Journal of Chromatography A, xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Bioprofiling of *Salvia miltiorrhiza* via planar chromatography linked to (bio)assays, high resolution mass spectrometry and nuclear magnetic resonance spectroscopy

Ebrahim Azadniya, Gertrud E. Morlock*

Chair of Food Science, Institute of Nutritional Science, Interdisciplinary Research Center (IFZ), Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32, 35392, Giessen, Germany

ARTICLE INFO

Article history:
Received 28 August 2017
Received in revised form 4 December 2017
Accepted 5 December 2017
Available online xxx

Keywords:
HPTLC
Direct bioautography (DB)
High resolution mass spectrometry (HRMS)
Preparative layer chromatography (PLC)
NMR
Salvia miltiorrhiza root (Danshen)

ABSTRACT

An affordable bioanalytical workflow supports the collection of data on active ingredients, required for the understanding of health-related food, superfood and traditional medicines. Targeted effect-directed responses of single compounds in a complex sample highlight this powerful bioanalytical hyphenation of planar chromatography with (bio)assays. Among many reports about biological properties of *Salvia miltiorrhiza* Bunge root (Danshen) and their analytical methods, the highly efficient direct bioautography (DB) workflow has not been considered so far. There was just one TLC-acetylcholinesterase (AChE) method with a poor zone resolution apart from our two HPTLC-DB studies, however, all methods were focused on the nonpolar extracts of Danshen (tanshinones) only. The current study on HPTLC-UV/Vis/FLD-(bio)assay-HRMS, followed by streamlined scale-up to preparative layer chromatography (PLC)-^{1}H-NMR, aimed at an even more streamlined, yet comprehensive bioanalytical workflow. It comprised effect-directed screening of both, its polar (containing phenolics) and nonpolar extracts (containing tanshinones) on the same HPTLC plate, the biochemical and biological profiling with four different (bio)assays and elucidation of structures of known and unidentified active compounds. The five AChE inhibitors, salvianolic acid B (SAB), lithiospermic acid (LSA) and rosmarinic acid (RA) as well as cryptotanshinone (CT) and 15,16-dihydrotanshinone I (DHTI) were confirmed, but also unidentified inhibitors were observed. In the polar extracts, SAB, LSA and RA exhibited free radical scavenging properties in the 2,2-diphenyl-1-picrylhydrazyl assay. CT, DHTI and some unidentified nonpolar compounds were found active against Gram-positive *Bacillus subtilis* and Gram-negative *Aliivibrio fischeri* (LOD 12 ng/band for CT, and 5 ng/band for DHTI). For the first time, the most multipotent unidentified active compound zone in the *B. subtilis*, *A. fischeri* and AChE fingerprints of the nonpolar Danshen extract was identified as co-eluted band of 1,2-dihydrotanshinone and methylenetanshinquinone in the ratio of 2:1.

1. Introduction

The unique hyphenation portfolio of high-performance thin-layer chromatography (HPTLC) with other complementary techniques makes HPTLC a versatile tool from bioactivity screening to structural characterization. All the relevant steps can be covered by the planar format, and thus, easily adapted and combined with (bio)assays, derivatization reagents and different detection techniques, *e.g.* UV/Vis/FLD/MS detection [1], nuclear magnetic resonance (NMR) [2–6] and attenuated total reflection Fourier transform infrared spectroscopies [7]. Exclusive features of HPTLC, like simple preparation of complex samples (keeping sample extracts as native as possible) and parallel matrix-robust analyses, allow an ideal direct comparison of the chromatographic fingerprints of complex sample extracts side by side. (HP)TLC is a cost-effective and time-saving method in the field of natural product analysis [8–12], and diverse publications in the field of bioautography [2,6,8–15] reported its benefits for chemical and biological profiling. Compared to contact and agar overlay bioautography, direct bioautography (DB) is a more streamlined workflow for bioprofiling, considering detectability, resolution and analysis time [12]. Different types of effect-directed analysis (EDA) combined with TLC/HPTLC have been employed, *e.g.*, to screen for

* Corresponding author.
E-mail addresses: Ebrahim.Azadniya@chemie.uni-giessen.de (E. Azadniya), Gertrud.Morlock@uni-giessen.de (G.E. Morlock).

https://doi.org/10.1016/j.chroma.2017.12.014
0021-9673/

enzyme inhibitors [4,15–17] as well as antibacterial [8,12], antifungal [6,14] and antioxidant compounds [10,11] in a wide range of different sample types.

