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Development and validation of a liquid chromatography/electrospray ionization time-of-flight mass spectrometry method for relative and absolute quantification of steroidal alkaloids in *Fritillaria* species

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Abstract

Steroidal alkaloids are naturally occurring nitrogen-containing compounds in many edible or medicinal plants, such as potato, tomato, *Fritillaria* and American hellebore, which possess a variety of toxicological and pharmacological effects on humans. The aim of this study is to explore the potential of liquid chromatography/electrospray ionization time-of-flight mass spectrometry (LC/ESI-TOF-MS) method in the determination of these important alkaloids in plant matrices. The application of this method has been proven through 26 naturally occurring steroidal alkaloids in *Fritillaria* species. Accurate mass measurements within 4 ppm error were obtained for all the alkaloids detected out of various plant matrices, which allowed an unequivocal identification of the target steroidal alkaloids. The bunching factor for mass spectrometer, an important parameter significantly affecting the precision and accuracy of quantitative method, was firstly optimized in this work and satisfactory precision and linearity were achieved by the optimization of that parameter. The ranges of RSD values of intra-day and inter-day variability for all alkaloids were decreased remarkably from 41.8–159% and 13.2–140% to 0.32–7.98% and 2.37–16.1%, respectively, when the value of bunching factor was optimized from 1 to 3. Linearity of response more than two orders of magnitude was also demonstrated (regression coefficient >0.99). The LC/TOF-MS detection method offered improvements to the sensitivity, compared with previously applied LC (or GC) methods, with limits of detection down to 0.0014–0.0335 μg/ml. The results in this paper illustrate the robustness and applicability of LC/TOF-MS for steroidal alkaloids analysis in plant samples. In addition, relative quantitative determination of steroidal alkaloid with one popular analyte verticinone which is commercially available was also investigated in order to break through the choke point of lack of standards in phytochemical analysis. The accuracies of relative quantitative method for steroidal alkaloids determinations with verticinone were 90.6–110.0% (average 98.5%) suggesting that it is feasible to quantify steroidal alkaloids by the proposed relative quantitative determination method within acceptable errors.

Keywords: Steroidal alkaloids; LC/ESI-TOF-MS; Quantification; Bunching factor; Correction factor

1. Introduction

Steroidal alkaloids are naturally occurring nitrogen-containing steroid derivatives present in many species of the family Apocynaceae, Buxaceae, Solanaceae and Liliaceae, including *Holarrhena*, *Buxus*, *Solanum*, *Lycopersicon*, *Fritillaria* and *Veratrum* species, etc. In *Holarrhena* species the major steroidal alkaloids are pregnane-type alkaloids, such as conssine; in *Buxus* species cyclopregnane-type alkaloids represented by cycloxbuxidine-F are present; while in *Solanum*, *Lycopersicon*, *Fritillaria* and *Veratrum* species cholestane-type alkaloids are predominant, such as solanidine and verticine [1–3]. It has been suggested that all these secondary metabolites produced

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by plants probably perform a multipurpose defence function (vs herbivores, bacterial and fungal infestation) which has developed during evolutionary time [4–6]. Some steroidal alkaloids also have toxicological effects on humans due to their significant teratogenic action, anticholinesterase activity and disruption function of cell membranes [6–9]. Taking steroidal glycoalkaloids in potato (*Solanum tuberosum*) for instance, acute toxicity syndromes in humans have been observed at steroidal glycoalkaloids levels of more than 2.8 mg/kg body weight [8]. An upper limit of 60–70 mg/kg for steroidal glycoalkaloids in potatoes has been proposed for human consumption [10]. On the other hand, steroidal alkaloids in many plants also have pharmacological effects on humans. For example, *Buxus*, *Fritillaria* and *Veratrum* species are traditionally used as herbal remedies in China, Japan, Turkey, Pakistan and south-east Asian countries, in which steroidal alkaloids have been proven to be responsible for their therapeutic effects [11]. For these reasons, it is important to develop a sensitive and selective analytical method to accurately evaluate the presence and content of the major and minor steroidal alkaloids in these plants.

