Journal of Chromatography A, 1345 (2014) 78–85

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Identification of effective combinatorial markers for quality standardization of herbal medicines

Zi-Qi Shi [1], De-Fang Song [1], Ruo-Qian Li, Hua Yang, Lian-Wen Qi, Gui-Zhong Xin, Ding-Qian Wang, Hui-Peng Song, Jun Chen, Haiping Hao *, Ping Li **

State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, China

ARTICLE INFO

Article history:
Received 13 February 2014
Received in revised form 7 April 2014
Accepted 7 April 2014
Available online 13 April 2014

Keywords:
Quality control
Effective combinatorial markers
Lycoridis Radiatae Bulbus
Alkaloids
Synergistic effects
Drug discovery

ABSTRACT

Quality standardization of herbal medicines (HMs) is an important task with great challenges. Selection of abundant compounds as markers is currently a major approach for the quality control of HMs; however, such marker compounds are irrelevant to the bioactivities in many cases. Taking Lycoridis Radiatae Bulbus (LRB) as an example, we proposed a universal strategy to identify the effective combinatorial markers (ECMs) that are representative of the bioactivities of HMs, and took them as chemical markers for quality standardization. Fingerprinting and quantification were employed to find out the common components in various batches of medicines. The contribution of each common compound to the overall bioactivity was determined through fingerprint–bioactivity modeling, which based on the absolute quantification of each compound and the acetylcholinesterase (AChE) inhibitory activity of LRB. Two most effective compounds, ungerimine and galanthamine, were therefore proposed as ECMs. Interestingly, these two compounds could synergistically inhibit AChE. This approach demonstrated its strong advantage of the bioactivity relevant quality assessment when compared with conventional methods. And the success of applying this ECMs-based method to the quality assessment of unknown LRB samples proved that our approach was reliable and reproducible. In conclusion, this approach is not only useful for the bioactivity relevant quality control of HMs but also helpful for the discovery of ECMs as new drug candidates.

1. Introduction

As the demands of herbal medicines (HMs) are increasing globally, their quality standardization and control have become more and more important in business and clinical applications [1]. Many factors such as geographic and environmental differences of growing conditions could affect the batch-to-batch uniformity of herbal products [2]. Unlike the western drugs, HMs contain relatively unrefined mixtures of phytochemical compounds and thus raising great challenges in selecting appropriate marker compounds for quality standardization [3].

Chemical marker-based methods are popular for the quality control of HMs. In these methods, selecting suitable markers that are representatives of bioactivities of the whole HMs from a large amount of candidates is the most crucial and challenging step for the quality control of HMs [4,5]. Currently, one or several abundant and bioactive components were selected as chemical markers for developing the quality standard in most cases [6,7]. However, the selection of such markers usually lack sufficient chemical and pharmacological evidence, resulting in poor bioactivity relevance in the quality control. It has been widely acknowledged that HMs take effects via a holistic mode of multiple-components and multiple-targets. Thus, it is important to identify a combination of chemical compounds which act together to elicit pharmacological effects and could be representative of the whole HMs. In this work, we proposed a method to discover most effective combinatorial markers (ECMs) as chemical markers in Lycoridis Radiatae Bulbus (LRB) for quality control. The major procedures of this method include (Fig. 1): (1) characterization of common peaks from fingerprints of 27 batches of LRB by high performance liquid chromatography-diode array detection (HPLC-DAD) profiling; (2) discovery and identification of markers that are most relevant with the AChE inhibitory activity of LRB by multiple linear regression (MLR) and Back propagation-Artificial Neural Network (BP-ANN); (3) the validation of using ECMs as chemical markers for the quality control of LRB samples by comparing with conventional markers.

* Corresponding author. Tel.: +86 25 83271060; fax: +86 25 83271060.
** Corresponding author. Tel.: +86 25 83271379; fax: +86 25 83271379.
E-mail addresses: hhp_770505@126.com (H. Hao), liping2004@126.com, yanghuacpu@126.com (P. Li).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.chroma.2014.04.015
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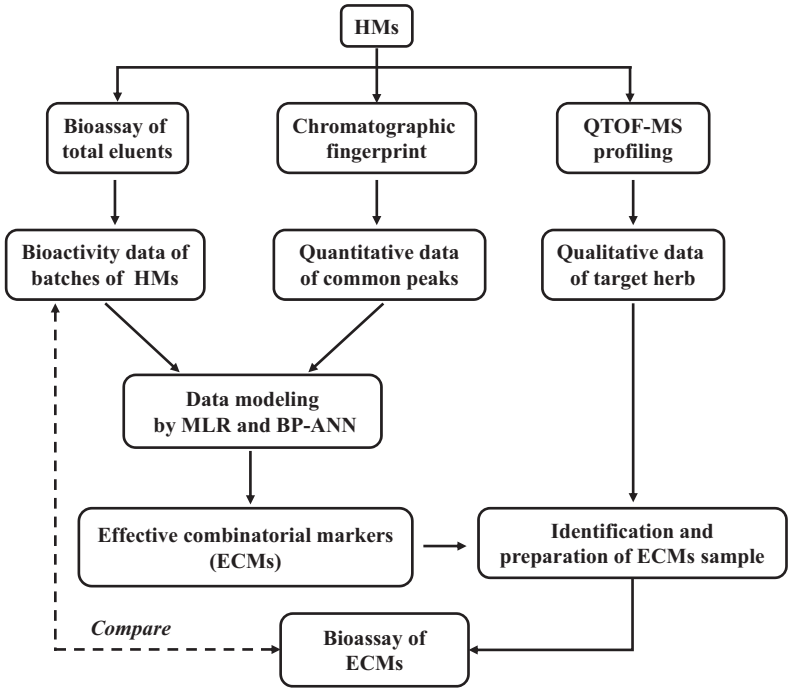