With regard to structure elucidation of unidentified active compounds, the hyphenation between TLC/HPTLC and NMR has not been considered enough due to the notable difference between the sensitivity of TLC/HPTLC (mainly ng/band) and NMR spectroscopy (hundreds of μg to some mg per vial) [18]. The use of an elution head-based interface for the zone collection from the plate for NMR [2,4] led to consume a high number of HPTLC plates to reach the required analyte amount. Thus subsequent to EDA, other separation techniques were mostly used for NMR compound collection, such as column chromatography (CC) [19], high-performance liquid chromatography (HPLC) [11] and low-flow LC-NMR with microfractionation [6].

In this study, *Salvia miltiorrhiza* Bunge root (Danshen) was selected, as one of the most commonly used traditional medicines in China, Japan, USA and other western countries [20]. It possesses several curative properties that have been applied to treat different diseases such as Alzheimer's, Parkinson's, cerebrovascular and coronary heart diseases, besides cancer, renal deficiency, bone loss, hepatocirrhosis, gynecologic disorders and skin lesions like chilblains, psoriasis, and carbuncle [20]. A variety of Danshen preparations and formulations are on the market, *i.e.* dripping pills, tablets, injections, capsules, syrups and sprays [21]. Danshen's well-known bioactive components include two major groups, hydrosoluble phenolics (phenolic acids) and lipophilic diterpenoid quinones (tanshinones). So far, 37 phenolics and 55 tanshinones have been reported for Danshen [22]. In order to obtain the chemical profiles of the main phenolics and/or tanshinones, several separation methods like HPLC-MS [23–25], HPLC-UV [25–27], countercurrent chromatography (CCC) [28], non-aqueous capillary electrophoresis [29] and HPTLC [26,30] were employed. Among the many reports about biological properties of Danshen and their analytical methods [20], DB has not been considered so far. To the best of our knowledge, only one biochemical assay (falsely named bioautographic method) was combined with TLC in 2009 [16]. Thus, for the first time in our previous studies [31,32], DB of the nonpolar extract of Danshen was shown using Gram-negative *Aliivibrio fischeri* and Gram-positive *Bacillus subtilis* bacteria for bioquantification. The directly obtained bioactive responses of single compounds in the complex Danshen samples encouraged us to proceed with this streamlined hyphenation of chromatography and bioassays to demonstrate an even more powerful bioanalytical option in contrast to the *status quo*, *e.g.*, analytical and fractional workflows followed by microtiter plate assays. This study aimed at developing a streamlined, yet comprehensive bioanalytical method, *i.e.* the fastest approach from screening to structures. Therefore, biological and biochemical profiling by HPTLC-(bio)assay-HRMS of both, the polar and the nonpolar Danshen extracts on the same plate, was followed by characterization and identification of known and unidentified bioactive compounds by a fast scale-up to preparative layer chromatography (PLC)-^{1}H-NMR spectroscopy.

