Journal of Chromatography A, xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Online screening of acetylcholinesterase inhibitors in natural products using monolith-based immobilized capillary enzyme reactors combined with liquid chromatography-mass spectrometry

Lvhuan Wang[a], Yumei Zhao[a], Yanyan Zhang[a], Tingting Zhang[a,b], Jeroen Kool[c], Govert W. Somsen[a,c], Qiqin Wang[a,b,*], Zhengjin Jiang[a,b,*]

[a] Institute of Pharmaceutical Analysis, College of Pharmacy, Jinan University, Guangzhou, 510632, China
[b] Guangdong Province Key Laboratory of Pharmacodynamic Constituents of Traditional Chinese Medicine & New Drug Research, Jinan University, Guangzhou, 510632, China
[c] Division of Bioanalytical Chemistry, AIMMS Research Group Biomolecular Analysis, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 28 March 2018
Received in revised form 26 May 2018
Accepted 29 May 2018
Available online xxx

Keywords:
Immobilized capillary enzyme reactor
Acetylcholinesterase
Comparative online ligand fishing
Monolith
Traditional Chinese medicines

ABSTRACT

In order to develop a direct and reliable method for discovering lead compounds from traditional Chinese medicines (TCMs), a comparative online ligand fishing platform was developed using immobilized capillary enzyme reactors (ICERs) in combination with liquid chromatography-mass spectrometry (LC–MS). Methacrylate-based monolithic capillaries (400 μm I.D. × 10 cm) containing epoxy reactive groups were used as support to immobilize the target enzyme acetylcholinesterase (AChE). The activity and kinetic parameters of the AChE-ICER were investigated using micro-LC-UV. Subsequently, ligand fishing and identification from mixtures was carried out using the complete AChE-ICER-LC–MS platform. For efficient distinction of true actives from false positives, highly automated comparative analyses were run alternatingly using AChE-ICERs and negative control-ICERs, both online installed in the system. After washing unbound compounds to the waste, bound ligands were eluted from the AChE-ICER to a trapping loop using a denaturing solution. The trapped ligands were further separated and identified using LC–MS. Non-specific binding to the monolith support or non-functional sites of the immobilized enzyme was investigated by exposing analytes to the negative control-ICER. The specificity of the proposed approach was verified by analyzing a known AChE inhibitor in the presence of an inactive compound. The platform was applied to screen for AChE inhibitors in extracts of *Corydalis yanhusuo*. Eight compounds (columbamine, jatrorrhizine, coptisine, palmatine, berberine, dehydrocorydaline, tetrahydropalmatine and corydaline) with AChE binding affinity were detected and identified, and their AChE inhibitory activities were further verified by an *in vitro* enzymatic inhibition assay. Experimental results show that the proposed comparative online ligand fishing platform is suitable for rapid screening and mass-selective detection of AChE inhibitors in complex mixtures.

1. Introduction

Traditional Chinese medicines (TCMs) are attracting increasing attention all over the world, due to their long historical clinical practice and appealing therapeutic efficacy. Moreover, TCMs possess high chemical scaffold diversity and can be considered as a huge and invaluable source of bioactive compounds for discovering promising new drugs [1,2]. However, because of the chemical complexity of TCMs, it is neither easy to identify bioactive constituents nor to elucidate their pharmacological mechanism. The conventional bioassay-guided fractionation approach has been a mainstream method for discovering bioactive compounds from natural products. Unfortunately, the isolation procedures central to this approach are usually labor-intensive, time-consuming and costly, and in many cases lead to the loss of bioactive compounds due to dilution and decomposition as well as sticking to vials, tubes, etc. [3]. Therefore, it is desirable to establish reliable and rapid methods for screening and identifying bioactive constituents from TCMs directly.

Taking advantage of good selectivity and high throughput, affinity-based approaches coupled to advanced chemical detectors have been frequently used to screen bioactive compounds in

* Corresponding authors at: Institute of Pharmaceutical Analysis, College of Pharmacy, Jinan University, Guangzhou, 510632, China
E-mail addresses: qiqinxtu@163.com (Q. Wang), jzjjackson@hotmail.com (Z. Jiang).

https://doi.org/10.1016/j.chroma.2018.05.069
0021-9673/

TCMs [4–7]. Ligand fishing is a well-developed affinity-based technique in which selective binding of ligands to target enzymes or receptors allows separation from unbound components of TCMs. The bound ligands are subsequently dissociated and identified using liquid chromatography-mass spectrometry (LC–MS). Up to now, ligand fishing experiments have been carried out in different formats, including ultrafiltration [8], equilibrium dialysis [9], nanotubes [10], magnetic beads [11,12], zeolite [13] and hollow fibers [14]. These methods are mostly applied in an offline mode, which often is tedious and suffers from time-consuming analytical steps involving incubation, separation, dissociation, and analysis. Online ligand fishing may be more attractive, since the incubation, ligand-enzyme/receptor complex isolation, dissociation and HPLC–MS analysis can be carried out in a continuous, automated fashion, which can greatly enhance the screening efficiency [15].

Affinity based solid-phase extraction columns, which use enzyme-functionalized media for capturing potential ligands, have been employed for online ligand fishing. Jonker et al. used dynamic protein-affinity chromatography solid-phase extraction (DPAC-SPE) combined with LC–MS for screening and identifying estrogen receptor alpha (ERα) ligands in complex mixtures. However, this DPAC-SPE method can only be used for fishing of His-tagged proteins [16]. Recently, Peng et al. established online coupling of an affinity SPE column with LC–MS/MS for fishing xanthine oxidase (XO) inhibitors which allowed rapid isolation and identification of inhibitors from complex mixtures [17]. However, the efficient packing of affinity SPE columns, particularly in micro- and capillary format, can be difficult. Polymeric monoliths have shown to be a highly useful alternative support material to immobilize proteins for e.g. proteomics studies [18], ligand-protein binding studies, and ligand affinity ranking studies [19–21]. So far, the use of monolith-based immobilized capillary enzyme reactors (ICERs) for online ligand fishing, particularly in relation to TCM profiling, has not been reported.

