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Review

Chromatographic analysis of *Fritillaria* isosteroidal alkaloids, the active ingredients of Beimu, the antitussive traditional Chinese medicinal herb

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Abstract

Bulbus Fritillariae derived from plants of various *Fritillaria* species is the most commonly used antitussive traditional Chinese medicinal herb and is called Beimu. Herbs derived from similar and/or different species of *Fritillaria* are also used in Japan and Turkey as traditional or folk medicines. Isosteroidal alkaloids are the main bioactive ingredients in *Fritillaria* species. As the contents and structure types of these bioactive alkaloids vary in different *Fritillaria* species, quality control of these active principles in herbal Beimu is very important to ensure its safe and effective clinical use. This review describes the development of chromatographic analyses for the simultaneous qualitative and quantitative determination of the main bioactive *Fritillaria* isosteroidal alkaloids in herbal and biological samples. The recently developed direct HPLC–evaporative light scattering detection method is the most simple, selective and sensitive assay, and is readily used as a suitable quality control method for the analysis of the active principles of herbal Beimu.

Keywords: Reviews; *Fritillaria* spp.; Pharmaceutical analysis; Alkaloids

Contents

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1. Introduction

1.1. Beimu (Bulbus Fritillariae)

Fritillaria is one of the largest genera in the plant family of *Liliaceae*. To date, about 130 species of *Fritillaria* have been identified worldwide. Until 1980, 20 species and two varieties of *Fritillaria* had been reported in China [1], and many new species of this genus have been discovered since then [2,3]. Many species of *Fritillaria* were traditionally used as herbal remedies in Japanese [4,5] and Turkish [6] folk medicines, while bulbs of many *Fritillaria* species growing in China have been used as antitussive and expectorant herbs using the Chinese name ''Beimu'' in traditional Chinese medicine (TCM) for more than 2000 years [7]. Officially, herbal Beimu is derived from the bulbs of nine *Fritillaria* species documented in China Pharmacopoeia (Year 2000 edition). These species include *Fritillariathunbergii* Miq., *F. cirrhosa* D. Don., *F. unibracteata* Hiao et Hsia, *F. przewalskii* Maxim ex Batal, *F. delavayi* Franch, *F. ussuriensis* Maxim., *F. walujewii*, *F. pallidiflora* Schrenk and *F. hupehensis* Hsiao et K.C. Hsia [8]. Amongst them, *F. hupehensis* is a species newly introduced in the Year 2000 edition of China Pharmacopoeia [8]. Furthermore, bulbs of some other *Fritillaria* species are often used as the plant sources for Beimu in different local regions in mainland China as the Chinese folk medicine [7]. For thousands of years of tradition in TCM practice till now, Beimu has been the most commonly used antitussive TCM herb.

Extensive chemical studies on various *Fritillaria* species, especially on the TCM herbal Beimu, have been conducted by several research groups [9–15]. Till the end of 1999, approximately 140 compounds have been isolated from 35 species of *Fritillaria* genus, and the types and quantities of these components present in different species vary significantly [10,16–21]. Amongst all compounds found from different *Fritillaria* species, the majority (72.7%) belong to isosteroidal alkaloids, while the rest are steroidal alkaloids (11.5%) and non-alkaloids (15.8%). Furthermore, pharmacological studies of various Beimu extracts and pure compounds isolated from different *Fritillaria* species have also been performed, and the results demonstrated that the major isosteroidal alkaloids present in different *Fritillaria* species are the primary active ingredients responsible for the antitussive activity [22–27]. Therefore, the development of quality control methods for both qualitative and quantitative determinations of the major active *Fritillaria* isosteroidal alkaloids in herbal Beimu is an essential issue for the effective and safe clinical use of this traditional herb.

1.2. Major active Fritillaria isosteroidal alkaloids

Based on the fundamental molecular moiety *Fritillaria* isosteroidal alkaloids are classified into three types: cevanine type, jervinine type and veratramine type. The structures of alkaloids in each of these three types correspond to cevane, jervine and veratraman, respectively. Cevanine type alkaloids are predominant in all isosteroidal alkaloids identified from genus of *Fritillaria* [28,29]. The representative alkaloids for each type are shown in Fig. 1. In the nine *Fritillaria* species officially used in China as the plant sources for Beimu, various compounds have been identified and the common major principles are seven cevanine type isosteroidal alkaloids namely

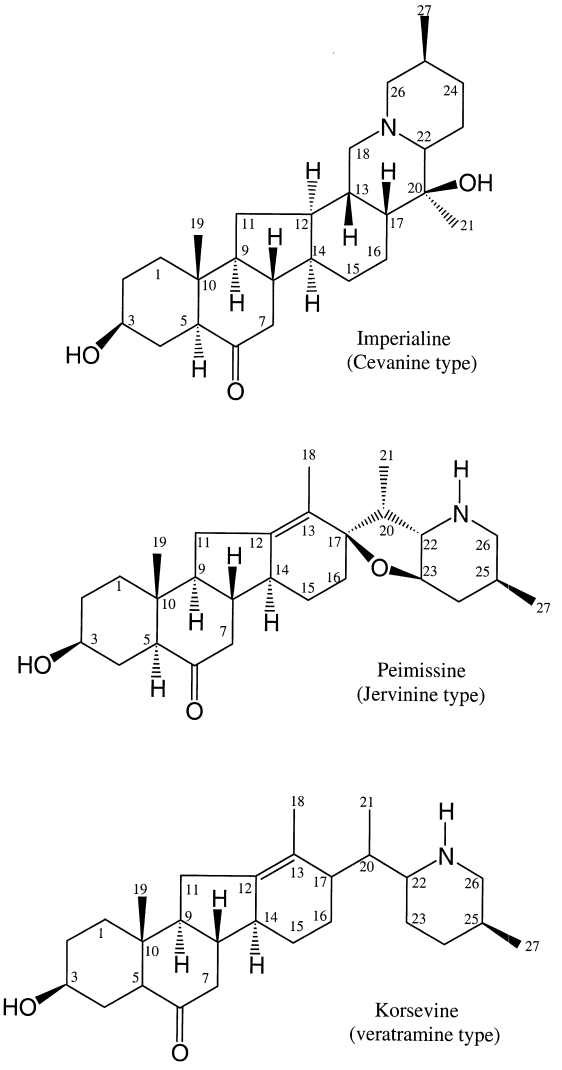


Fig. 1. Representatives of three types of *Fritillaria* isosteroidal alkaloids.

imperialine, verticine, verticinone, isoverticine, ebeiedine, ebeiedinone, and ebeienine, plus one jervinine type isosteroidal alkaloid peimissine [16–21,30–32]. In addition, hupehenine, a cevanine type isosteroidal alkaloid, has only been identified in *F. hupehensis* and is one of the main ingredients in this species [26]. Structures of these seven major ingredients plus peimissine and hupehenine are illustrated in Table 1. Furthermore, the plant species from which these major ingredients were found are also shown in Table 1.

