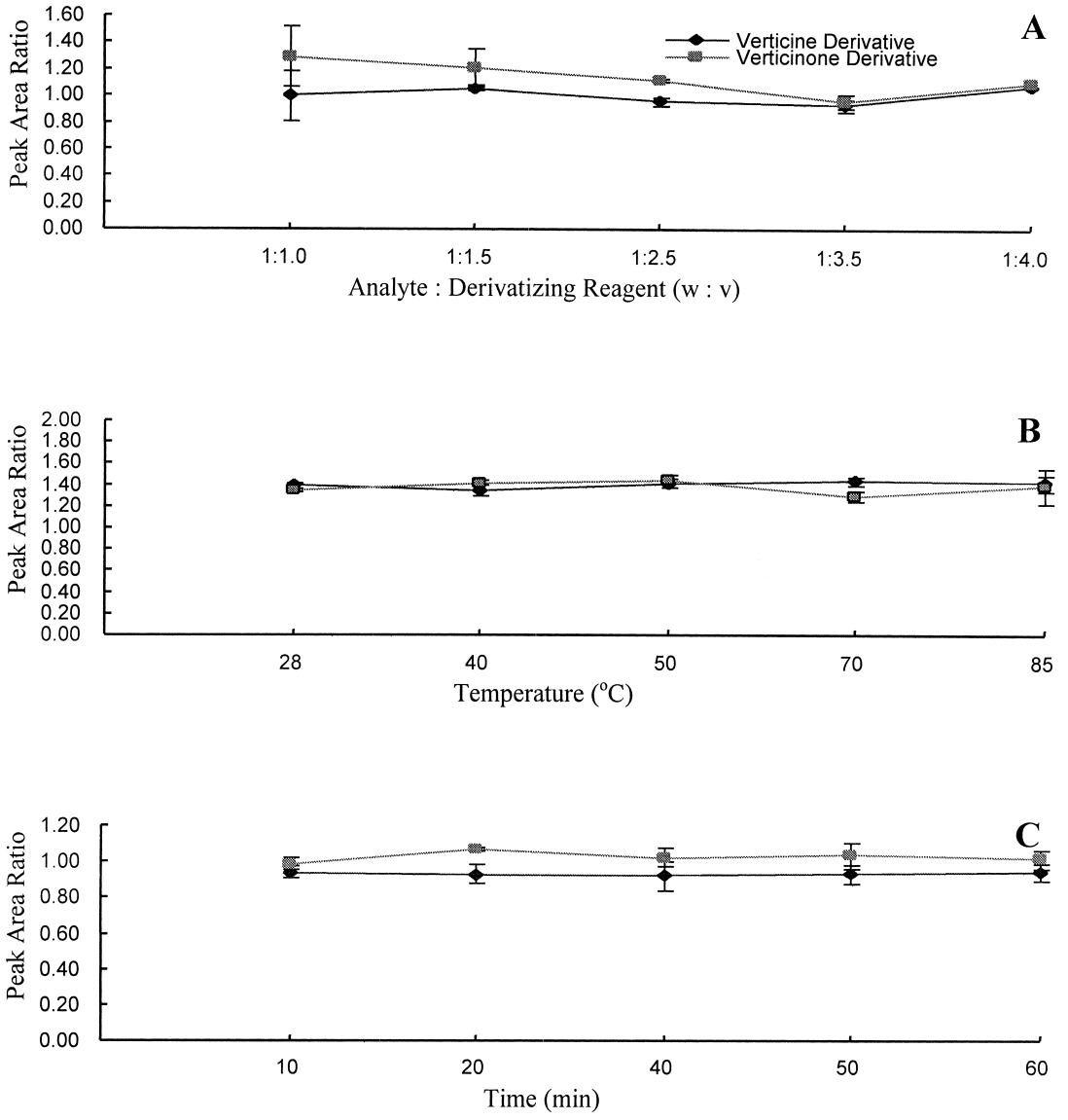

Fig. 3. Influence of concentration of derivatizing reagent (A), reaction temperature (B) and reaction duration (C) on the derivatization of verticine and verticinone.

