Journal of Chromatography A, 1345 (2014) 50–56

Contents lists available at ScienceDirect

Journal of Chromatography A

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

High-performance liquid chromatography coupled with post-column dual-bioactivity assay for simultaneous screening of xanthine oxidase inhibitors and free radical scavengers from complex mixture

D.Q. Li[a,b], J. Zhao[a], S.P. Li[a,*]

[a] State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, China
[b] Department of Pharmacy, The Second Hospital of Hebei Medical University, Shijiazhuang, China

ARTICLE INFO

Article history:
Received 12 November 2013
Received in revised form 25 March 2014
Accepted 25 March 2014
Available online 3 April 2014

Keywords:
Xanthine oxidase inhibitors
Free radical scavengers
Post-column dual-bioactivity assay

ABSTRACT

Xanthine oxidase (XO) can catalyze hypoxanthine and xanthine to generate uric acid and reactive oxygen species (ROS), including superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide. XO inhibitors and free radical scavengers are beneficial to the treatment of gout and many related diseases. In the present study, an on-line high-performance liquid chromatography (HPLC) coupled with post-column dual-bioactivity assay was established and successfully applied to simultaneously screening of XO inhibitors and free radical scavengers from a complex mixture, *Oroxylum indicum* extract. The integrated system of HPLC separation, bioactivity screening and mass spectrometry identification was proved to be simple and effective for rapid and sensitive screening of individual bioactive compounds in complex mixtures.

1. Introduction

Xanthine oxidase (XO) can catalyze the oxidation of hypoxanthine and xanthine to uric acid with the generation of reactive oxygen species (ROS) including superoxide anion radical and hydrogen peroxide [1]. The overproduction of uric acid (hyperuricemia) in the body is a key risk factor for the development of gout and ROS generated in the enzymatic process are involved in oxidative damage which is linked to many pathological processes such as renal dysfunction, cardiovascular diseases, hypertension, hyperlipidemia, cancer, and diabetes, etc. [2]. Therefore, XO inhibitors and free radical scavengers have been proposed as potential preventative or therapeutic agents for gout and/or many other related diseases. In clinic, allopurinol is most commonly used for the treatment of gout, although remarkably effective, sometimes the serious adverse effect of hypersensitivity syndrome will limit its usage [3]. As a consequence, the recent trend has been toward developing safer and natural inhibitors as alternatives to allopurinol.

Until now, several methods have been reported for the assessment of XO inhibitory activity such as spectrophotometric [4] and HPLC [5] methods and superoxide scavenging activity like chemiluminescence [6], respectively. However, all the methods determined the XO inhibitory and superoxide scavenging activities individually. Recently, Liu et al. [7] developed an ultra high-performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (UHPLC–QqQ–MS) method for simultaneous screening of XO inhibitors and superoxide anion scavengers. Although this improved method enabled to determine these two activities of some herbal extracts or pure compounds from natural products in a single analysis, to elucidate the activity of separated compounds in complex mixtures was not possible. Besides, TLC (thin-layer chromatography) autographic method was developed for simultaneous detection of XO inhibitors and superoxide scavengers from natural product [8]. However, this method has two main disadvantages of the low separation ability of analytes and the absence of compounds identification. In recent years, on-line HPLC coupled with post-column bioactivity detection methods for screening of enzymatic inhibitors or antioxidants from complex mixtures have been established [9,10]. By combination of HPLC separation, bioactivities measurement and rapid identification, this technique allows to rapidly screen active compounds from complex mixture. The post-column antioxidant activity-based detection has been broadly used for screening of potential antioxidants and most of them used DPPH or ABTS as radicals [10]. On the other hand, for inhibitors screening, the on-line HPLC coupled with biochemical detection has been only described for few target enzymes, such as acetylcholinesterase [11], *alpha*-glucosidase [12] and human cytochrome P450 1A2 [13]. To

* Corresponding author. Tel.: +853 8397 4692; fax: +853 2884 1358.
E-mail addresses: zhaojing.cpu@163.com (J. Zhao), lishaoping@hotmail.com, SPLI@UMAC.MO (S.P. Li).

http://dx.doi.org/10.1016/j.chroma.2014.03.065
0021-9673/

the best of our knowledge, the on-line method for XO inhibitors screening has not been installed previously.

In the present study, an analytical method using on-line HPLC coupled with post-column dual-bioactivity assay (XO inhibition and antioxidant assays) was developed to reveal the XO inhibitory and free radical scavenging activities of individual compounds from complex mixture. The applicability of the on-line high-throughput screening was demonstrated by the analysis of a natural product, *Oroxylum indicum* extract. The compounds with the activities of XO inhibition and/or radical scavenging were simultaneously and rapidly screened and then identified by comparing their UV and MS data with that of standard substances and/or previous reports.

2. Materials and methods

2.1. Materials and reagents

Xanthine oxidase (EC 1.1.3.22) from bovine milk, xanthine, allopurinol and uric acid (UA) were purchased from Sigma (St. Louis, MO, USA). Rutin, quercetin and baicalein were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 99%) was purchased from International Laboratory (San Bruno, CA, USA). Potassium persulfate (99%) was from Fluka (Seelze, Germany). HPLC grade formic acid, acetonitrile (ACN), methanol (MeOH) and ethanol were purchased from Merck (Darmstadt, Germany); water was obtained from Milli-Q water purification system (Milford, MA, USA); solvents and all other chemicals not mentioned were of analytical grade.

