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High-throughput and sensitive screening of compounds with deoxyribonucleic acid-binding activity by a high-performance liquid chromatography–tandem mass spectrometry-fluorescence detection technique using palmatine as a fluorescence probe

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ABSTRACT

A high-throughput biochemical detection method based on the combination of high-performance liquid chromatography (HPLC), multiple-stage mass spectrometry (MS^n) and DNA-binding activity assay was developed and validated for the simultaneous screening and identification of DNA-binding compounds in complex samples. Palmatine was used as a sensitive, nontoxic and environmentally friendly DNA fluorescence probe. HPLC fingerprints, ultraviolet absorption spectra, MS^n fragments of components, and DNA-binding activity profiles could be simultaneously recorded during real-time analysis. Using the proposed method, 25 compounds were identified from *Lophatherum gracile* Brongn extracts, of which 18 were novel compounds first identified in these extracts. Nineteen compounds showed DNA-binding activity, most of which were flavone glycosides, with distinct dose-effect and structure–activity relationships. The method was validated and was proven to have a good linearity in the range of concentrations used in the study. The limit of detection was 0.2020 nmol. Our study indicated that the proposed method was sensitive, accurate, precise and reliable to be used for simultaneous screening and identification of DNA-binding compounds in complex samples.

1. Introduction

Over the past few decades, much attention has been paid to the binding of small molecules with DNA. Drug-DNA binding interactions, including insertions, groove bindings and alkylations [1], play important roles in the mechanisms of action of many drugs, such as antitumor, antibacterial and antiviral drugs. Therefore, DNA-binding compounds, whether natural or synthetic, have received considerable attentions as promising drug candidates.

It is generally believed that embedding of planar aromatic structures in DNA through insertion or/and groove binding can result conformational changes in the DNA double helix by causing relaxation or even unwinding of double-stranded DNA. These changes then interfere with DNA replication, transcription and repair processes. Such effects are conducive to cell apoptosis or death and therefore may be used to kill bacteria or viruses [2]. Since DNA intercalators and groove agents can kill fast-growing cells, bacteria and viruses [3], they have long been considered as promising drug candidates for the treatment of cancers, bacterial infections and viral infections [4]. However, currently used DNA-intercalators and groove agents are highly neurotoxic. This has been a major barrier to their clinical uses [5]. Thus, researchers are turning to medicinal herbs to obtain new natural DNA intercalators and groove agents.

Ethidium bromide (EB) is the earliest and most widely used fluorescent probe. EB can insert into the base pairs of DNA, causing a strong fluorescence signal. An intercalator with a DNA-binding ability greater than that of EB can displace EB from the DNA-EB complex, and consequently quench the fluorescence signal [6]. However, EB is an unstable, highly toxic, mutagenic, and carcinogenic reagent [7]. Improperly handled laboratory waste containing EB, may cause long-term environmental damage. Thus, development of new types of fluorescent probes with no toxicity, few side effects, and environment-friendly properties has become a necessity. Palmatine (PAL), an isoquinoline alkaloids, is present in many kinds of plants of the Berberidaceae family and has been reported to be able to interfere with DNA [8]. The fluorescence intensities of PAL and DNA are both very weak, but when they bind,

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a remarkable increase in fluorescence intensity is observed [9]. This could be used to study the interactions between small molecules and DNA. Because it is nontoxic, safe, and has a low environmental impact, PAL appears to be a suitable fluorescence probe [8].

Traditional procedures for the discovery of active compounds in complex mixtures are time-consuming, labor-intensive, and expensive strategies that can sometimes lead to loss of drug activity during the process of isolation and purification. Recent developments in high-performance liquid chromatography–multiple-stage mass spectrometry (HPLC–MS^n) make it possible to simultaneously identify multiple active ingredients in complex mixtures [10,11]. Nonetheless, HPLC–MS^n cannot provide the activity profiles of compounds. Fortunately, technology for the online detection of activity of compound, which combines HPLC–MS^n and biochemical detection methods, can make it come true by permitting rapid identification of active individual compounds and providing structural determination without the need for purification. The active assay of individual compounds and studies of the mechanisms, structure–activity relationships and concentration–effect relationships are also feasible by using this technology [12,13]. Moreover, the chromatographic separation step usually leads to sample clean up, reducing the matrix effect and improving the reproducibility of the biochemical assay [14]. Furthermore, problems of compound degradation and introduction of impurities prior to MS measurements and activity assays are avoided, and cross-reactivity of sample compounds is easier to handle [14]. In the present study, we combined HPLC–MS^n and online DNA-binding activity detection technology for the first time and established a high-performance liquid chromatography-diode array detection–multiple-stage mass spectrometry and fluorescence detection of PAL-DNA (HPLC-DAD–MS^n-DNA-PAL-FLD detection system) for rapid isolation, structure identification and activity screening of compounds from natural herbs simultaneously. Subsequently, we applied the developed online detection method for screening active DNA-binding compounds from *Lophatherum gracile* Brongn (*L. gracile*).