by GC–MS analysis with both EI and CI ionisation interfaces. As shown in Fig. 4, the EI-mass spectrum of ebeiedine derivative exhibited a peak with a highest mass unit at m/z 559 corresponding to the molecular ion $[M^+]$ of bis(trimethylsilyl)ebeiedine. Therefore, two hydroxyl groups at 3 and 6 positions in ebeiedine were silylated and the derivative was assigned as 3β,6β-bis(trimethylsilyl)ebeiedine. The EI-mass spectrum of ebeiedinone derivative showed the molecular ion at m/z 485, which corresponds to the trimethylsilylation of one hydroxyl group, thus 3β-trimethylsilylebeiedinone was obtained. In the case of verticine derivative, the ion with the highest mass unit at m/z 647 was observed in the EI–MS indicating that all three hydroxyl groups in verticine were derivatized, and thus 3β,6α,20β-tris

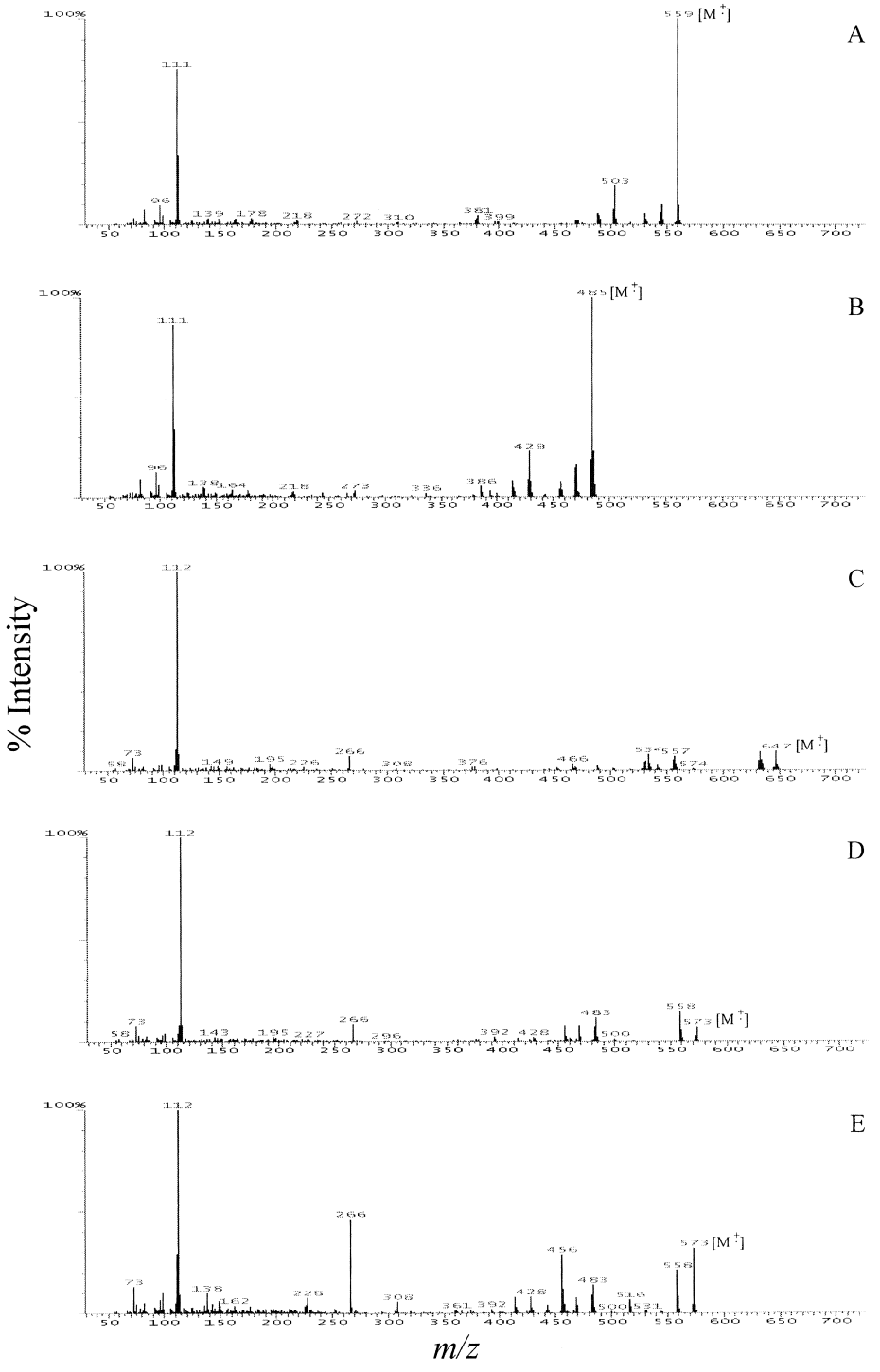

Fig. 4. EI–MS for trimethylsilylated derivatives of ebeiedine (A), ebeiedinone (B), verticine (C), verticinone (D) and imperialine (E).

(trimethylsilyl)verticine was assigned as verticine derivative. The EI-mass spectra of verticinone and imperialine derivatives both exhibited the highest mass unit ion at m/z 573 corresponding to the molecular ion with two trimethysilylated hydroxyl groups. Therefore, the derivatives of verticinone and imperialine were elucidated as 3β,20β-bis-(trimethylsilyl)verticinone and 3β,20β-(trimethylsilyl)imperialine, respectively.

Furthermore, since the intensity of molecular ions of verticine, verticinone and imperialine exhibited in the EI–MS spectra were very low, the derivatives of these three alkaloids were also analyzed by CI–MS. The corresponding protonated molecular ions $[M+H]^+$ at m/z 648 for verticine derivative (intensity 18.79%) and at m/z 574 for both verticinone (intensity 15.54%) and imperialine (intensity 22.10%) derivatives, respectively, were observed. Therefore, the identities of the derivatives of these three alkaloids were further confirmed.