Table 1
Structures of the main *Fritillaria* isosteroidal alkaloids and some of *Fritillaria* species from which these alkaloids were identified

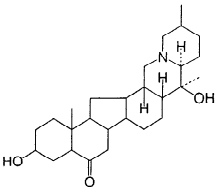
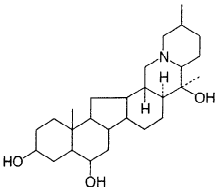
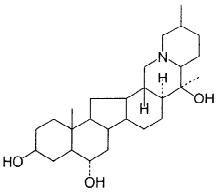
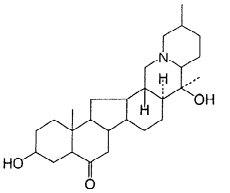
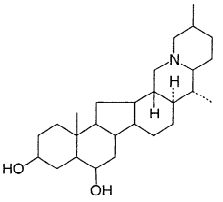
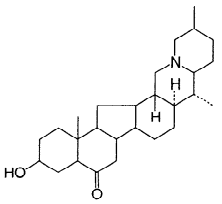
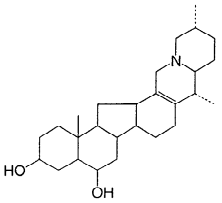
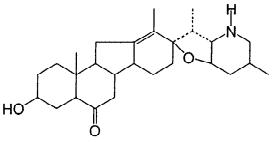
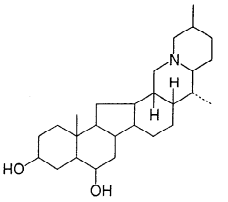
Alkaloid	Plant sources
 Imperialine (Sipeimine) $C_{27}H_{43}NO_3$ (61825-98-7)	*F. ussuriensis* [57] *F. pallidiflora* [22] *F. wabuensis* [58] *F. delavayi* [59] *F. walujewii* [60] *F. taipaiensis var. ningxiaensis* [61] *F. cirrhosa* [62]
 Isoverticine $C_{27}H_{45}NO_3$ (23496-43-7)	*F. thunbergii* [63,64] *F. ebeiensis var. purpurea* [65] *F. thunbergii var. chekiangensi* [64] *F. ningguoensis* [66] *F. wabuensis* [58] *F. anhuiensis* [67] *F. taipaiensis var. ningxiaensi* [61]
 Verticine (Peimine) $C_{27}H_{45}NO_3$ (23496-41-5)	*F. thunbergii* [68] *F. ussuriensis* [69] *F. hupehensis* [70] *F. ebeiensis var. purpurea* [26] *F. thunbergii var. chekiangensis* [64] *F. ningguoensis* [66] *F. monantha* [71] *F. wuyangensis* [72]
 Verticinone (Peiminine) $C_{27}H_{43}NO_3$ (18059-10-4)	*F. thunbergii* [68] *F. hupehensis* [70] *F. ebeiensis var. purpurea* [26] *F. thunbergii var. chekiangensis* [64] *F. ningguoensis* [66] *F. wuyangensis* [72] *F. anhuiensis* [73] *F. taipaiensis var. ningxiaensis* [74] *F. monantha* [71]
 Ebeiedine $C_{27}H_{45}NO_2$ (25650-70-8)	*F. ebeiensis* [75] *F. ebeiensis var. purpurea* [65]

Table 1. Continued

Alkaloid	Plant sources
	*F. ebeiensis* [75]
Ebeiedinone $C_{27}H_{43}NO_2$ (25650-68-4)	*F. ebeiensis var. purpurea* [65]
	*F. ebeiensis var. purpurea* [21]
Ebeienine $C_{27}H_{43}NO_2$ (123116-14-3)	
	*F. ussuriensis* [76] *F. ningguoensis* [66] *F. wabuensis* [58] *F. taipaiensis var. ningxiaensis* [74] *F. anhuiensis* [73] *F. dajinensis* [51]
Peimissine $C_{27}H_{41}NO_3$ (19773-24-1)	*F. mellea* [51] *F. monantha* [71]
	*F. hupehensis* [26,77]
Hupehenine $C_{27}H_{45}NO_2$ (98243-57-3)	

The CA (Chemical Abstract) registry number for each compound is presented in parentheses.

The pharmacological studies of these major alkaloids present in nine *Fritillaria* species officially used as the plant sources for Beimu have demonstrated that except ebeienine and hupehenine, all other six cevanine-type isosteroidal alkaloids produce antitussive activity in both in vitro and in vivo animal models [22–27], although the potency of these alkaloids varies. For example, a recent in vitro study of tracheal and bronchial relaxation effects of four main bioactive isosteroidal alkaloids carried out by our research team suggested that the rank order of potency was imperialine>verticine≥verticinone> ebeiedine [22]. As shown in Table 1, in seven main cevanine-type isosteroidal alkaloids, only ebeienine contains a double bond in piperidine ring, which may lead to its stereo configuration and biological activity being significantly different from other saturated cevanine-type isosteroidal alkaloids. However, there are no published data reporting the pharmacological activity of ebeienine. Furthermore, biological activities of hupehenine, which is only present in one *Fritillaria* species: *F. hupehensis*, and peimissine, the jervinine-type isosteroidal alkaloid, have not been reported. Therefore, saturated cevanine-type isosteroidal alkaloids are generally recognized as the primary antitussive active principles present in herbal Beimu, and most of the analytical methods have been developed for the analysis of these active ingredients [30–34].

Fritillaria isosteroidal alkaloids lack conjugated unsaturation and thus they do not display strong ultraviolet (UV) absorption. This structural property of *Fritillaria* isosteroidal alkaloids obscures the use of liquid chromatographic techniques coupled with conventional UV detection for common quality control analysis of these herbal medicines. Furthermore, except peimissine and ebeiedinone, all seven major *Fritillaria* isosteroidal alkaloids have at least two hydroxyl groups. The substituted hydroxyl groups increase the polarity of these alkaloids and may also generate internal hydrogen bonds if these hydroxyl groups substitute in suitable positions. Thus, this type of isosteroidal alkaloid is also difficult to elute from the conventional GC column in gas chromatographic analysis. Consequently, the development of appropriate analytical methods for the quality control of *Fritillaria* isosteroidal alkaloids in herbal Beimu has long been a challenge to scientists. Recently, several analytical methods using different chromatographic techniques have been developed mainly by our research team and other groups for the analysis of various *Fritillaria* isosteroidal alkaloids. This review focuses on the development of chromatographic methods for the simultaneous qualitative and quantitative analysis of the major pharmacologically active *Fritillaria* isosteroidal alkaloids.

2. Thin-layer chromatography scanning

TLC scanning is the first analytical method developed for qualitative and quantitative determination of the major *Fritillaria* isosteroidal alkaloids in herbal Beimu. Various TLC scanning methods using silica gel normal-phase TLC plate with different solvent developing systems have been established in the 1980s and early 1990s [35–44]. Three commonly used mobile phase systems for different *Fritillaria* species are: ethyl acetate–methanol–ammonium hydroxide (17:2:1) [35,36], benzene or cyclohexane–ethyl acetate–diethylamine (6:4:1) [37–42] and diethyl ether–ethanol (100:3) saturated with ammonia vapour, respectively [39,43,44]. For the detection of *Fritillaria* isosteroidal alkaloids, Dragendorff reagent is firstly sprayed on the developed TLC plates, and colour spots generated are then scanned by a double beam scanner with sample wavelengths at 495–540 nm and reference wavelengths at 600–650 nm. Seven cevanine-type isosteroidal alkaloids, including verticine and verticinone [39–41,43–45], isoverticine [39], imperialine [35–37,41,42], hupehenine [38], wanpeinine A [40], and chuanbeinone [39,42], have been reported to be quantified individually by various TLC scanning methods. However, most of the reported TLC scanning methods were not able to simultaneously determine all major *Fritillaria* isosteroidal alkaloids in Beimu extracts. The poor separation is mainly due to the structural similarity of the major *Fritillaria* isosteroidal alkaloids, since several alkaloids present in Beimu are isomers or stereoisomers and unlikely to be well-separated using a single mobile phase composition on a one-dimensional TLC plate. Furthermore, due to a general limitation of relatively low sensitivity and poor reproducibility in TLC scanning quantification, apparently the TLC scanning technique should not be a suitable quality control method for a simultaneous quantitative determination of the major active ingredients in herbal Beimu.

