Journal of Chromatography B 1083 (2018) 102–109

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Screening and isolation of potential neuraminidase inhibitors from leaves of *Ligustrum lucidum* Ait. based on ultrafiltration, LC/MS, and online extraction-separation methods

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ARTICLE INFO

Keywords:
Ligustrum lucidum
Neuraminidase inhibitor
Ultrafiltration
Pressurized liquid extraction
Centrifugal partition chromatography
Preparative liquid chromatography

ABSTRACT

Ultrafiltration liquid chromatography-mass spectrometry (ultrafiltration LC/MS) is introduced as an efficient method that can be applied to rapidly screen and identify ligands from the leaves of *Ligustrum lucidum* Ait. Using this method, we identified 13 compounds, including organic acids, flavonoids, and glycosides, as potent neuraminidase inhibitors. A continuous online method, employing pressurized liquid extraction followed by parallel centrifugal partition chromatography and preparative liquid chromatography PLE-(parallel-CPC/PLC), was developed for the efficient, scaled-up production of 12 compounds with high purities. The bioactivities of the separated compounds were assessed by an *in vitro* enzyme inhibition assay. The use of ultrafiltration LC/MS combined with PLE-(parallel-CPC/PLC), and an *in vitro* enzyme inhibition assay facilitated the efficient screening and isolation of neuraminidase inhibitors from complex samples, and could serve as an important platform for the large-scale production of functional ingredients.

1. Introduction

Neuraminidase is an enzyme present on the surface of influenza virus. It is an attractive target for agents against influenza infections, and its inhibitors have been widely used as anti-influenza drugs [1–4]. *Ligustrum lucidum* Ait. (Oleaceae) is a commonly used plant in China [5]. The leaves are the important part of *L. lucidum*, and have been used widely in China. The main chemical compounds present in the leaves of *L. lucidum* are flavonoids [6], secoiridoids [6,7], and glycosides. Recently, secoiridoids were shown to have potent *in vitro* anti-bacterial [8] and anti-influenza virus activities [9]. Flavonoids have been shown to have anti-influenza virus [10–12] and anti-inflammatory activities [13–15]. The identification and isolation of phenylethanoid glycosides and flavonoids from the leaves of *L. lucidum* are therefore important for the development of natural anti-influenza viral foods or drugs.

The leaves of *L. lucidum* are usually used in China to combat the influenza virus. Based on activity screening and medicinal plant data, we found that extracts from the leaves of *L. lucidum* have strong neuraminidase inhibitory activities (inhibition rate of 17.06% at a concentration of 10 μg/mL). Therefore, we investigated their chemical compositions and analyzed the neuraminidase inhibitory activities of the constituents. In the fast drug screening domain, receptor–ligand affinity ultrafiltration combined with HPLC/MS has been shown to be a powerful tool. This method has certain advantages including low sample consumption, no immobilization, reuse of enzymes, as well as high-throughput screening and identification of active compounds [16,17]. Moreover, ultrafiltration combined with LC/MS has been used to screen bioactive compounds [18].

CPC served as the liquid-liquid partition chromatography technology without a solid matrix. The principles behind CPC are similar to those of counter current chromatography (CCC) [19,20]. CPC has some advantages in the separation of glycosides; for example, when the mobile phase of CCC is *n*-butanol or another solvent with high viscosity, the retention of the stationary phase is low, which affects chromatographic peak resolution. Issues surrounding the low retention of the stationary phase with viscous solvent systems in CPC have largely been overcome [21]. CCC has usually been used to separate flavonoids. In addition to flavonoids, target compounds in this study also included phenylethanoid glycosides; these glycosides are insoluble in ethyl acetate, so a solvent system based on n-butanol and water was required,

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https://doi.org/10.1016/j.jchromb.2018.03.006
Received 21 August 2017; Received in revised form 23 January 2018; Accepted 5 March 2018
Available online 06 March 2018

followed usually by CPC separation [22–24]. However, CPC still has some disadvantages. When the rotation speed of the CPC column is fast, or the flow rate of the mobile phase is fast, the pressure in the CPC system will be large. Therefore, the rotation speed of the CPC column and the flow rate of the mobile phase cannot both be fast, as this would lead to a low separation efficiency, and other chromatography techniques would be required for the large-scale preparation of chemical components with a wide range of polarities [25]. The separation precision of PLC is much greater than that of CPC. For some complex mixtures, neither PLC nor CPC can purify all of the active target compounds in one step; PLC and CPC can be combined to purify target bioactive compounds with different polarities because of their complementary and orthogonal properties [26]. The combined use of PLC and other separation techniques is also carried out [27–29].

The aim of this study was to use neuraminidase binding combined with ultrafiltration LC/MS to screen neuraminidase inhibitors from *L. lucidum* leaves. The target bioactive compounds were then extracted and isolated online by PLE coupled with CPC and PLC (PLE-(parallel-CPC/PLC)).

2. Experimental

2.1. Instruments

A Dionex ASE 150 Accelerated Solvent Extraction System (ThermoScientific, Waltham, MA, USA) with a 100 mL stainless steel ASE vessel was used. A SIC CPC-240 system (System Instruments Co., Ltd., Hachioji, Japan) was refitted and used to carry out CPC. The CPC column was formed by stacking the circular partition disk rotor (2136 cells; ~240 mL). Two high-pressure rotary seals were used to connect the CPC column with the injector and detector. Semi-preparative high performance chromatography was performed using a 2545 Quaternary Gradient Module pump, Fraction Collector III, Waters 2489 UV/Vis detector, and semi-prep SunFire™ C_{18} Column (100 × 19 mm, I.D. 5 μm, Waters Corporation, Milford, MA). A Waters Acquity H-class instrument containing a Waters PDA detector (Milford, CT, USA) was used to perform UPLC. A Q-Exactive orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for mass spectrometry (MS) measurements.