3.5. Stability of the derivatives

Verticine and verticinone again were chosen for the assay of stability of the derivatives. The amounts of the derivatives of these two alkaloids determined by GC did not change when the samples were kept under controlled moisture at room temperature for 20 h after derivatization.

3.6. Calibration Curves

The standard curves for all five alkaloids showed good linearity within the test ranges. The statistical relationships, test ranges and other details of the calibration curves of each analyte are summarised in Table 1.

3.7. Validation of the assay

The accuracy of the determination of five alkaloidal derivatives was higher than 94% and 96% for intra- and inter-day assays, respectively. Furthermore, the intra-day variation was between 1.8% and 3.8%, and the inter-day variation was between 1.0% and 4.1%, respectively (Table 2). The results indicated that the developed GC method is reproducible with a good accuracy.

3.8. Analysis of Beimu alkaloids from plant materials

Three commonly used Beimu in traditional Chinese medicine: Chuanbeimu (*F. cirrhosa*), Zhebeimu (*F. thunbergii*) and Yibeimu (*F. pallidiflora*), were analyzed. As shown in Table 3, the established GC analytical method successfully applied for a simultaneous determination of the five biologically active *Fritillaria* alkaloids in different Beimu species. All five alkaloids were detected in *F. cirrhosa*, although their quantities were very low from trace amount to 24.8 μg/g of the dried herb. For *F. thunbergii*, verticine and verticinone were found as the major components accounting for 1.015 mg/g and 0.708 mg/g of the dried herb, respectively. While relatively lower contents of ebeiedine (0.062 mg/g) and ebeiedinone (0.148 mg/g) were determined in this herb. In the case of *F. pallidiflora*, imperialine was found as a major component (0.750 mg/g), whereas the other four alkaloids were not detected under the present conditions (Table 3).