3. Gas chromatography

3.1. Pre-column derivatization gas chromatography

GC analytical methods for the determination of the major active *Fritillaria* isosteroidal alkaloids have not been reported until recently when a pre-column derivatization with GC analysis was developed by our research team [33]. Since, except for ebeiedinone, all the major active *Fritillaria* isosteroidal alkaloids contain at least two polar hydroxyl groups and are not resolved well by conventional GC columns, derivatization was introduced to solve the problem by producing the corresponding less polar derivatives prior to column separation.

3.1.1. Pre-column derivatization

In order to achieve a reproducible derivatization, the selection of an appropriate derivatizing reagent and optimizing the reaction conditions are critical. In the developed method [33], trimethylsilylation and acetylation, the two most commonly used derivative reactions for the analytes containing hydroxyl groups, were examined using various derivatizing reagents at different reaction conditions. Amongst six derivatizing reagents tested, namely *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, trifluoroacetamide, bis(trimethylsilyl)acetamide, trimethylchlorosilane, bis(trimethylsilyl)trifluoroacetamide, and trimethylsilylimidazole (TMSI), derivatization with TMSI resulted in a rapid and completed trimethylsilylation of all hydroxyl groups in each isosteroidal alkaloid tested. Furthermore, excess amounts of derivatizing reagent were required for the completion of reactions of all alkaloids present in the herbal extract. Subsequently, the optimal derivatizing condition was obtained by reaction of the analytes present in Beimu extract with at least a 2.5-fold molar excess of TMSI at 40°C for 20 min. Aliquots of the derivatized mixture were then directly subjected to GC analysis.

Characterization of the derivatives was performed by on-line GC–MS analysis using both electron impact (EI) and chemical ionization interfaces. The mass spectra (Fig. 2) of the derivatives of all *Fritillaria* isosteroidal alkaloids analyzed confirmed that all hydroxyl groups in each alkaloid were converted to *O*-trimethylsilylate. Therefore, as illustrated in Fig. 3, depending on the structure of each individual *Fritillaria* isosteroidal alkaloid, trimethylsilylation of either one, two or three hydroxyl groups occurred in the corresponding trimethylsilyl (TMS) derivatives. The results demonstrated a complete *O*-trimethylsilylation of all *Fritillaria* isosteroidal

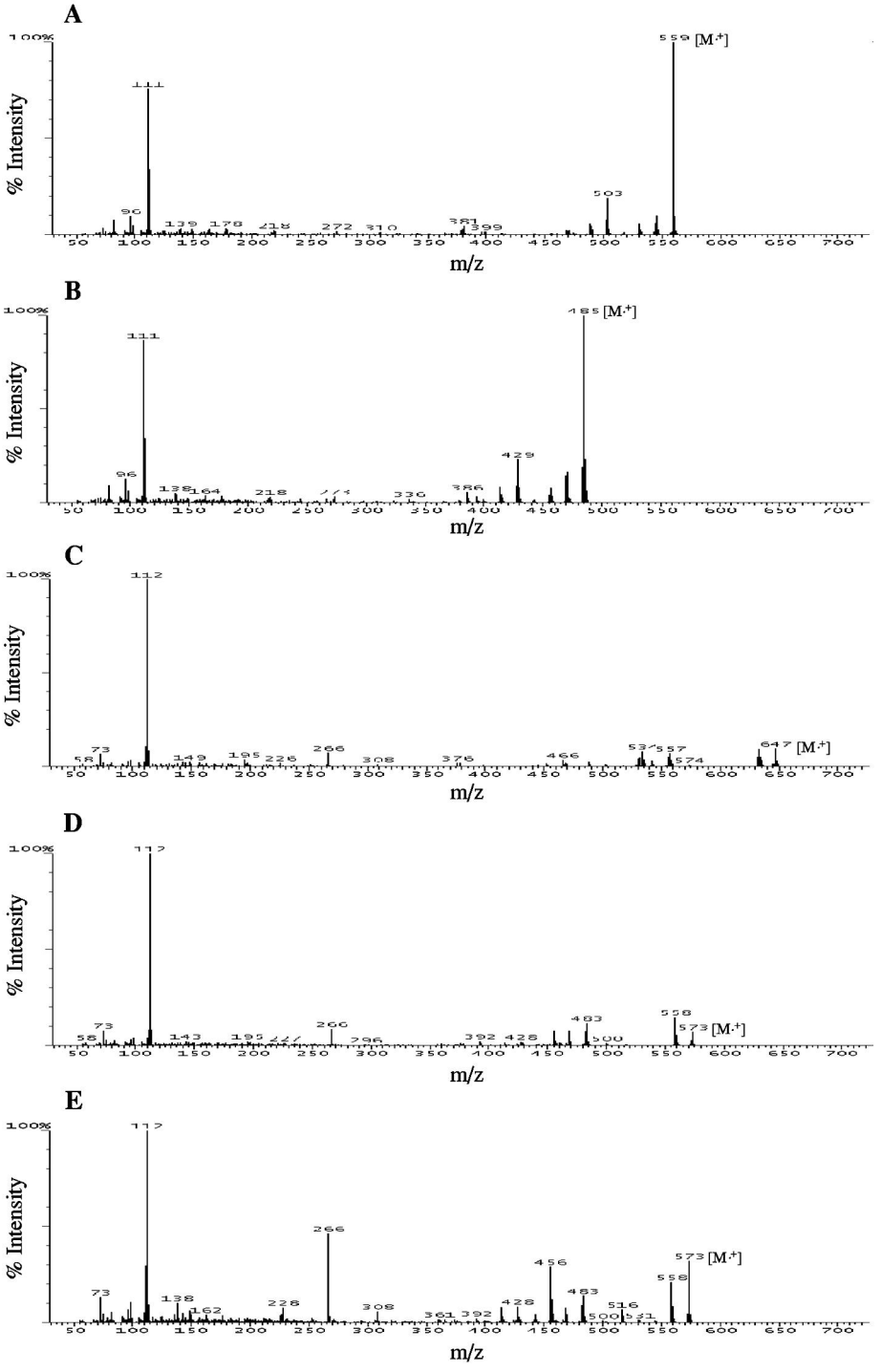
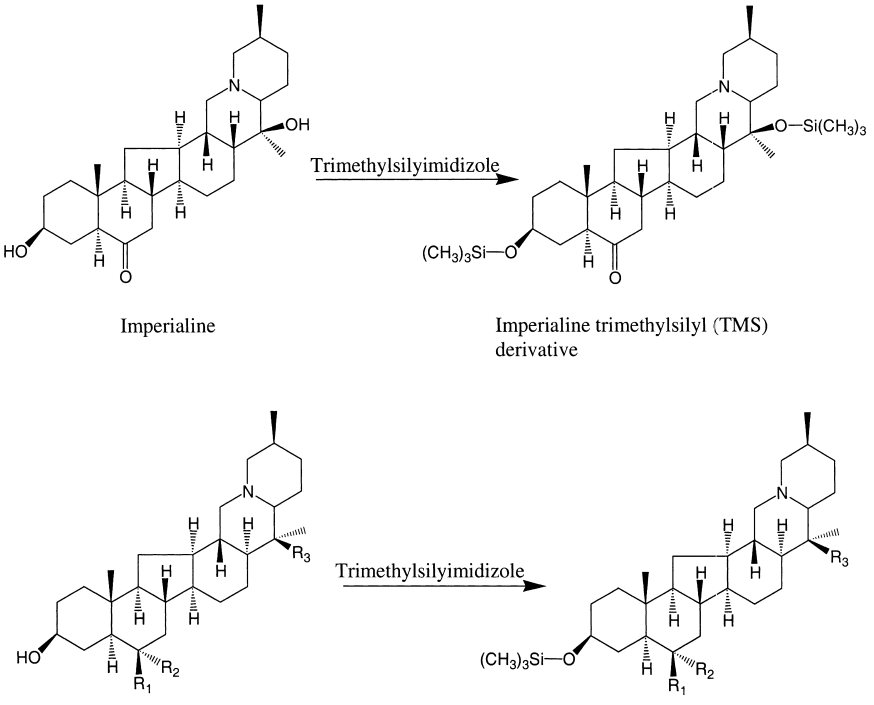


Fig. 2. EI-MS for trimethylsilyl derivatives of ebeiedine (A), ebeiedinone (B), verticine (C), verticinone (D) and imperialine (E). (From reference [33] with permission from Elsevier Science).