2.2. Reagents and materials

L. lucidum leaves were obtained from the YuYanFang Medicinal Store on July 14, 2017 (Anguo, Hebei, China, batch number 20170702). Neuraminidase was purchased from Yuanye (enzyme of activity of 2.5 U, Shanghai, China), and phosphate-buffered saline (PBS) was purchased from Bueke, Switzerland. Microcon YM-10 ultrafiltration chambers with a molecular weight cutoff of 10,000 Da were obtained from Millipore (Bedford, MA, USA). HPLC-grade acetonitrile was obtained from Fisher Scientific (Shanghai, China). *n*-Butanol, *n*-hexane, and ethanol were of analytical grade (Beijing Chemicals, Beijing, China). Water with a resistivity of 18.2 MΩ/com was purified using a Labostar water purification system (Siemens, Munich, Bayern, Germany).

2.3. Screening procedure using neuraminidase inhibition assay and ultrafiltration LC/MS

The screening experiments were conducted using ultrafiltration LC/MS [1]. The incubation mixtures were prepared by mixing 60 μL of the 10 mg/mL sample solution, 60 μL of 20 μM neuraminidase, 0.3 M ethanesulfonic acid hydrate, and 4 mM calcium chloride in the buffer solution (total volume ~120 μL). The solvents were mixed on a plate shaker for 30 s. After incubating for 30 min at 37 °C, the mixture was transferred into an ultrafiltration chamber and centrifuged at 13000 × *g* for 15 min at 25 °C. Unbound materials on the filter were removed by centrifuging with 100 μL deionized water at room temperature. The bound ligands were released by centrifuging 3 times for 10 min with 200 μL methanol at 13000 × *g*. The solvent was removed under vacuum and the filtrate was redissolved in 50% methanol for LC analysis. Control experiments were performed with a denatured enzyme. The binding degree of the compounds to neuraminidase was based on the following equation:

$$\text{Binding degree}\ (\%) = (A_b - A_c)/A_a \times 100$$

where A_a refers to the peak area of the compound after addition to the buffer without interactions. A_b and A_c refer to the peak areas of the compounds that interact with the active or denatured neuraminidase.

We reduced the combined ultrafiltrates under vacuum and performed LC/MS analysis for the released ligands. Before the screening experiments, control experiments without neuraminidase were carried out.

A Waters Acquity BEH C_{18} column (50 × 2.1 mm, 1.7 μm) was adopted to isolate the bioactive compounds. The mobile phase contained water (solvent A) and acetonitrile (solvent B). The gradient program was set as 0–20 min, 93–30% A, with a flow rate of 0.4 mL/min, injection volume of 4 μL; the peaks of interest were evaluated at 245 nm. The mass spectrometer was operated in negative-ion mode for data collection. The samples were injected using an online LC system. The capillary voltage and spray voltage were 5 V and 4.0 kV, respectively. The capillary temperature was 320 °C. The desolvation gas flow rate was 15.0 L/min. Fragment ions were obtained using the in-source collision induced dissociation (CID) method.

2.4. Neuraminidase inhibition assay

The neuraminidase-inhibitory abilities of the CPC and PLC fractions were tested. Neuraminidase inhibition was determined using a modified version of a previously described spectrophotometric method [30]. A reaction mixture containing 70 μL of the reaction buffer solution, 10 μL of neuraminidase, and 10 μL of standard samples in 10% methanol were added to each well. Vibration mixing was carried out for approximately 1 min, and the samples were incubated at 37 °C for 2 min, so that the neuraminidase and standard samples could interact. Next, 10 μL of the fluorescent substrate was added to obtain a total volume of 100 μL. The entire mixture was thoroughly mixed under vibration for approximately 1 min and the plate was incubated at 37 °C for 20 min. The fluorescence was read on a TECAN A-5082 microplate reader instrument (Tecan Group Ltd., Austria), with an excitation wavelength of 360 nm and emission wavelength of 440 nm. The controls contained the same reaction mixture and same volume of water as the standard samples. The inhibition of neuraminidase activity was calculated using the equation:

$$\text{Inhibition}\% = (A_1 - A_2)/A_1 \times 100$$

where A_1 is the absorbance of the control and A_2 is the absorbance of the sample. IC_{50} was determined by plotting the percentage of neuraminidase activity against inhibitor concentration using manufacturer-provided software.

2.5. Measurement of partition coefficient values of the counter current chromatographic solvents

The composition of the two-phase solvent system was tested based on the partition coefficient (*K*) of the target compound. In each experiment, ~2.0 mg of the raw sample was placed into a test tube, and mixed with the equilibrated two-phase solvent system (1.0 mL of each phase), which was based on combinations of *n*-hexane, *n*-butanol, acetonitrile, water. The tube was then shaken to ensure thorough partitioning. When equilibrium was reached, the phases were isolated and evaporated under N_2 gas until completely dry. The residue was removed by dissolving in methanol and was characterized by UPLC. The