Simultaneous determination of the major isosteroidal alkaloids and their glucosides in the bulbs of *Fritillaria* by high performance liquid chromatography coupled with evaporative light scattering detection

LI Ping[1*] , ZENG Ling-jie[1] , LI Song-lin[1] , BI Zhi-ming[1] , LIN Ge[2]

(1. *Department of Pharmacognosy , China Pharmaceutical University , Nanjing 210009 , China ;*
2. *Department of Pharmacology , Faculty of Medicine , Chinese University of Hong Kong , Hong Kong , China*)

Abstract: **Aim** To establish an HPLC-ELSD method for the simultaneous determination of five major bioactive isosteroidal alkaloids and gluco-alkaloids in the bulbs of *Fritillaria* namely peimissine , imperialine , sinpeinine A, imperialine-3β-glucoside and yibeinoside A. **Methods** A Nova-Pak C_{18} column (150 mm × 3.9 mm ID) was used. The chromatography was carried out with a linear gradient programming. The mobile phase was acetonitrile-water (containing 0.1 % diethylamine) and the flow rate was 1.0 mL·min^{-1}. **Results** The linear range of peimissine was 13.1 - 288.2 mg·L^{-1} (r^2 = 0.997 5) , imperialine-3β-glucoside 7.7 - 169.4 mg·L^{-1} (r^2 = 0.999 3) , yibeinoside A 7.3 - 160.6 mg·L^{-1} (r^2 = 0.999 7) , imperialine 16.5 - 363.0 mg·L^{-1} (r^2 = 0.999 2) , sinpeinine A 8.7 - 191.4 mg·L^{-1} (r^2 = 0.994 2) . **Conclusion** The method is accurate with overall intra- and inter-day variation less than 5 % and recovery more than 95 %. The method was successfully applied to analyze five major bioactive alkaloids and gluco-alkaloids in three *Fritillaria* bulbs.

Key words: *Fritillaria* ; isosteroidal alkaloids ; gluco-alkaloids ; HPLC-ELSD
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HPLC-ELSD法测定贝母中异甾类生物碱及糖苷类成分的含量

李 萍[1*] , 曾令杰[1] , 李松林[1] , 毕志明[1] , 林 鸽[2]

(1. 中国药科大学 生药学教研室 , 江苏 南京 210038 ; 2. 香港中文大学 药理系 , 香港)

摘要: 目的 建立同时测定贝母中5种异甾类生物碱——peimissine , imperialine , sinpeinine A, imperialine-3β-glucoside 和 yibeinoside A含量的HPLC分析方法。方法 C_{18}柱;流动相:乙腈-水(含0.1 %二乙胺);梯度洗脱,流速1.0 mL·min^{-1};检测器:Alltech 500 蒸发光散射检测器(ELSD)。结果 线性范围为 peimissine 13.1 ~ 288.2 mg·L^{-1} (r^2 = 0.997 5) , imperialine-3β-glucoside 7.7 ~ 169.4 mg·L^{-1} (r^2 = 0.999 3) , yibeinoside A 7.3 ~ 160.6 mg·L^{-1} (r^2 = 0.999 7) , imperialine 16.5 ~ 363.0 mg·L^{-1} (r^2 = 0.999 2) , sinpeinine A 8.7 ~ 191.4 mg·L^{-1} (r^2 = 0.994 2) 。5个化合物的精密度和重现性RSD均 < 5 %。结论 本方法简便,有效,可行,可用于贝母中5种异甾类生物碱的含量测定。

关键词: 贝母 ; 异甾类生物碱 ; 糖苷类生物碱 ; HPLC-ELSD

The bulbs of many *Fritillaria* species growing in China have been used as antitussive and expectorant herbs with Chinese name "Beimu" in traditional Chinese medicine (TCM) for more than 2000 years[1]. Widely chemical and pharmacological studies on various *Fritillaria* species have been conducted by several research groups[2-4], and the results indicated that the major isosteroidal alkaloids present in different *Fritillaria* species are the active ingredients responsible for the

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* Corresponding author Tel: 86 - 25 - 5322256, Fax: 86 - 25 - 5322448, E-mail: lipingli@publicl.ptt.js.cn

antitussive and expectorant activity[5,6]. Therefore the quantity control of the major active alkaloids in this herb have always been an important issue to ensure its effective and safe clinical use.