	R_1	R_2	R_3		R_1	R_2	R_3
Verticine:	H	OH	OH	Verticine TMS derivative:	H	O-Si$(CH_3)_3$	O-Si$(CH_3)_3$
Verticinone:	R_1R_2=O		OH	Verticinone TMS derivative:	R_1R_2=O		O-Si$(CH_3)_3$
Ebeiedine:	OH	H	H	Ebeiedine TMS derivative:	O-Si$(CH_3)_3$	H	H
Ebeiedinone:	R_1R_2=O		H	Ebeiedinone TMS derivative:	R_1R_2=O		H

Fig. 3. Pre-column derivatization of five major bioactive *Fritillaria* isosteroidal alkaloids with trimethylsilylimidazole.

alkaloids tested, thus the reproducible yields and production of identical derivatives in this pre-column derivatization were well controlled.

3.1.2. Gas chromatography

The resultant TMS derivatives should be resolved well by varieties of packed and capillary GC columns. In the reported method [33], amongst various GC columns examined, such as packed columns: Dexsil 300 (0.5 m×2 mm I.D.), OV-7 (2 m×2 mm I.D.) and OV-1 (2 m×2 mm I.D.), and capillary columns: OV-1 (12 m×0.53 mm I.D.), OV-17 (15 m×0.53 mm I.D.) and SE-54 (15 m×0.53 mm I.D.), the OV-1 capillary column was most suitable for the separation of the TMS derivatives of the major bioactive *Fritillaria* isosteroidal alkaloids. As illustrated in Fig. 4, the TMS derivatives of five isosteroid-

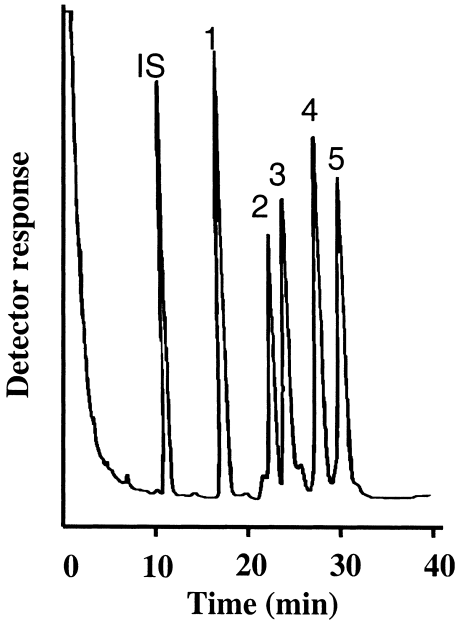


Fig. 4. Representative GC chromatogram of a derivatized mixture of five major bioactive *Fritillaria* isosteroidal alkaloids. An OV-1 (12 m×0.53 mm, 0.33 μm capillary column) was used. The injector and detector temperatures were set at 260°C. Column temperature was programmed from 210 to 245°C at 1°C/min and held at 210°C for 5 min. Hydrogen was used as carrier gas with a flow-rate of 35 ml/min. 1, Ebeiedine TMS derivative; 2, ebeiedinone TMS derivative; 3, verticine TMS derivative; 4, verticinone TMS derivative; 5, imperialine TMS derivative; IS, internal standard. (From reference [33] with permission from Elsevier Science).

al alkaloids namely verticine, verticinone, ebeiedine, ebeiedinone and imperialine, were separated well on the OV-1 capillary column using H_2 carrier gas with a gradient temperature program and flame ionization detection (FID). The limits of detection for all five alkaloids determined are summarized in Table 2, which provided adequate sensitivities for the analysis of all five major bioactive *Fritillaria* isosteroidal alkaloids present in different Beimu herbs.

The established pre-column derivatization GC–FID method was successfully applied by our research team to a simultaneous qualitative and quantitative determination of the five main bioactive *Fritillaria* isosteroidal alkaloids in 16 *Fritillaria* species collected from different regions in China [46]. The results demonstrated, for the first time, that both structural types and quantities of the major bioactive *Fritillaria* isosteroidal alkaloids present in different Beimu herbs were significantly influenced by the environments of plant growth [46,47].

3.2. Direct gas chromatography

Obviously inclusion of pre-column derivatization has several disadvantages, for example it is more time consuming and requires well-controlled reaction conditions to produce reproducible yields and identical derivatives for each analyte in the mixture. Therefore, our research team has further investigated

Table 2
Limits of detection for the different chromatographic analytical methods

Fritillaria alkaloids	Limit of detection (μg/g of dried herb)			
	GC–FID for trimethylsilylated derivatives	Direct GC–FID	HPLC–UV for 1-naphthoate derivatives	Direct HPLC–ELSD
Verticine	44.0	25.0[a]	83.3	35.0
Verticinone	60.0	25.0[a]	83.3	31.5
Ebeiedine	48.5	26.0	83.3	35.0
Ebeiedinone	49.5	26.0	83.3	–
Ebeienine	–	15.0	–	–
Isoverticine	–	22.0	83.3	31.5
Imperialine	61.0	21.5	–	35.0
Peimissine	–	–	–	38.5
Hupehenine	–	17.5	–	–

–, not applicable.
[a] Quantified as the sum of two alkaloids.

possible direct GC methods for the analysis of the bioactive *Fritillaria* isosteroidal alkaloids. With increases in various new commercially available GC columns, recently a direct GC–FID assay for the analysis of eight *Fritillaria* isosteroidal alkaloids was developed by our research team [30]. In this direct GC study, two commercially available capillary columns were examined, namely the Supelco SAC-5 column (30 m×0.25 mm I.D.) specifically designed for the analysis of steroids, and the HP-1 column (12.5 m×0.22 mm I.D.) for compounds with hydroxyl groups. The results demonstrated that the Supelco SAC-5 capillary column was more suitable for the separation of main *Fritillaria* isosteroidal alkaloids. As illustrated in Fig. 5, although verticine and verticinone co-eluted, the other six isosteroidal alkaloids, including ebeiedine, ebeiedinone, ebeienine, isoverticine, imperialine, and hupehenine, were resolved well with base-line separations using N_2 as a carrier gas and an isocratic oven temperature at 295°C.

The developed direct GC–FID method enabled a simultaneous analysis of seven major *Fritillaria* isosteroidal alkaloids namely ebeiedine, ebeiedinone, ebeienine, isoverticine, verticine, verticinone and imperialine, plus hupehenine, the major isosteroidal alkaloid found in *F. hupehensis* only, although contents of verticine and verticinone were determined as a sum of these two alkaloids [30]. The limits of detection for all alkaloids analyzed are also listed in Table 2, which were markedly improved compared with those obtained by pre-column derivatization GC analysis [33]. Apparently, direct GC analysis has several advantages over pre-column derivatization GC method. For example, the direct GC assay is much more simple, less time consuming and easy to control for providing good reproducibility and accuracy.