2. Material and methods

2.1. Chemicals and materials

Solvents were of analytical grade. toluene, ethyl acetate, chloroform, methanol, formic acid (96%), ammonia (25%), sulfuric acid (98%), petroleum ether (60–80 °C), cyclohexane, Müller–Hinton broth, anisaldehyde, protocatechuic aldehyde (PCAD) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, ≥98%) were purchased from Carl Roth (Karlsruhe, Germany). Fast Blue B salt, α-naphthyl acetate, acetylcholinesterase (AChE) lyophylisate (*Electrophorus electricus*, electric eel), hydrochloric acid (32%), sodium chloride, sodium monohydrogen phosphate, pentyl acetate, bovine serum albumin, tris(hydroxymethyl)aminomethane (TRIS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) were provided from Sigma–Aldrich (Steinheim, Germany). *B. subtilis* spores (BGA, ATCC 6633), citric acid as well as HPTLC (0.2 mm layer thickness) and PLC (0.5 mm layer thickness) plates silica gel 60, also with F_{254}, were delivered by Merck (Darmstadt, Germany). Luminescent marine bacteria *A. fischeri* (DSM no. 5171) were obtained from the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Bidistilled water was prepared with a Destamat Bi 18 E (Heraeus, Hanau, Germany). The standard compounds of rosmarinic acid (RA), caffeic acid (CA), tanshinone I (TI), tanshinone IIA (TIIA), cryptotanshinone (CT), 15,16-dihydrotanshinone I (DHTI) were purchased from Sigma-Aldrich, whereas salvianolic acids A (SAA) and B (SAB) as well as lithospermic acid (LSA) were provided by Phytolab (Vestenbergsgreuth, Germany). NMR tubes (5 mm) were purchased from VWR (Darmstadt, Germany), and deuterated methanol (D ≥99.80%, H_2O <0.03%) from Eurisotop (Saarbrücken, Germany). Two Danshen samples were purchased from pharmacies (sample A, HerbaSinica Hilsdorf, Rednitzhembach, Germany, and sample B, meine-teemischung, Hofapotheke St. Afra, Augsburg, Germany).

2.2. Standard solutions and sample preparation

Methanolic solutions of DHTI (20 ng/μL), CT (100 ng/μL), TI (100 ng/μL), TIIA (100 ng/μL), SAB (1000 ng/μL), LSA (500 ng/μL), SAA (500 ng/μL), PCAD (500 ng/μL), CA (250 ng/μL) and RA (150 ng/μL) were prepared. For subsequent sample preparation, the following steps were in common for all the different extracts (Table S-1 with concentrations of Danshen in mg/mL solvent): 15 min in the ultrasonic bath at room temperature, centrifugation at 756 × *g* for 5 min and vortexing for 1 min.

2.2.1. Polar extracts 1–3

Milled Danshen (1 g each of samples A and B) were decocted on the hotplate stirrer with 50 mL distilled water at 70 °C for 30 min, followed by ultrasonication and centrifugation (20 mg/mL). For the first extract (1), the resulting supernatant was acidified with hydrochloric acid (32%, 12 M) to pH 3.0. After centrifugation, the supernatant was transferred into a 50-mL volumetric flask, filled up to the mark with bidistilled water and extracted 5 times with 5 mL ethyl acetate (40 mg/mL). After centrifugation, 15 mL of this upper ethyl acetate phase was concentrated to dryness under a nitrogen stream. The residue was dissolved in 2 mL ethyl acetate (300 mg/mL).

For the second extract (2), 10 mL aqueous supernatant of sample B (20 mg/mL) was acidified by adding 200 μL hydrochloric acid (32%). After centrifugation, 0.5 g sodium chloride was added (salting-out effect). After vortexing, this suspension was extracted with 4 mL of a 1:1 (*V*/*V*) mixture of ethyl acetate (extractant) and acetone (disperser solvent) as dispersive liquid-liquid extraction (DLLE), vortexed and centrifuged. The upper ethyl acetate phase was used (2 mL, 100 mg/mL). For the third extract (3) as a reference, milled Danshen (1 g) was extracted two times with 5 mL methanol (100 mg/mL), vortexed, ultrasonicated and centrifuged. The supernatant was used.

2.2.2. Non-polar extracts 4 and 5

The fourth extract (4) was extracted as for (3), but two times with 5 mL ethanol - pentyl acetate (4:1, *V*/*V*). A 5-mL aliquot of the supernatant (100 mg/mL) was evaporated to dryness. The residue

Please cite this article in press as: E. Azadniya, G.E. Morlock, Bioprofiling of *Salvia miltiorrhiza* via planar chromatography linked to (bio)assays, high resolution mass spectrometry and nuclear magnetic resonance spectroscopy, J. Chromatogr. A (2017), https://doi.org/10.1016/j.chroma.2017.12.014