Several chromatographic techniques have been developed for analysis of various *Fritillaria* isosteroidal alkaloids[7-10]. These methods, however, suffer from limited applicability for compounds with a wide range of polarity or a relatively low sensitivity or a time-consuming sample preparation process.

The aim of this study is to develop a simple and sensitive direct HPLC-ELSD method for the simultaneous determination of the major active free isosteroidal alkaloids and their glucosides in *Fritillaria* bulbs, which have been found existing commonly in the same species of *Fritillaria* and also be tested pharmacologically as the primary active ingredients responsible for the activity, especially expectorant activity.

Material and methods

Chemicals and materials HPLC grade acetonitrile was purchased from Tedia (Fairfield, OH, USA). Distilled water was supplied by Wahaha (Hangzhou, China) and diethylamine (AR) was obtained from Shanghai Chemical Company (Shanghai, China). Five authentic alkaloids (peimissine, imperialine, imperialine-3β-glucoside, sinpeinine A and yibeinoside A) were isolated from *F. pallidiflora* Schrenk., the purity of the isolated alkaloids was shown to be higher than 99 % by HPLC analysis, and their structures were confirmed by IR, ^{1}HNMR, ^{13}CNMR and MS analysis. Sulforidazine (internal standard) was purchased from Sigma Chemical Company (St. Louis, MO, USA). The analyzed *Fritillaria* bulbs were collected from a native population in Xinjiang uighur Autonomous Region of China, and authenticated by Prof. Li P. The voucher specimens were deposited at the herbarium of the China Pharmaceutical University.

Apparatus and conditions Analyses were performed on a Agilent 1100 Chromatograph system (California, USA) equipped with an Alltech 500 (Deerfield, IL, USA) evaporative light-scattering detector. A Waters Nova-Pak (Milford, USA) reversed-phase C_{18} column (150 mm × 3.9 mm ID) together with a C_{18} guard column was used during the whole analysis. The mobile phase consisted of acetonitrile and water (containing 0.1 % diethylamine), and the elution was designed as follows: 0 - 8 min, isocratic, acetonitrile-water (40∶60); 8 - 25 min, gradient, the amount of acetonitrile was increased linearly from 40 % to 90 %; 25 - 35 min, isocratic, the proportion of water to acetonitrile was kept as 10 to 90, the flow rate was 1.0 mL•min^{-1}. The temperature of the drift tube of the ELSD was set at 90 ℃ and the nitrogen gas flow was adjusted to 2.0 L•min^{-1}.

Results

1 Calibration curves and test limits

Methanol stock solutions containing peimissine, imperialine, imperialine-3β-glucoside, sinpeinine A, yibeinoside A, were prepared and diluted to a series of appropriate concentration for the construction of calibration curves and for the limits of detection. Each calibration curve was performed with six different concentrations in triplicate. A 50 μL (1 g•L^{-1}) volume of the internal standard, sulforidazine, was added in each concentration. The peak area ratio of the standard to the internal standard versus the standard concentration calibration curves were constructed. Results were shown in Table 1. All the calibration curves were linear within the test range and followed the equation of the type ($Y = aX + b$) with high correlation. The limit of detection for each alkaloid was calculated at a signal-to-noise ratio of 5 (Table 1).

2 Precision and accuracy

Known quantities of the five alkaloids and 50 μL internal standard (1 g•L^{-1}) were added to the sample powder (500 mg), extracted using the method described as "**Analysis of five alkaloids in the bulbs of *Fritillaria***," and analysed as above. The total content of each alkaloid was determined by using the corresponding calibration curve, while the content of each spiked alkaloid was calculated by subtraction of the detected amount of the corresponding alkaloid present in the original sample powder from the total content. Consequently, the recovery was defined as the percentage of the spiked amount that could be determined and considered as a measure of accuracy. The relative standard derivation (RSD) was taken as a measure of precision. The precision and accuracy of the established method were shown in Table 2.