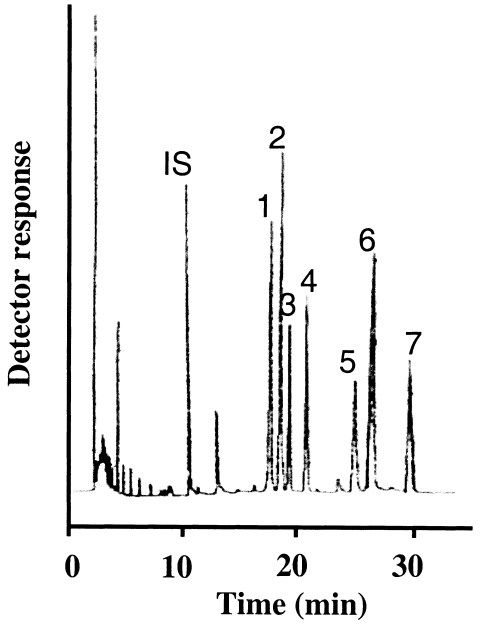


Fig. 5. Representative GC chromatogram of a mixture of eight main *Fritillaria* isosteroidal alkaloids. An SAC-5 (30 m×0.25 mm, 0.25 μm capillary column) was used. The injector and detector temperatures were set at 310°C. Column temperature was set at 295°C. Nitrogen was used as carrier gas with a flow-rate of 30 ml/min. 1, Ebeiedine; 2, ebeiedinone; 3, ebeienine; 4, hupehenine; 5, isoverticine; 6, verticine and verticinone; 7, imperialine; IS, internal standard.

4. High-performance liquid chromatography

4.1. Pre-column derivatization high-performance liquid chromatography

As described in the Introduction, most of the *Fritillaria* isosteroidal alkaloids do not contain a strong chromophore for UV absorption, which limits the sensitivity and selectivity of conventional HPLC–UV analysis for the determination of such alkaloids. Therefore, ''adding'' UV-absorbing chromophore(s) via a pre-column derivatization of these isosteroidal alkaloids appeared a useful technique for HPLC analysis coupled with a common multiple wavelength UV detector or photo diode-array detector. Recently, a few pre-column derivatization HPLC methods have been developed for the determination of the major bioactive *Fritillaria* isosteroidal alkaloids in both Beimu extracts [48,49] and biological samples obtained from laboratory animals treated with these alkaloids [50].

4.1.1. Pre-column derivatization

The first pre-column derivatization HPLC–UV

method was developed by our research team in 1996, and five major bioactive *Fritillaria* isosteroidal alkaloids present in extracts of different *Fritillaria* species were simultaneously identified and quantified by this method [34]. The pre-column derivatization was conducted via esterification of hydroxyl groups in the alkaloids using 1-naphthoyl chloride with thionyl chloride as a catalyst. UV-absorbing 1-naphthoate group(s) were introduced to the secondary hydroxyl groups in each isosteroidal alkaloid (Fig. 6), thus the resultant derivatives could be detected by a conventional UV detector. In order to obtain an optimal derivatizing condition, a systematic evaluation of variables of different catalysts, derivatizing reagent concentrations, reaction durations, and reaction temperatures was performed. The optimal derivatization was achieved by heating the analytes in the extracts with approximately 20-fold

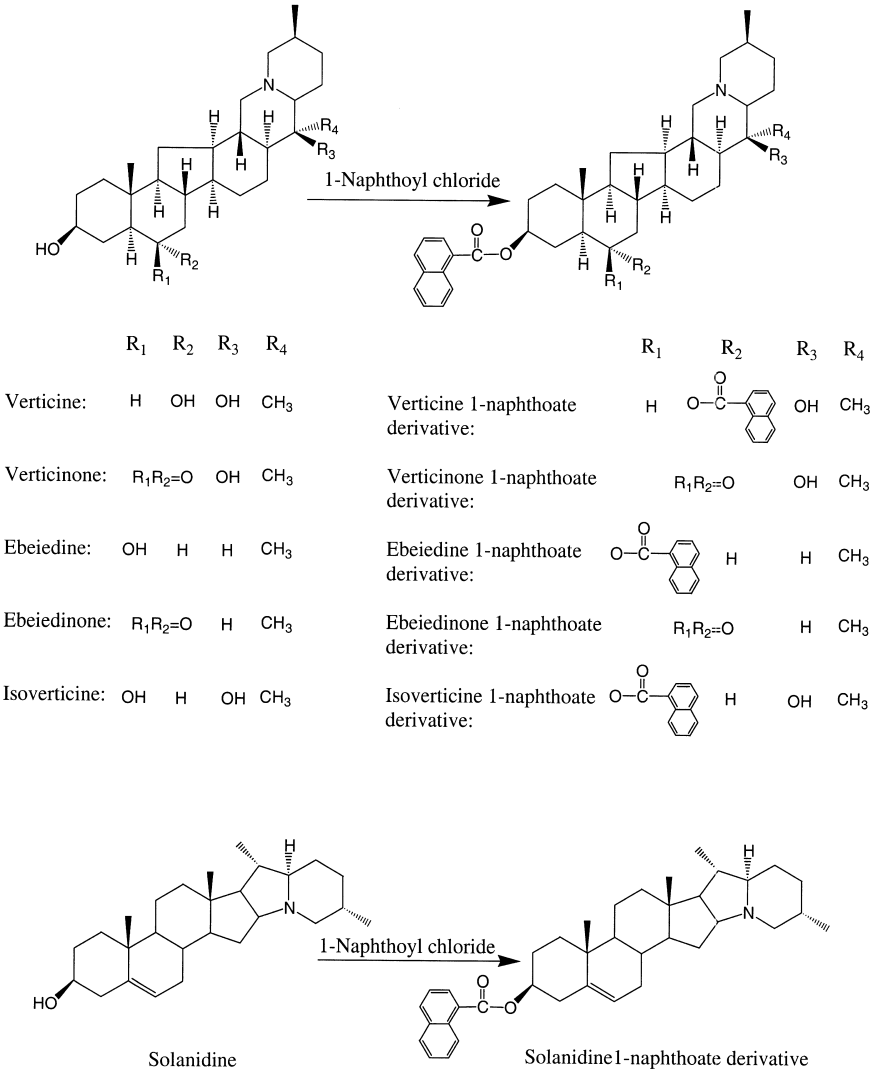


Fig. 6. Pre-column derivatization of five major bioactive *Fritillaria* isosteroidal alkaloids and the internal standard, solanidine, with 1-naphthoyl chloride.