Table 1
Calibration curve date for the quantification of ebeiedine, ebeiedinone, verticine, verticinone and imperialine

Analyte	R_t (min)	Standard curve (r^2)[a]	Test range (μg/ml)	Test limit (μg/ml)
Ebeiedine derivative	17.37	$Y = 0.0441x - 0.0190$ (0.9990)	19.4–582.0	0.97
Ebeiedinone derivative	23.16	$Y = 0.0296x - 0.0610$ (0.9991)	19.8–594.0	0.99
Verticine derivative	24.91	$Y = 0.0459x - 0.0360$ (0.9990)	17.6–528.0	0.88
Verticinone derivative	28.82	$Y = 0.0408x - 0.0103$ (0.9994)	24.0–720.0	1.20
Imperialine derivative	31.66	$Y = 0.0378x - 0.0949$ (0.9992)	24.4–732.0	1.22

[a] Y: peak area ratio (analyte:internal standard); x: concentration of analyte (μg/ml)

Table 2
Intra- and inter-day variability for the assay of *Fritillaria* alkaloids

Alkaloids added (μg/ml)	Intra-day variability			Inter-day variability		
	Detected (μg/ml, $n=3$)	RSD (%)[a]	Accuracy (%)[b]	Detected (μg/ml, $n=3$)	RSD (%)[a]	Accuracy (%)[b]
Ebeiedine 77.60	82.47±1.51	1.8	93.7	80.64±2.40	2.9	96.1
Ebeiedinone 79.20	75.27±2.41	3.2	95.0	76.66±1.21	1.6	96.8
Verticine 70.40	69.79±1.86	2.7	99.1	70.80±1.24	1.8	99.4
Verticinone 96.00	95.65±1.01	1.1	96.6	96.20±0.98	1.0	99.8
Imperialine 97.60	92.11±3.48	3.8	94.4	95.53±3.92	4.1	97.9

[a] RSD (%) (relative standard deviation)=(SD/mean)×100.
[b] Accuracy (%)=[1−(mean concentration measured−concentration spiked)/concentration spiked]×100.

In the practice of traditional Chinese medicine, Chuanbeimu, Zhebeimu and Yibeimu are used in different cough status regarding to their potencies [20], variations of the major pharmacologically active alkaloids in three different Beimus may be responsible for the different potencies of these medicinal herbs. However, the relationship between quantity and type of active *Fritillaria* alkaloids and their pharmacological activities needs to be clarified. The presently established precolumn derivatization GC analytical method for *Fritillaria* alkaloids is compatible with a previously developed HPLC assay by our research team [12]. It also provides an alternative analytical method for the quantification of the major active ingredients in Beimu, a most popularly utilised antitussive traditional Chinese medicine. Further studies on the quantitative determinations of the major alkaloids in other commonly used *Fritillaria* species and in biological samples by using the presently developed GC method are currently in the progress in our laboratories.

4. Conclusions

Results obtained from the present study showed that the developed GC analytical method with precolumn derivatization is a very simple, sensitive and

Table 3
Contents of ebeiedine, ebeiedinone, verticine, verticinone and imperialine in three *Fritillaria* species

Alkaloid	Derivatized hydroxyl group	*F. cirrhosa*	*F. thunbergii* (μg/g of dried herb)	*F. pallidiflora*
Ebeiedine	3β, 6β	trace[a]	62.12±3.00	nd
Ebeiedinone	3β	19.15±1.43	147.83±2.24	nd
Verticine	3β, 6α, 20β	26.10±2.02	1015.61±6.01	nd
Verticinone	3β, 20β	20.48±0.61	708.14±13.61	nd
Imperialine	3β, 20β	24.80±2.84	nd[a]	749.90±15.33

[a] trace: content of the alkaloid was lower than the test limit. nd: not detected.

reproducible technique for the determination of the major biologically active alkaloids in *Fritillaria* species. The established GC method can be readily utilised for a simultaneous quantification of the five major biologically active *Fritillaria* alkaloids in clinically used Beimu species.

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