3 Analysis of five alkaloids in the bulbs of *Fritillaria*

To the grounded dried bulbs (500 mg) pre-alkalized with ammonia hydroxide, 5 mL of dichloromethane-methanol (4∶1) and 50 μL internal standard solution (1 g•L^{-1}) were added. After ultrasonicated for 2 h, 0.5 mL of the extract organic solvent was taken into a tube and evaporate to dryness, the residues were re-dissolved in 500 μL methanol. The resultant solution was filtered

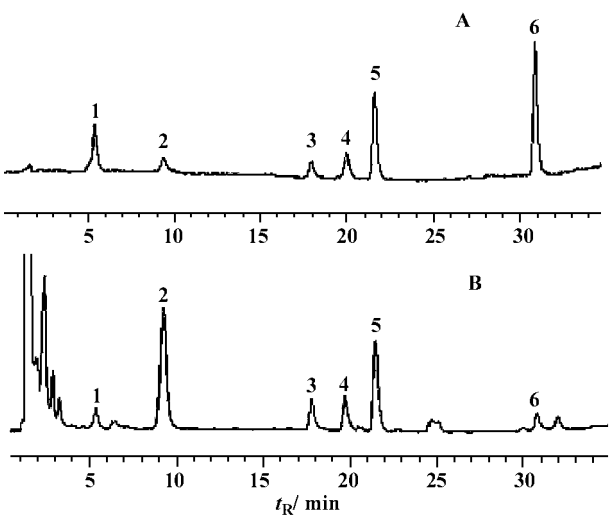
through syringe filter (0.45 μm) , and 20 μL of each filtrate was subjected to HPLC-ELSD analysis (Figure 1) . The content of each alkaloid was calculated from the corresponding calibration curve (Table 3) .

Table 1 Calibration curves of five alkaloids

Alkaloid	Retention time/ min	Calibration curve	r^2	Linear range/ mg·L^{-1}	Limit of detection/ mg·L^{-1}
Peimissine	5.36	$Y = 0.013\,9X - 0.210\,7$	0.997 5	13.1 - 288.2	0.94
Imperialine-3β-glucoside	9.22	$Y = 0.030\,4X - 0.051\,6$	0.999 3	7.7 - 169.4	0.56
Yibeinoside A	17.75	$Y = 0.046\,2X - 0.079$	0.999 7	7.3 - 160.6	0.63
Imperialine	21.46	$Y = 0.015\,5X - 0.265\,6$	0.999 2	16.5 - 363.0	0.80
Sinpeinine A	30.76	$Y = 0.012\,1X - 0.067\,2$	0.994 2	8.7 - 191.4	0.50

Table 2 Precision and accuracy for the assay of five alkaloids in the bulbs of *Fritillaria* ($n = 3$, $\bar{x} \pm s$)

Alkaloid	Intra-day variability			Inter-day variability		
	Added/ μg	Detected/ μg	RSD/ %	Accuracy/ %	Detected/ μg	RSD/ %	Accuracy/ %
Peimissine	37.24	52.8 ±1.7	3.1	100 ±5	52.7 ±1.8	3.4	100 ±3
	74.48	91.2 ±1.2	1.3	101.7 ±1.7	91.3 ±1.3	1.5	101.9 ±2.5
Imperialine-3β-glucoside	7.11	51.7 ±1.1	1.4	104 ±12	51.8 ±1.4	2.6	104 ±3
	14.22	59.8 ±0.8	2.2	105.2 ±0.5	59.6 ±1.3	2.2	104.7 ±1.2
Yibeinoside A	14.26	21.8 ±1.1	4.9	100 ±7	21.5 ±0.5	2.3	99 ±5
	28.52	37.2 ±0.9	2.4	104 ±4	37.5 ±1.4	3.7	104 ±4
Imperialine	91.24	150.6 ±2.5	1.6	99.0 ±2.5	151.0 ±2.7	1.8	100 ±4
	182.48	242 ±8	3.1	100 ±4	242 ±10	4.0	100 ±4
Sinpeinine A	72.07	82.6 ±1.7	2.1	99.9 ±2.5	82.4 ±1.7	2.1	98.7 ±2.6
	144.14	155.1 ±4	2.6	100.3 ±2.9	155 ±4	2.8	101.4 ±2.3