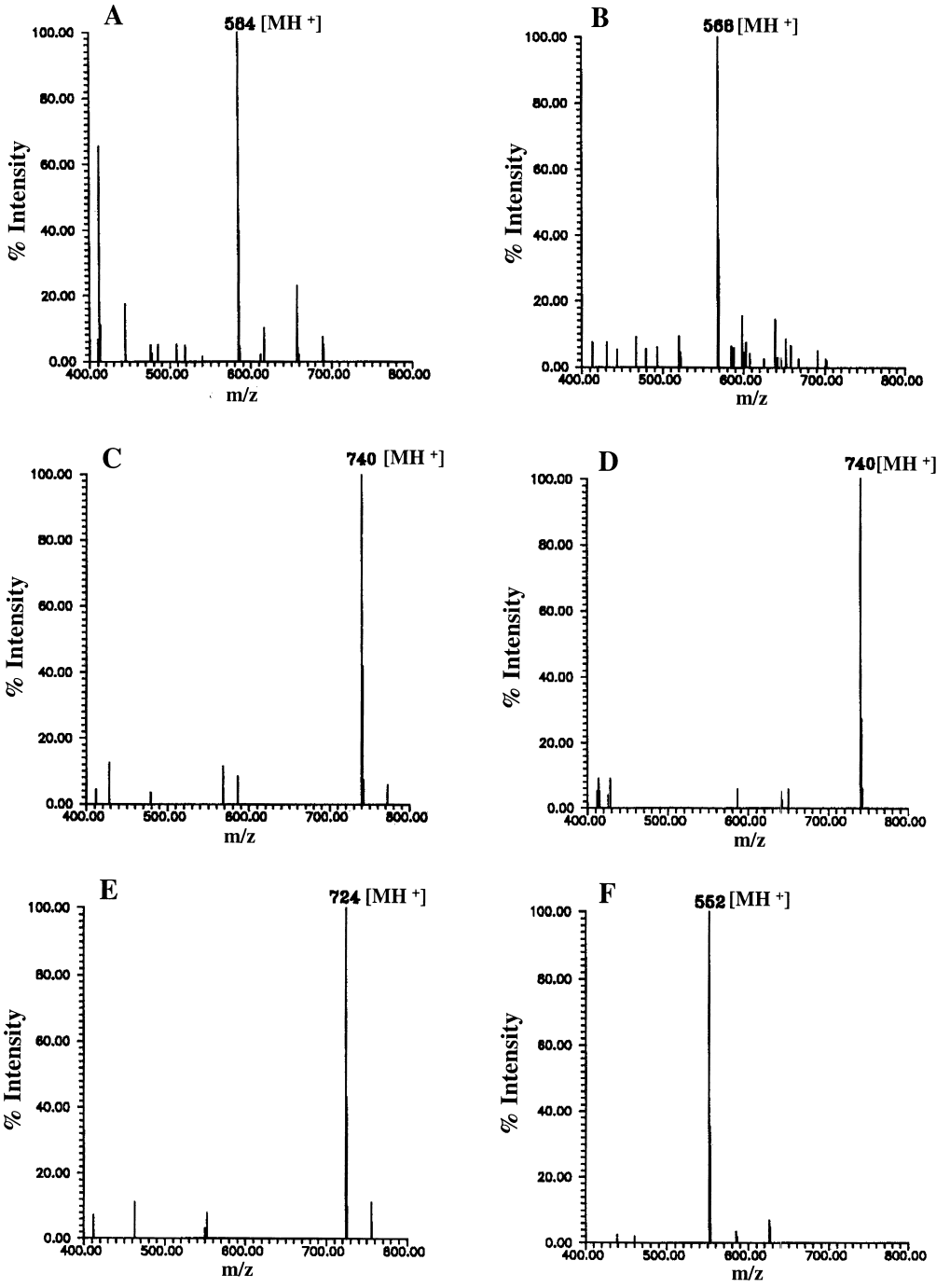


Fig. 7. ESI-MS for 1-naphthoate derivatives of verticinone (A), ebeiedinone (B), isoverticine (C), verticine (D), ebeiedine (E) and solanidine (F).

excess of 1-naphthoyl chloride in anhydrous acetonitrile at 88°C for 2 h in the presence of the catalyst thionyl chloride.

1-Naphthoate derivatives were identified by on-line HPLC–MS using an electrospray ionization (ESI) interface. The mass spectrometric data (Fig. 7) exhibited that 1-naphthoation only reacted on the secondary hydroxyl group, whereas the tertiary hydroxyl group was intact in all isosteroidal alkaloids examined (Fig. 6). In addition, the internal standard solanidine was also quantitatively derivatized in the pre-column derivatization. The mass spectrum (Fig. 7) suggested 1-naphthoation of the secondary hydroxyl group at the 3 position of solanidine as showed in Fig. 6. The yield of derivatization for each alkaloid and the internal standard proved to be quantitative and reproducible under the optimal reaction condition. However, the derivatized mixtures should be analyzed within 24 h due to a gradual degradation of 1-naphthoate derivatives occurring at 24 h after termination of the derivatization.

Using benzoyl chloride as a derivatizing reagent, Li et al. [49] established another pre-column derivatization technique for the HPLC analysis of imperialine (also called sipeimine) in bulbs of *F. pallidiflora*. The optimal derivatization was performed by reacting imperialine with an excess of benzoyl chloride in anhydrous pyridine at ambient temperature for 12 h. Direct probe mass spectrometric analysis of the derivative confirmed that esterification occurred on the secondary hydroxyl group of imperialine to form the corresponding UV-absorbing benzoate ester as illustrated in Fig. 8. Benzoate derivative was demonstrated to be stable for 72 h at 4°C.

Recently, a new pre-column derivatization HPLC assay for the pharmacokinetic study of verticinone (also named as peiminine) in mice was reported by Zhang et al. [50]. In this newly developed pre-column derivatization method, the ketone group in verticinone was converted to a strong UV-absorbing 2,4-dinitrophenylhydrazone group via reaction with 2,4-dinitrophenylhydrazine as shown in Fig. 9. Similarly, derivatizing conditions were optimized and the optimal reaction was conducted by heating plasma extract with an excess of 2,4-dinitrophenylhydrazine under acidic conditions (pH 2, adjusted with 2 *M* HCl) at 50°C for 30 min.

4.1.2. Analysis by high-performance liquid chromatography with UV detection

Without further extraction, all derivatized mixtures produced by the three different pre-column derivatization techniques described above were directly subjected to HPLC analysis. For the 1-naphthoate derivatives [34], the derivatives of the five main bioactive *Fritillaria* isosteroidal alkaloids were resolved well on a Nova-Pak C_{18} reversed-phase column (150×3.9 mm I.D., 4 μm) using a simple isocratic mobile phase of methanol containing 0.2% diethylamine, and detected at 224 nm (Fig. 10). However, under the developed condition, peaks 1 and 2 corresponding to verticinone 1-naphthoate derivative and ebeiedinone 1-naphthoate derivative were eluted in the tail of the front solvent peak. This HPLC condition needs to be further modified for the

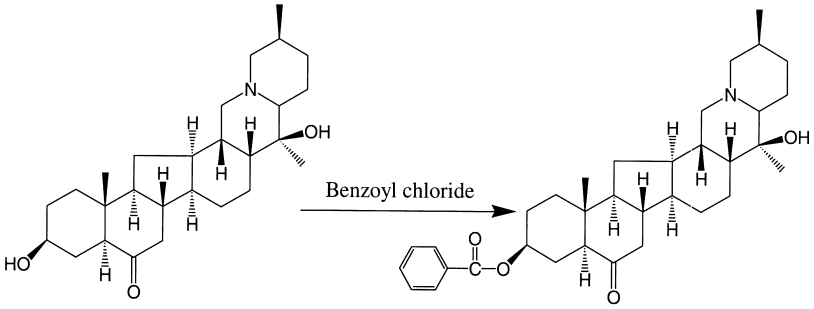


Fig. 8. Pre-column derivatization of imperialine with benzoyl chloride.

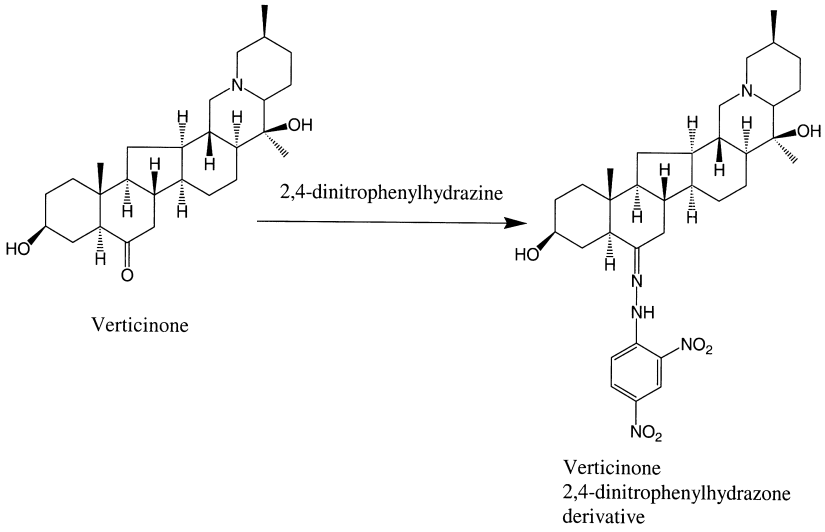


Fig. 9. Pre-column derivatization of verticinone with 2,4-dinitrophenylhydrazine.

