Journal of Pharmaceutical and Biomedical Analysis 115 (2015) 10–19

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Discovery of characteristic chemical markers for classification of aconite herbs by chromatographic profile and probabilistic neural network

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A R T I C L E I N F O

Article history:
Received 4 March 2015
Received in revised form 10 June 2015
Accepted 17 June 2015
Available online 23 June 2015

Keywords:
Chemical marker
Species classification
Processed aconite roots
Alkaloids
Probabilistic neural network
High performance liquid chromatography

A B S T R A C T

Most *Aconitum* species, also known as aconite, are extremely poisonous, so it must be identified carefully. Differentiation of *Aconitum* species is challenging because of their similar appearance and chemical components. In this study, a universal strategy to discover chemical markers was developed for effective authentication of three commonly used aconite roots. The major procedures include: (1) chemical profiling and structural assignment of herbs by liquid chromatography with mass spectrometry (LC-MS), (2) quantification of major components by LC-MS, (3) probabilistic neural network (PNN) model to calculate contributions of components toward species classification, (4) discovery of minimized number of chemical markers for quality control. The MS fragmentation pathways of diester-, monoester-, and alkyloyamine-diterpenoid alkaloids were compared. Using these rules, 42 aconite alkaloids were identified in aconite roots. Subsequently, 11 characteristic compounds were quantified. A component–species modeling by PNN was then established combining the 11 analytes and 26-batch samples from three aconite species. The contribution of each analyte to species classification was calculated. Selection of fuziline, benzoylhypaconine, and talatizamine, or a combination of more compounds based on a contribution order, can be used for successful categorization of the three aconite species. Collectively, the proposed strategy is beneficial to selection of rational chemical markers for the species classification and quality control of herbal medicines.

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1. Introduction

The plants in the genus *Aconitum* (family Ranunculaceae) are widely distributed. Most *Aconitum* species are extremely poisonous and must be dealt with carefully. A soaking or boiling pretreatment is usually required to decrease their toxicities. Since ancient time, the processed aconite roots have been used for therapeutic purpose because of extensive pharmacological effects such as cardiotonic, anti-rheumatism and analgesic action [1,2]. Clinical observations demonstrated that the processed aconite roots had a narrow therapeutic window. They showed pharmacological activities at the low dose, and cause neurotoxicity and cardiotoxicity at a relatively high dose [3,4].

Chemical studies showed that aconite roots mainly contained C_{19}-diterpenoid alkaloids. On the basis of the substituent groups, they could be divided into three types: diester-diterpenoid alkaloids (DDAs), monoester- diterpenoid alkaloids (MDAs), and alkyloyamine-diterpenoid alkaloids (ADAs) [5,6]. The toxicity of aconite roots was largely attributed to DDAs, such as aconitine, mesaconitine and hypaconitine. After processing, the content of DDAs in roots greatly decreased and the content of MDAs increased [7,8].

Three processed aconite roots derived from *Aconitum* genus are usually applied in clinic, including the processed mother root of *Aconitum kusnezoffii* (PAK), processed mother root and processed lateral root of *Aconitum carmichaelii* (PMAC and PLAC) [1]. Structural characterization and toxicities of C_{19}-diterpenoid alkaloids have been widely studied, the differentiation of *Aconitum* species is challenging because of their similar appearance and chemical components [9,10].

Recently, the supervised machine learning methods have been a powerful technique for data classification and estimation of variable contributions in various fields, such as species classification

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http://dx.doi.org/10.1016/j.jpba.2015.06.021
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[11], pharmaceutical research [12], credit rating analysis [13] and food science [14]. The elucidation of relationships between variables, often complicated and highly non-linear, are more objective by mathematical modeling methods rather than by subjective analysis. Since the secondary metabolites of plant may change to be specific by processing, it is possible to explore the potential chemical markers and to classify the similar herbs by components-species relationship.

In this work, a novel and practical strategy was developed based on chemical profile and supervised machine learning methods for the exploration of potential chemical markers toward species classification (Fig. 1). This strategy uses liquid chromatography coupled with multiple detection methods for chemical profiling and peak assignment in herbal extracts, and then major and characteristic components were quantitated. Subsequently, a component–species modeling based on probabilistic neural network was established to calculate contributions of components toward species classification. Eventually, minimized number of chemical markers were discovered for quality control. Compared with the untargeted metabolomic analysis, this strategy is easy to find the optimum combination of chemical marker for species authentication. Taking processed aconite herbs for example, three components including fuziline, benzoylhypaconine and talatizamine were optimized as the chemical markers to categorize 26-batch samples.

2. Materials and methods

2.1. Chemicals and reagents

Reference compounds, including aconitine, mesaconitine, hypaconitine, benzoylaconine, benzoylmesaconine, benzoylhypaconine and telepathine (IS) were purchased from National Institute for the Control of Pharmaceutical and Biological Product (Beijing, China). Talatizamine, neoline, fuziline, 8-OEt-14-benzoylmesaconine, songorine, napelline, chuanfumine, hetisine, 3-epiignavinol and guan fu base H were previously isolated from the lateral roots of *A. carmichaeli* Debx. by the authors, and their structures were elucidated by ^{1}H NMR and ^{13}C NMR spectroscopic method with a purity of more than 95%.