Various analytical techniques have been used for the determination of steroidal alkaloids, including capillary electrophoresis coupled with mass spectrometry (CE–MS) [10], isotachophoresis [12], thin-layer chromatography [13], colorimetry [14], gas chromatography [15,16], counter current chromatography [17], high-performance liquid chromatography (HPLC) [18–25] and immunoassays [26,27]. Among these methods, HPLC-UV was more frequently used, although UV detection was often performed at short wavelengths from 200 to 215 nm, due to the skeleton of steroidal alkaloids lacking any useful chromophore. An alternative to UV detection at short wavelength was using the evaporative light scattering detection (ELSD) [24,25], a universal detection suitable for the analysis of non-chromophoric compounds. However, low sensitivity and possible uncertainty of chromatographic peak identification (mainly by retention time) made HPLC-UV or -ELSD methods unlikely match the routine analysis of interested steroidal alkaloids, especially for the minor ones in complex plant extracts.

Recent success with the use of liquid chromatography combined with time-of-flight mass spectrometry (LC/TOF-MS) for characterizing and quantifying a wide variety of compounds in complex samples [28–31] suggests that LC/TOF-MS might also be a powerful technique in the comprehensive determination of multiple steroidal alkaloids in complex plant extracts. One of the main attributes of TOF-MS that makes it an attractive analytical technique is its accurate mass measurement. By the accurate mass measurement, LC/TOF-MS can alleviate the matrix interferences from coeluting impurities, which are often encountered by conventional LC-UV, LC-ELSD and LC–MS methods and preclude reliable quantification. In addition, LC/TOF-MS instruments offer the capability of unequivocal identification (provided by accurate mass measurements and retention time match) of target compounds from complex matrices, as well as the possibility of quantitation at low-level concentrations in real samples. Yet, no attempts have been made to develop a method of steroidal alkaloids analysis based on accurate mass measurements using LC/TOF-MS.

Therefore, the aim of this study is to show the potential of LC/TOF-MS for multiple steroidal alkaloids analysis in complex plant samples in regard to confirmation and quantification of target analytes. On the other hand, due to the lack of standards, most biologically significant steroidal alkaloids could not be quantified in many edible or medicinal plants (e.g. potato, tomato and American hellebore). For this reason, another aim of this work is to demonstrate the feasibility of relative quantitative method for steroidal alkaloids determinations with a common one which is commercially available. As an illustrative case study, 26 naturally occurring steroidal alkaloids have been investigated in different *Fritillaria* species, a group of important medicinal plants used as antitussive and expectorant remedies in herbal medicines. To the best of our knowledge, the new method presented here provides the best sensitivity and specificity for determination of the steroidal alkaloids so far.

2. Experimental

2.1. Chemicals and reagents

The steroidal alkaloid standards, verticine N-oxide (**1**), puqienine F (**2**), verticinone N-oxide (**3**), puqienine C (**4**), puqienine D (**5**), peimisine (**6**), imperialine-3-β-D-glucoside (**7**), puqienine E (**8**), puqienine B (**9**), puqienine A (**10**), puqiedine-7-ol (**11**), puqietinonoside (**12**), hupeheninoside (**13**), yibeinoside A (**14**), imperialine (**15**), verticine (**16**), *N*-demethylpuqietinone (**17**), verticinone (**18**), ebeienine (**19**), isoverticine (**20**), puqiedinone (**21**), puqietinone (**22**), puqiedine (**23**), ebeiedinone (**24**), ebeiedine (**25**) and puqietinedinone (**26**) (Fig. 1), were isolated from several *Fritillaria* species in our laboratory [32–37], and their identities were confirmed by IR, ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR), MS analyses. The purity of these steroidal alkaloids were determined to be more than 98% by normalization of the peak areas detected by HPLC with ESI/MS, and showed very stable in methanol solution at 4 °C in refrigerator. Solasodine (internal standard, I.S.) was purchased from Sigma (St Louis, MO, USA). HPLC-grade acetonitrile was supplied by Merck (Darmstadt, Germany) and purified water was provided by a Millipore water purification system (Millipore, Bedford, MA, USA). Diethylamine of analytical grade was purchased from Shanghai Lingfeng Chemical Reagent Co. (Shanghai, China).

Stock standard solutions of selected steroidal alkaloids were prepared in methanol at a final concentration of 400 μg/ml. These solutions were stored at 4 °C. Working solutions, used for LC/TOF-MS analysis, were obtained by diluting the stock solutions with methanol. Individual I.S. (solasodine) stock solution of 40 μg/ml was prepared in methanol and stored at 4 °C until use, and the final concentration of the solasodine (I.S.), was 4 μg/ml in all calibration standard solutions.

2.2. Sample preparation

A total of 12 *Fritillaria* species (including *F. ussuriensis*, *F. unibracteata*, *F. przewalskii*, *F. delavayi*, *F. cirrhosa*, *F. pallidiflora*, *F. walujewii*, *F. thunbergii*, *F. ebeiensis*, *F.*