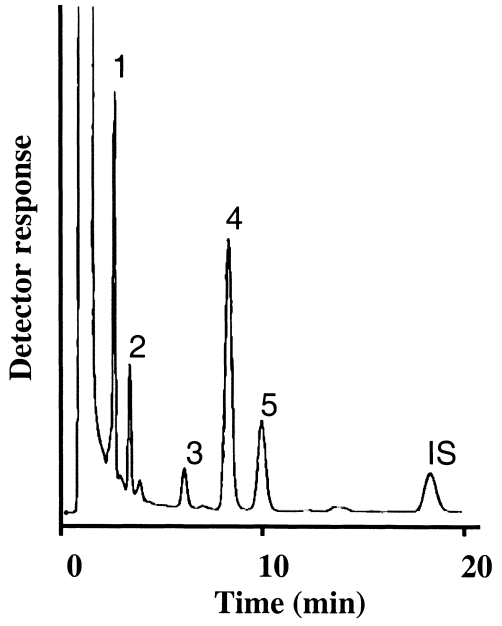


Fig. 10. Representative HPLC–UV chromatogram of a derivatized mixture of five major bioactive *Fritillaria* isosteroidal alkaloids. A Nova-Pak C_{18} column (150×3.9 mm I.D., 4 μm) was used. The mobile phase consisted of methanol containing 0.2% diethylamine with a flow-rate of 1.2 ml/min. The peaks were detected at 224 nm. 1, Verticinone 1-naphthoate derivative; 2, ebeiedinone 1-naphthoate derivative; 3, isoverticine 1-naphthoate derivative; 4, verticine 1-naphthoate derivative; 5, ebeiedine 1-naphthoate derivative; IS, internal standard.

base-line separation of all major active *Fritillaria* isosteroidal alkaloids in Beimu extracts. Nevertheless, the method developed was successfully applied to a simultaneous qualitative and quantitative analysis of five major bioactive *Fritillaria* isosteroidal alkaloids, including verticine, verticinone, isoverticine, ebeiedine and ebeiedinone, in the crude extracts of different *Fritillaria* species [34]. The limit of detection for all five alkaloids was 83.3 μg/g of the dried herbal extract, which is relatively less sensitive than those provided by three other chromatographic methods summarized in Table 2. More discussions on the relatively high values of the limits of detection are described in Section 4.2.2.

In the case of benzoate derivative of imperialine [49], the HPLC–UV determination was conducted on a reversed-phase C_{18} column (250×4 mm I.D., 5 μm) eluted with the mobile phase methanol–water (90:10, v/v) containing 0.4% triethylamine. The benzoate derivative of imperialine was detected at its maximum absorption wavelength at 227 nm. The developed method was used to analyze the most potent bioactive *Fritillaria* isosteroidal alkaloid imperialine in bulbs of *F. pallidiflora*; however, other bioactive isosteroidal alkaloids were not analyzed.

The 2,4-dinitrophenylhydrazone derivative of verticinone was also analyzed with a Hypersil ODS2 column (150×4.6 mm I.D., 5 μm) [50]. A mobile

phase consisting of acetonitrile–0.01% ammonium acetate (pH 5.0) (36:61, v/v) was used and the 2,4-dinitrophenylhydrazone derivative was detected at 375 nm. This method was applied to the analysis of verticinone in the plasma samples obtained from mice pretreated with verticinone. However, only ketone-containing compounds can be converted to the corresponding 2,4-dinitrophenylhydrazone derivatives; the developed pre-column 2,4-dinitrophenylhydrazine derivatizing HPLC method is unsuitable for the analysis of the majority of bioactive *Fritillaria* isosteroidal alkaloids due to a lack of ketone groups in their structures (Table 1).

4.2. Direct high-performance liquid chromatography

4.2.1. Direct high-performance liquid chromatography–UV detection

Only one direct HPLC–UV method was reported by Chao et al. in 1993 to analyze *Fritillaria* isosteroidal alkaloids in different *Fritillaria* species [51]. In this reported method, a reversed-phase Shimpack CLC-ODS column (150×6 mm I.D., 5 μm) was employed using a mobile phase of methanol–water (69:31, v/v) containing 7.5 m*M* sodium dodecyl sulfate. UV detector set at 205 nm was used to monitor the analytes. Three isosteroidal alkaloids including verticine, verticinone and peimissine were attempted to be determined simultaneously. However, due to the low absorption and interference with absorptions of solvents in the mobile phase, verticine and verticinone could not be selectively detected, whilst only peimissine, a double bond-containing isosteroidal alkaloid (Table 1), was quantified with a relatively lower sensitivity. Consequently, only one *Fritillaria* alkaloid peimissine was quantified in 12 different *Fritillaria* species examined.

4.2.2. Direct high-performance liquid chromatography–evaporative light scattering detection

A marked increase in the use of HPLC analysis coupled with evaporative light scattering detection (ELSD) in a recent decade demonstrated that ELSD is an excellent detection method for the analysis of non-chromophoric compounds [52–54]. This new universal detector provides a possibility for the direct HPLC analysis of *Fritillaria* isosteroidal alkaloids, since the response of ELSD depends on the size, shape, and number of eluate particles rather than the structure and/or chromophore of analytes. More recently, our research team established a direct HPLC–ELSD method for simultaneous determination of eight *Fritillaria* isosteroidal alkaloids, namely peimissine, verticine, verticinone, imperialine, isoverticine, ebeiedinone, ebeiedine and hupehenine [31].

In this newly developed method, after assessment of various chromatographic conditions, such as different columns and mobile phase systems, a reversed-phase Supelco C_8 column (150×4.5 mm I.D., 3 μm) with a simple isocratic mobile phase consisting of acetonitrile–methanol–water (66.5:3.5:30, v/v/v) containing 0.006% triethylamine was chosen for the excellent separation and well-defined peaks for all alkaloids analyzed and the internal standard as illustrated in Fig. 11.

Unlike UV detection, ELSD response mainly depends on the size, shape, and number of eluate particles. Amongst various factors affecting ELSD response, the flow-rate of nebulizing gas and the temperature of drift tube in ELSD chamber are the most important parameters and should be adjusted to allow solvents in the mobile phase to be completely vaporized when the residual droplets reach the light scattering cell [54]. Therefore, the mobile phase containing high aqueous composition is less compatible with this detector. Recently a lower temperature adaptor (LTA) was designed by Alltech Associates Inc. to remove large droplets in the nebulized effluents from the nebulization chamber in LTA directly into the waste outlet. Subsequently, the temperature required to evaporate the remaining droplets prior to reaching the light scattering cell in ELSD is significantly reduced and thus high aqueous-containing mobile phase can be adopted [55]. In the reported method, on-line use of LTA-ELSD with temperature at 65°C for both LTA and drift tube in ELSD and a nebulizing nitrogen flow of 2.64 SLPM (standard liters per min) well adopted the design mobile phase containing 30% water, and exhibited an excellent baseline stability as shown in Fig. 11.