Fig. 1. Strategy to the characterization of effective combinatorial markers (ECMs) as the activity markers for quality standardization of herbal medicines.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (ACN) of HPLC grade was purchased from Merck (Darmstadt, Germany), ethylenediamine, chloroform and methanol of analytical grade was purchased from Jiangsu Hanbang Chemical Reagent Co., Ltd. (Nanjing, China). AChE, Acetylthiocholine iodide (ATCh), 5,5-dithiobis (2-nitro) benzoic acid (DTNB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Deionized water (18 MΩ) was prepared by distilled water through a Milli-Q system (Millipore, Milford, MA, USA). Other reagents were of analytical purity.

The standard alkaloid reference compounds, ungerimine, lycorine, dihydrolycorine, lycorenine, lycoramine, galanthamine, tazettine and *O*-methyllycorenine were purchased from Yangling Dongke Maidisen Medical Pharmaceutical Co., Ltd. (Xi'an, China). Hydroxyvittatine, natalensine, hippeastrine and sanguinine were generous gifts from Professor Wencai Ye. Their identities were confirmed by IR, ^{1}H and ^{13}C NMR and MS analysis. The purity of these alkaloids was determined to be more than 98% by LC-UV and LC-QTOF/MS methods. The structures of these reference compounds are shown in Fig. S1. Stock standard solutions of 12 reference alkaloids were prepared in methanol at a final concentration of 25 μg/ml. These solutions were stored at 4 °C for further study.

2.2. LRB sample preparation

A total of 27 batches of LRB were collected from various areas of China and authenticated by Professor Ping Li. The dried LRB were powdered and sieved through a No. 60 mesh, then dried at 50 °C in the oven for 4 h. Dried bulb powders (1 g) were pre-alkalized with 3 ml ammonia solution for 30 min, and immersed in 40 ml chloroform: methanol (4:1, v/v) mixture and weighed, then ultrasonicated for 90 min. The extract (20 ml) was evaporated and the residue was dissolved with 0.1 M HCl and transferred to 2 ml volumetric flask. The solution was shaken up and filtered with 0.45 μm millipore filter. The solution (500 μl) was then processed with cation-exchange/reversed-phase columns (Oasis MCX, 1 cc/30 mg, Waters, USA) [8]. The eluents were dried with nitrogen, dissolved with methanol and diluted 36 times for UV spectrometry and 125 times for HPLC injection.

2.3. Chromatography and mass spectrometry conditions

Chromatographic analysis was performed on an Agilent 1200 Series (Agilent Technologies, Germany) LC system equipped with an online degasser, a binary pump, an auto sampler, a thermostatically controlled column compartment and a diode array detector. Chromatographic separation was achieved at 25 °C on an Agilent ZorBax Extend-C_{18} column (4.6 mm × 250 mm, 5 μm). The separation was obtained using a gradient mobile phase consisting of water (0.05% ethylenediamine) (A) and acetonitrile (B). The gradient elution was set at: 8% B from 0–5 min, 8–11% B from 5–10 min, 11–21% B from 10–15 min, 21% B from 15–20 min, 21–23% from 20–25 min, 23% B from 25–30 min, 23–25% B from 30–45 min, 25–32% B from 45–50 min, 32–34% B from 50–65 min, 34–100% B from 65–80 min, 100% B from 80–95 min. A 20-min post run time back to the initial mobile phase composition was used after each analysis. The flow rate was kept at 0.5 ml/min, and was split at the column outlet to allow 50% eluent to flow into the mass spectrometer. The sample volume injected was set at 2 μl.

Mass spectrometry was performed on an Agilent 6530 QTOF MS (Agilent Corp, USA) equipped with an electrospray ionization (ESI) interface. The operating parameters were as follows: drying gas (N_2) flow rate, 11.0 L/min; drying gas temperature, 325 °C; nebulizer, 45 psig; capillary, 3500 V; skimmer, 65 V; OCT 1 RF Vpp, 750 V; fragmentor voltage, 120 V. The sample collision energy was set at 30 V. All the operations, acquisition, and analysis of data were operated under MassHunter Workstation Software Version B.02.00 (Agilent Technologies). Each sample was analyzed in positive mode to provide information for structural identification. Mass range was set at m/z 100–1000.