When applying ligand fishing methods, due attention should be paid to the prevention of false positives caused by non-specific binding of compounds to the support material and/or non-functional sites of the enzyme [11,22]. Recently, Chen et al. developed an online comparative cell membrane chromatography (CMC) method by simultaneously using CMC columns packed with normal and pathological tissue-derived silica. This approach effectively increased the specificity of the screening results through visualized comparison of the chromatographic affinity behaviors between normal and pathological CMC columns [23].

Acetylcholinesterase (AChE) can terminate nerve impulse by hydrolyzing active neurotransmitter acetylcholine (ACh) in central nervous system (CNS) [24]. The inhibition of AChE from breaking down acetylcholine (ACh) is one of the most important therapeutic strategies in Alzheimer's disease treatment. Furthermore, AChE inhibitors can be used as insecticides to kill insects [25,26]. It is of importance to find new inhibitors that could modulate AChE activity. Some AChE-based immobilized enzyme reactors (AChE-IMERs) have already been developed for screening AChE inhibitors from pure compound library or assessing the overall inhibitory activity of natural products [27–30]. For example, Bartolini et al. developed a human recombinant AChE micro-immobilized enzyem reactor (hrAChE-IMER) by immobilizing hrAChE on monolithic disk (12mm × 3mmi.d.) [31]. The prepared hrAChE-IMER allowed to screen potential hrAChE inhibitors rapidly from pure compound library, but it was not used as SPE column to directly fish ligands in natural products.

In this study, AChE-ICERs and control-ICERs were prepared through immobilizing AChE onto the surface of a poly (glycidyl methacrylate-*co*-ethylene dimethacrylate) (poly (GMA-*co*-EDMA)) monolithic support through a ring opening reaction between epoxy groups and amine groups. The resulting AChE-ICER and control-ICER were installed in parallel as SPE columns to establish a comparative online ligand fishing platform for rapid separation and identification of AChE ligands in TCMs (as shown in Fig. 1). With this system, ligands are first captured on the AChE-ICER, while inactive compounds are flushed to waste by washing buffer. For identification, the bound ligands are desorbed and eluted to LC–MS through valve switching. Parallel comparison is conducted by performing two subsequent analytical runs on the different SPE columns to eliminate false results caused by non-specific binding. The applicability of this comparative online ligand fishing platform was tested by screening AChE inhibitors from extracts of *Corydalis yanhusuo*. The activity of the found ligands was verified by an AChE inhibitory assay.

2. Experimental

2.1. Chemicals and materials

Acetylcholinesterase from *Electrophorus electricus* (eelAChE) type VI-S, acetylthiocholine iodide (ATCh) and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) were purchased from Sigma-Aldrich (Shanghai, China). 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS), 2,2′-azobisisobutyronitrile (AIBN), glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), galantamine, 1,4-butanediol, 1-propanol and ammonium acetate were all purchased from Aladdin Chemicals (Shanghai, China). Acebutolol hydrochloride was obtained from the Guangdong Institute for Food and Drug Control (Guangzhou, China). Coptisine, berberine, jatrorrhizine hydrochloride, palmatine hydrochloride, columbamine, dehydrocorydaline, tetrahydropalmatine and corydaline were purchased from Shanghai Yuanye Bio-Technology Co. Ltd. HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Merck (Shanghai, China). The fused-silica capillaries with an inner diameter of 400 μm (800 μm O.D.) were purchased from Polymicro Technologies Ltd. (Phoenix, AZ, USA).

2.2. Instrumentation

Enzyme immobilization was carried out by using a precise peristaltic pump (Baoding Longer Pump Company, Hebei, China) with a 2 mLsyringe. A Jinghong DK-S22 water bath (Shanghai, China) was used for the thermally initiated co-polymerization. All scanning electron microscopy (SEM) experiments were performed on a Leo 1530 VP Field Emission Scanning Electron Microscope equipped with Oxford INCA 400 energy dispersive X-ray microanalysis (Oberkochen, Germany) at an acceleration voltage of 1 kV. The *in vitro* AChE inhibitory assays were conducted on a microplate reader (Biotek Instrument, USA).

The immobilized enzyme catalytic activity assays were performed by installing the ICERs on a self-assembled micro-LC system, which is composed of a DiNa-S nano pump (Tokyo, Japan), a Dionex Spark 920 LC Packing Famos Autosampler (Emmen, Netherlands) with 1 μL sample loop and an Applied Biosystems 785A UV–vis detector (USA). LC–MS experiments were carried out on an Agilent 1260 Infinity Series HPLC analytical system coupled to Agilent 6540 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The 1260 infinity HPLC analytical system consisted of a quaternary pump, a degasser, an autosampler with 5 μL sample loop, a column oven, a six-port dual-position valve and a diode array detector.

The online platform is an integration of some modules of the 1260 infinity HPLC analytical system (Agilent Technologies, USA) and a PU-1580 Jasco binary pump (Kyoto, Japan). Two additional Valco two position switching valves with a four-port and a six-port (Houston, TX, USA), respectively, were used for con-

Please cite this article in press as: L. Wang, et al., Online screening of acetylcholinesterase inhibitors in natural products using monolith-based immobilized capillary enzyme reactors combined with liquid chromatography-mass spectrometry, J. Chromatogr. A (2018), https://doi.org/10.1016/j.chroma.2018.05.069