The dry stems and leaves of *L. gracile* are commonly used in traditional Chinese medicine for the treatment of fever, pain during urination, polydipsia and mouth sores [15]. However, few data are available regarding the active ingredients of *L. gracile* and the action mechanisms of these compounds. Although some reports [16,17] have described the 4 classic flavone glycosides (also known as orientin, isoorientin, vitexin and isovitexin) in *L. gracile*, *L. gracile* possesses wide variety of biological and pharmacological activities, such as antimicrobial activity and cardiovascular protection [18]. The precise mechanisms underlying these activities are still not fully understood. The present study provided a comprehensive analysis of the constituents of *L. gracile* and their DNA-binding properties. Results of this study could offer a theoretical foundation for the clinical use of *L. gracile*.

2. Experimental

2.1. Materials and reagents

Leaves of *L. gracile* were collected from Zhejiang City, China. The sample was authenticated by Professor Hong Wang of the Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University. Quercetin, 3-caffeoylquinic acid (3-CQA), 5-caffeoylquinic acid (5-CQA), vitexin, isovitexin, orientin, and isoorientin were obtained from the National Institute for the Control of Pharmaceutical (Beijing, China). PAL was purchased from the National Institute of Control of Pharmaceutical and Biological Products. The purity of all chemicals was more than 98%, as determined by HPLC. DNA from fish sperm was purchased from Sigma–Aldrich (Sigma Code D-3159, Germany).

HPLC-grade methanol (MeOH, Merck, Darmstadt, Germany) and acetonitrile (ACN, Merck, Darmstadt, Germany), analytical-grade formic acid (FA) and tetrahydrofuran (THF) from Suzhou Yacoo Chemical Reagent Corporation (Jiangsu, China) were used in the preparation of the mobile phase. Deionized water (18 MΩ) was prepared by filtering distilled water through a Milli-Q system (Millipore, MA, USA). Analytical-grade MeOH (Beijing Chemical Works, Beijing, China) was used for sample preparation.

2.2. Sample preparation

Powdered leaves of *L. gracile* (2.0 g, 60 mesh) were accurately weighed and extracted with 20 mL of 50% MeOH in an ultrasonic bath (28 kHz, 250 W) for 30 min. The extract solution was filtered through a 0.22 μm membrane prior to use, and a 10 μL aliquot was injected into the HPLC system for analysis.

2.3. Online detection of solutions

Stock solutions of DNA and PAL were prepared by directly dissolving the DNA and PAL in deionized water, and solutions were then stored at −4 °C in the dark. The concentration was determined to be 2.1×10^{-3} mol L^{-1} by absorption spectrometry, using the absorptivity $\varepsilon_{260} = 6600$ L mol^{-1} cm^{-1}. The purity of the DNA preparation was checked by monitoring the absorption spectrum and the ratio of the absorbances at 260 and 280 nm (A_{260} and A_{280}, respectively). The solution gave a ratio of $A_{260}/A_{280} > 1.8$, indicating that DNA was sufficiently free from protein [19]. Quercetin, 5-CQA, vitexin, and isoorientin were prepared as MeOH solutions with concentrations of 3.31×10^{-4}, 3.50×10^{-4}, 7.06×10^{-4}, and 2.23×10^{-4} mol L^{-1}, respectively. A 0.05 mol L^{-1} Tris–HCl buffer (pH 7.40) was used to control the pH of the reaction system.

2.4. HPLC and electrospray ionization-ion trap-time-of-flight (ESI-IT-TOF) mass spectrometry (MS) conditions

The chromatographic separation was carried out using an Intertsil ODS-4C_{18} column (5 μm, 150 mm × 4.6 mm, Shimadzu, Tokyo, Japan), at a temperature of 35 °C. Linear-gradient elution was performed using a mobile phase composed of A (MeOH containing 0.1% FA and 1% THF) and B (water containing 0.1% FA), with the following conditions: 0–5 min, 10% A; 5–10 min, 10–20% A; 10–20 min, 20% A; 20–30 min, 20–28% A; 30–35 min, 28% A; 35–45 min, 28–29.3% A; 45–55 min, 29.3–50% A; 55–65 min, 50–60% A; 65–70 min, 60–70% A. The flow rate was 1 mL min^{-1}, the online ultraviolet (UV) spectrum was recorded in the 200–400 nm range, and diode array detection (DAD) was set at 331 nm.

The effluent from the HPLC gradient was split and introduced into the ESI source at a flow rate of 0.2 mL min^{-1}. Optimized MS operating conditions were as follows: negative and positive ionization mode, scan spectra from m/z 100 to 700, and nebulizing of flow rate of nitrogen at 1.5 L min^{-1}. The curved desolvation line (CDL) temperature and block-heater temperature were maintained at 200 °C. The capillary voltage, CDL voltage, and detector voltage were fixed at 4.5 kV, 10 V, and 1.7 kV, respectively. Data were acquired and processed by LCMS Solutions software (version 3, Shimadzu, Kyoto, Japan), which included a predictor for chemical formulas. The data-dependent acquisition was set such that the most abundant ions in the full-scan MS would trigger multiple-stage mass spectrometry (MS^n, $n = 2$–4). In the automatic mode, all ions were first accumulated in the octopole and then rapidly pulsed into the ion trap for MS^n analyses according to the criteria settings. The collision energy for MS^n was adjusted to 50% in the HPLC–MS analysis, and the isolation width of precursor ions was 3.0 U. All ions produced were introduced into the TOFMS instrument for accurate mass determination.