1 : Peimissine ; 2 : Imperialine-3β-glucoside ; 3 : Yibeinoside A; 4 : Internal standard ; 5 : Imperialine ; 6 : Sinpeinine A

Figure 1 Representative HPLC chromatograms of the standards (A) and the extract of *F . pallidiflora* (B)

Table 3 Five alkaloids content in the bulbs of *Fritillaria* (μg·g^{-1} , $n = 3$)

Sample	Peimissine	Imperialine-3β-glucoside	Yibeinoside A	Imperialine	Sinpeinine A
F . pallidiflora	288 ±12	1120 ±53	341 ±4	826 ±29	197 ±4
F . yuminensis	866 ±5	136.0 ±1 .9	259 ±14	1 914 ±9	548 ±34
F . maximowiczii	-	206 ±9	-	-	-

Discussion

Compared with our previous study[9-11] , there are larger differences in polarity amongst present compounds analyzed which include alkaloids and their glucosides . Therefore optimal chromatographic condition was obtained after testing different mobile phase systems and its elution manner(isocratic or gradient) with two reversed-phase types (C_8 and C_{18}) . The mobile phase system established was an isocratic and gradient elution system with two solvents : acetonitrile and water (containing 0.1 % diethylamine) on the Nova-Pak C_{18} column . In the case of the Suplco C_8 column , free alkaloid and its glucoside , could not be completely separated . On the other hand , three alkaloids , two gluco-alkaloids and internal standard could be resolved as a baseline separation with C_{18} as shown in Figure 1 . The results revealed that the alkaloids and their glucosides could be separated successfully on the reversed-phase C_{18} column with the isocratic and gradient elution .

For internal standard compound , solanidine was also considered as a selection just like the developed method previously , but under the present conditions , its retention time lagged to 40 minutes , and its peak is tailing with bad symmetry . So finally after several alkaloids have being tested , sulforidazine was selected for the internal standard

in the assay for its similar solubility to the determined alkaloids and its suitable polarities. Using this internal standard method good reproducibility and accuracy for the quantification of five major bioactive *Fritillaria* isosteroidal alkaloids and gluco-alkaloids in herbal Beimu were obtained, and the overall intra- and inter-day variation was less than 5 % with the overall recovery of higher than 95 % for this analysis. Good linear calibrations and adequate sensitivity were obtained for all compounds examined.

The developed HPLC-ELSD assay was successfully applied to the simultaneous quantification of the five isosteroidal alkaloids and gluco-alkaloids presented in *Fritillaria* bulbs. Representative chromatograms of the extract of Beimu samples and authentic samples are shown in Figure 1, and their contents are showned in Table 3.

The results indicate that the contents of five alkaloids in the bulbs of *Fritillaria pallidiflora* are higher than previously reported. And about the five alkaloids, the contents of imperialine-3β-glucoside (1.12 $mg \cdot g^{-1}$) or imperialine (0.84 $mg \cdot g^{-1}$), which are the main constituents in the bulbs of *F. pallidiflora*, are 2 - 3 times higher than previously reported. This result is compatible with the study of the relationship between quantity and type of active *Fritillaria* alkaloids and their pharmacological activities by our research team recently (published separately). And our current investigation of correlation between chemical ingredients and their activities revealed that the total alkaloids of the bulbs of *Fritillaria pallidiflora* possess a higher antitussive and expectorant activity than that of others and two major compounds, imperialine and imperialine-3β-glucoside, have significant antitussive and expectorant activity. The antitussive effect produced by these two alkaloids is compatible with codeine, a most effective antitussive agent. Owing to their potent antitussive and expectorant activity, imperialine and imperialine-3β-glucoside may be developed as a new natural drug for the treatment of cough and are worthy of further investigation.

In conclusion, the established HPLC-ELSD method is reproducible with good accuracy and sensitivity for simultaneously quantitative analysis of alkaloids and gluco-alkaloids in *Fritillaria* bulbs. It provides a suitable quality control method for Beimu samples and can be readily utilized for the determination of the major biologically active ingredients in Beimu, the most commonly used antitussive and expectorant TCM herb. The alkaloids and their glycosides in the *Fritillaria* species were simultaneously determined by this method for the first time.

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