This newly developed direct HPLC–ELSD analysis enabled a simultaneous analysis of eight major

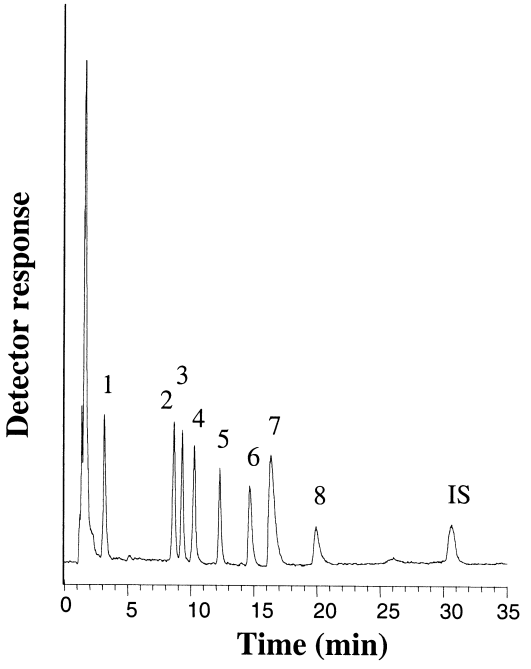


Fig. 11. Representative HPLC–ELSD chromatogram of a mixture of eight *Fritillaria* isosteroidal alkaloids. A Supelco C_{18} column (150×4.6 mm I.D., 3 μm) was used. The mobile phase consisted of acetonitrile–methanol–water (65.5:3.5:30, v/v) containing 0.006% triethylamine with a flow-rate of 1 ml/min. 1, Peimissine; 2, verticine; 3, verticinone; 4, imperialine; 5, isoverticine; 6, ebeiedinone; 7, hupehenine; 8, ebeiedine; IS, internal standard. (From reference [31] with permission from Elsevier Science).

Fritillaria isosteroidal alkaloids. Six of them including peimissine, verticine, verticinone, imperialine, isoverticine and ebeiedine were quantified, whereas ebeiedinone and hupehenine were only qualitatively determined due to a lack of sufficient authentic samples for the construction of corresponding calibration curves. Successful application of this direct HPLC method achieved simultaneous qualification and quantification of the major bioactive *Fritillaria* isosteroidal alkaloids in various *Fritillaria* species [31]. In addition, a parallel study using both direct HPLC–ELSD and previously developed direct GC method [30] was performed to compare accuracies for the quantification of alkaloids present in three different *Fritillaria* species. The results demonstrated that both analytical methods provided adequate accuracies for the quantification of the major bioactive *Fritillaria* isosteroidal alkaloids in Beimu herbs [31]. Furthermore, the detection limits of this direct HPLC–ELSD analysis for all isosteroidal alkaloids tested are compatible with previously developed GC analyses (Table 2).

As summarized in Table 2, all four analytical methods exhibited relatively high values of limits of detection, especially in the pre-column 1-naphthoate derivatization HPLC–UV analysis. This might be mainly due to the simple one-step extraction procedure used. In all cases, herbal powders were simply extracted with alkalized diethyl ether without further purification. Consequently, the resultant extracts contained the major isosteroidal alkaloids as well as all other diethyl ether soluble compounds, which subsequently increased the background of noise peaks and decreased the sensitivity for the analysis of analytes. In the cases of pre-column derivatization, the derivatized extracts were directly subjected to the HPLC or GC analysis. This might further reduce the sensitivity of detection, since the derivatized extracts contained more impurities such as the excess derivatizing reagent, catalyst (in 1-naphthoate derivatization HPLC–UV), and even some derivatives of other non-isosteroidal alkaloids present in the original crude extracts. Therefore, modification of the reported extraction procedure with further purification of the crude extracts by either liquid–liquid extraction with pH changes or solid-phase extraction should be possible solutions to increase the sensitivity of these methods. On the other hand, as described in all four developed methods [30,31,33,34], the reported limits of detection are adequate and sensitive enough for the analysis of all major *Fritillaria* isosteroidal alkaloids in herbal Beimu. Therefore, the reported simple one-step extraction has less time-consuming and more cost-effective advantages.

Recently in the pharmacokinetic study of imperialine in rats carried out by our research team, another direct HPLC–ELSD method was established for the analysis of imperialine in biological samples [48]. Similarly, a reversed-phase Supelco C_8 column (150×4.5 mm I.D., 3 μm) was used, while the mobile phase system was modified to be a gradient elution with three solvents: (A) distilled water, (B) acetonitrile and (C) methanol containing 0.6% triethylamine. The gradient programme is as follows: 0–6 min A/B/C=7:35:58; 6–7 min linear increase

to A/B/C=0:42:58 and maintain for 25 min; 25–30 min return to the initial conditions. Since relatively low aqueous composition was used in this gradient mobile phase system, for example 7% as the highest, ELSD was used without connection with LTA and set with nebulizing nitrogen gas flow of 2.00 SLPM and drift tube temperature at 72°C. This direct HPLC method provided an acceptable detection limit for imperialine at 50 ng/ml of blood sample, and was demonstrated to successfully determine imperialine in plasma samples collected from rats at different time periods after intravenous or oral administration of imperialine [48].

4.3. Construction of calibration curves with an internal standard method

In general, for the study of herbal materials, calibration curves are normally conducted without using the internal standard method since blank controls are unavailable. Therefore, reproducibility and extraction yield become critical for the quantification of the principal components in herbs. In order to solve this problem, a method for the construction of calibration curves with an internal standard has been previously developed by our research team [56]. This method was successfully adopted in the direct HPLC–ELSD [31] and the direct GC [30] analytical method described above for the determination of major bioactive *Fritillaria* isosteroidal alkaloids in different Beimu herbs. For the construction of calibration curves in the direct HPLC–ELSD assay, a spiked herbal extract was prepared by spiking both internal standard and analytes tested into the herbal powders prior to extraction. In addition, control herbal extract was also conducted by spiking the internal standard only to the similar herbal powders prior to extraction. The simple one-step extraction produce described in the above section was used. Briefly the prepared samples were extracted with 5 ml of diethyl ether–mmonium hydroxide (50:1, v/v) by vortex shaking for 2 h, then centrifuged at 1780 *g* for 10 min. The supernatants were evaporated to dryness. The residues were reconstituted into appropriate volumes of methanol, filtered through a syringe filter (0.45 μm), and then analyzed chromatographically in the same manner. The peak area ratio of analyte over the internal standard was calculated for each analyte in all extracts analyzed. Calibration curves were then constructed as a function of the concentration of analyte versus the peak area ratio differences (peak area ratio$_{spiked}$ − peak area ratio$_{control}$) between spiked and control herbal extracts. Good linear calibrations were obtained for all six *Fritillaria* isosteroidal alkaloids determined in the direct HPLC–ELSD [31]. Using this internal standard method, good reproducibility and accuracy for the quantification of six major bioactive *Fritillaria* isosteroidal alkaloids in herbal Beimu were obtained, and the overall intra- and inter-day variations were less than 11% with an overall accuracy of higher than 90% for direct HPLC–ELSD analysis [31].

5. Conclusions

In this review, we have mainly described the simultaneous qualitative and quantitative analysis of the major bioactive *Fritillaria* isosteroidal alkaloids by various chromatographic techniques. Both recently developed direct GC–FID and HPLC–ELSD assays are simple, sensitive and selective analytical methods with good accuracy and reproducibility. However, since the two major bioactive *Fritillaria* isosteroidal alkaloids verticine and verticinone could not be quantified separately in the reported direct GC–FID method [30], consequently, the most recently developed HPLC–ELSD assay [31] is the most suitable and readily adoptable quality control method for the simultaneous qualitative and quantitative determination of the main bioactive *Fritillaria* isosteroidal alkaloids in the most commonly used antitussive TCM herbal Beimu.

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