Seeds of *Oroxylum indicum* were collected from Foshan, Guangdong Province, China. The species was authenticated by Dr. Chunfeng Qiao, and voucher specimens were deposited at ICMS, University of Macau, Macao, China.

2.2. Preparation of Oroxylum indicum extract

The dried plant materials were pulverized, and 1 g power was extracted with methanol (10 mL) for 30 min at room temperature under ultrasonication. After centrifugation at 4000 × *g* for 5 min (Allegra X-15R, Beckman Coulter, Fullerton, CA), the supernatants were filtered through a 0.45 μm filter (Agilent Technologies) before injection into HPLC system for analysis.

2.3. Preparation of $ABTS^{\bullet+}$ solution

ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS solution (7 mM in water) with 2.5 mM potassium persulfate (final concentration) for 12 h with a ratio at 2:1 (v/v) at 4 °C in dark (stock solution). Then the $ABTS^{\bullet+}$ stock solution was diluted with ethanol to an absorbance of approximately 1.0 at 750 nm, which was stable for at least 2 days in the dark.

2.4. Determination of XO inhibition using microplate-based HPLC

The XO inhibition was determined by a HPLC method based on the assay developed by Nagao et al. [5]. The substrate (0.25 mM) and enzyme (0.1 U/mL) were dissolved in 50 mM phosphate buffer (pH 7.5). The assay was performed by mixing 50 μL of buffer, 20 μL of substrate solution with 20 μL of inhibitor solution (or phosphate buffer for control). Then 10 μL of XO was added and they were incubated at room temperature of 26 °C for 10 min with continuous agitation at 450 rpm (Thermomixer comfort, Eppendorf, Germany). The reaction was stopped by addition of 10 μL of 5 M hydrochloric acid and the solution was centrifuged at 1.2×10^5 rpm (Centrifuge 5415D, Eppendorf, Germany) before being analyzed by reversed-phase HPLC.

Table 1
The chromatographic conditions for sample separation.

Selected analytes[a]		*Oroxylum indicum* extract[b]	
Time	ACN (%)	Time	ACN (%)
0	5	0	18
5	15	15	25
10	25	20	35
15	30	30	60
20	45	35	60
30	50		

[a] The other mobile phase was 0.2% formic acid.
[b] The other mobile phase was 0.3% formic acid.

An Agilent series 1100 (Agilent Technologies) liquid chromatography, equipped with a vacuum degasser, a quaternary pump, an autosampler, a diode array detector controlled by Agilent Chemstation software were used for the UA analysis. Separation was performed on a Zorbax XDB C_{18} column (150 mm × 4.6 mm i.d., 5 μm) with a Zorbax XDB C_{18} guard column (12.5 mm × 4.6 mm i.d., 5 μm) column. The mobile phase was 10 mM potassium dihydrogen phosphate solution (pH 4.7). The flow rate was 0.6 mL/min and the absorbance detector was set at 290 nm. After injection, the UA peak areas of control and the assay with inhibitor were obtained and the percentage of inhibition was calculated according to the equation: $\text{inhibition\%} = [1 - (A_{\text{inhibitor}}/A_{\text{control}})] \times 100\%$, where $A_{\text{inhibitor}}$ and A_{control} were the UA peak areas of the assay with inhibitor and of the control sample without inhibitor, respectively.

2.5. Continuous xanthine oxidase assay

A conventional flow injection analysis (FIA) manifold with diode array detector used as the biochemical detector was constructed (Fig. S1A). A double-infuse syringe pump equipped with two 20 mL plastic syringes was used. One was filled with enzyme (XO in PBS, 0.08 U/mL) and the other was filled with substrate (1.0 mM xanthine in PBS). The two syringes were connected to a static mixer and infused at a flow rate of 50 μL/min each. An Agilent HPLC pump (carrier pump) was connected to the mixer as well and operated at a flow rate of 100 μL/min of phosphate buffer. A 0.25-mm internal diameter (I.D.) reaction coil made of 0.3 m PEEK tubing was connected to the mixing device. After mixing in a knitted reaction coil, the substrate xanthine was converted into UA which was detected at 295 nm.

Positive drug, allopurinol, at different concentrations was also injected into the carrier stream. It can compete with xanthine for the active site of XO so as to cause a temporarily decreased production of UA monitored by biochemical detector and IC_{50} was determined.

2.6. On-line HPLC coupled with post-column dual-bioactivity assay

Fig. S1B (supplementary material) illustrated the scheme of post-column dual-bioactivity assay coupled with on-line HPLC separation method for screening of XO inhibitors and free radical scavengers. Sample separation was performed on an Agilent series 1200 liquid chromatography (Agilent Technologies), equipped with a vacuum degasser, two quaternary pumps, an autosampler, two diode array detectors and an ion-trap mass spectrometer with electrospray ionization interface, controlled by Agilent LC/MSD Trap software. A Zorbax SB-Aq C_{18} column (150 mm × 4.6 mm i.d., 5 μm) with a Zorbax SB-Aq C_{18} guard column (12.5 mm × 4.6 mm i.d., 5 μm) was used. The separation was achieved by a gradient elution of formic acid aqueous (A) and acetonitrile (B) at a flow rate of 1 mL/min (Table 1). To maintain a constant concentration