HPLC grade acetonitrile (ACN) and formic acid were obtained from Merck (Darmstadt, Germany) and ROE (Newark, New Castle, USA), respectively. Deionized water was purified with a Millipore Milli-Q water system (Milford, MA, USA). Ammonium formate, ammonia solution, isopropanol and ethyl acetate were of analytical grade.

2.2. Materials and sample preparation

Twenty-six batches of *Aconitum* samples containing PAK, PMAC and PLAC were purchased from pharmacy in China (Table S1). All samples were authenticated by Prof. Ping Li and the corresponding voucher specimens were deposited in the Department of Pharmacognosy, China Pharmaceutical University.

The samples were pulverized into powder and sieved through a No. 40 mesh. The dried powder (1.0 g) of each batch was alkalized with 2 mL ammonia solution and ultrasonically extracted (100 Hz) with 30 mL mixtures of ethyl acetate–isopropanol (1:1) for 30 min. After restoring the lost weight by adding extracted solution, 5 mL of the extract was evaporated under vacuum at 35 °C. Subsequently, the dried residue was redissolved and made up to exactly 10 mL with 50% ACN (v/v) containing a final concentration of 1 μg/mL telepathine (IS). After centrifugating at 13,000 rpm for 10 min, the supernatant solution 5 μL was subjected to analysis.

2.3. HPLC-MS conditions

The chromatography was carried out on an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of a binary pump, an online degasser, an auto sampler and a thermostatically controlled column compartment. An Agilent Zorbax SB-C18 column (5 μm, 250 mm × 4.6 mm I.D.) was utilized at a column temperature of 35 °C for separation at a flow rate of 1 mL/min. Solvent A (water containing 10 mM ammonium formate) and solvent B (acetonitrile containing 0.1% formic acid) were used as mobile phase. Optimized gradient elution program was 10–14% B at 0–10 min, 14-–47% B at 10–25 min, 47-–48% B at 25–27 min, 48–75% B at 27–32 min, 75–100% B at 32–33 min and kept 100% B for 6 min. The HPLC system was connected to the mass spectrometer with a post-column split (1:1).

Qualitative and quantitative analysis was performed by an Agilent 6520 quadrupole time-of-flight mass spectrometer (QTOF MS) with a Dual ESI source and an Agilent G1946D quadrupole mass spectrometer (Q MS) with an ESI source, respectively. Both the conditions were adjusted as follows: drying gas (N_2) flow rate, 10 L/min; drying gas temperature, 350 °C; fragmentor voltage, 120 V; nebulizer pressure, 40 psig; capillary voltage, 3500 V. All data were acquired in positive mode. The QTOF MS were operated in high resolution mode (4 GHZ) within the range *m*/*z* 100–2000 Da and 80–1500 Da respectively for MS and MS/MS data acquisition. The optimized collision energy was 45 V. The TOF was calibrated before data acquisition for accurate mass (<3 ppm) by using tuning mix solution (Agilent Technologies) contains the standard masses (*m*/*z* 118.0863, 322.0481, 622.0290, 922.0098, 1221.9906, 1521.9715, 1821.9523, 2121.9332, 2421.9140 and 2721.8948) and recalibrated of each acquired MS spectrum using the reference masses (*m*/*z* 121.0509 and 922.0098). The system was operated under MassHunter workstation acquisition software, version B.04.00 (Agilent Technologies). The QMS data was acquired in selected ion monitoring (SIM) mode by using a time program according to the retention time and quasi- molecular ion ($[M+H]^+$) of each analyte. The QMS data were acquired and analyzed by Chemstation software, version A 09.03 (Agilent Technologies).

2.4. Statistic analysis

All statistical algorithms were carried out by Matlab software (7.8.0, 2009a, Mathworks).

3. Results and discussion

3.1. Optimization of extraction method and chromatographic conditions

The extraction for *Aconitum* samples was carried out based on the procedure described in the Chinese Pharmacopoeia (2010 edition) with slight modifications. The optimal sample preparation procedure was detailed in Section 2.2. Since some aconite alkaloids were unstable in water and methanol [15], the dried extract was redissolved in 50% ACN (*v*/*v*).

To obtain satisfactory separation, HPLC conditions including mobile phase, column and column temperature were optimized. Different mobile phases such as acetonitrile and methanol were tested with some modifiers including formic acid, acetic acid, triethylamine and ammonium acetate. Results showed that 10 mM ammonium formate water and 0.1% formic acid (v/v) ACN produced a good separation for alkaloids and was compatible to MS analysis. Agilent Zorbax SB-C18 column with a temperature at 35 °C generated a better resolution than Agilent Zorbax Extend-C18 column

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