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Short communication

An approach to speed up the isolation of hydrophilic metabolites from natural sources at semipreparative level by using a hydrophilic–lipophilic balance/mixed-mode strong cation exchange–high-performance liquid chromatography/mass spectrometry system

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ABSTRACT

An approach to speed up the isolation of hydrophilic metabolites in complex natural matrixes by using a HLB/MCX–HPLC/MS system based on the retention properties of hydrophilic–lipophilic and cation exchange polymeric cartridges was developed. This methodology was successfully applied to the re-isolation of small water soluble compounds with completely different structures from two different natural extracts such as a dipeptide (vanchrobactin) from a bacterium culture broth and a pyrrolidine bearing a carboxylic acid moiety (clionapyrrolidine A) from a sponge. This method improved not only the efficiency of the isolation methodology but also the isolation time in relation to the existing methods.

1. Introduction

In recent decades considerable progress has been made in the search for bioactive secondary metabolites from microbial, plant and marine sources. This is a challenging task but is also rewarding because natural products have been the single most productive source of leads for the development of new drugs [1–4]. Advances in high-performance liquid chromatography (HPLC) and other chromatographic techniques for semi-preparative separation such as high-speed countercurrent chromatography, gel permeation chromatography and solid phase extraction (SPE), have enabled chemists to separate even the most complex mixtures of both non-volatile and volatile compounds into their individual components [5,6]. In addition, considerable progress has been made in detection and hyphenation in analytical techniques. The direct coupling of HPLC with either mass spectrometry (MS) or NMR and GC–MS now allows the quick and straightforward identification and quantification, with high sensitivity and selectivity, of individual components in complex mixtures [7–10].

Despite the remarkable advances in purification methods, the isolation of pure metabolites frequently remains not only highly labour-intensive and time-consuming but is also expensive and contaminating in terms of environmental conservation. Furthermore, as Shimizu and Li stated "the isolation of small water-soluble molecules still remains a mystery for natural-product researchers" [11]. Indeed, aqueous and other polar extracts, e.g. from the marine environment, are known to contain high levels of salts and such samples require extensive desalting and fractionation processes. The standard methods for desalting involve the use of reversed-phase columns (e.g. C-18 silica gel), organic polymer resins (e.g. XAD) or size-exclusion columns (e.g. Sephadex) while for fractionation implies in most cases the use of size exclusion, ion exchange chromatography or reversed phase silica gel based supports. But very often these methods lead to poor recoveries and, at worst, a loss of activity in the extract. Attempts to deal with these issues have included efforts not only to develop faster and more convenient extraction and fractionation approaches for purification of these water-soluble compounds, but also to hyphenate these steps with instrumental techniques to enhance the isolation of low

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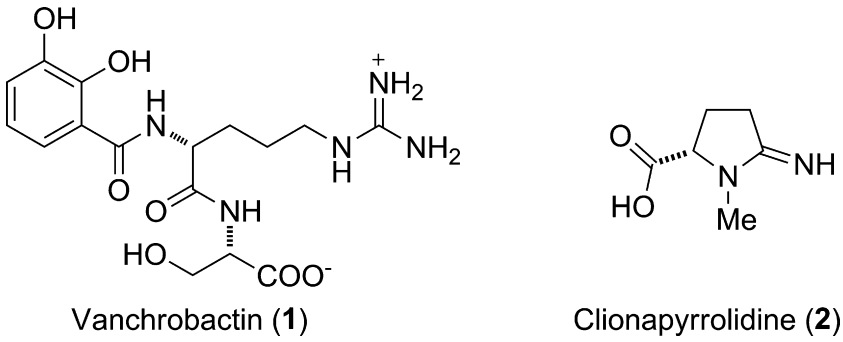

Fig. 1. Structure of the small water-soluble molecules isolated by this approach.

abundance secondary metabolites prior to their final purification by HPLC.

In an effort to address the challenges associated with the isolation and purification of hydrophilic metabolites from natural sources, we introduce here an approach to speed up the isolation of this type of natural products using a HLB/MCX–HPLC/MS system (hydrophilic–lipophilic balance/mixed-mode strong cation exchange–liquid chromatography/mass spectrometry) based on the retention properties of HLB and MCX polymeric cartridges. This approach involves a quick and straightforward fractionation step on solid phase extraction (SPE) polymeric cartridges with the optimal combination of two highly orthogonal and complementary retention mechanisms (reversed-phase and/or mixed-mode cation exchange-reversed-phase and/or hydrophilic interaction chromatography HILIC) followed by purification on a RP-HPLC column. The entire process is monitored by HPLC/MS. The nature and the high specific surface area of these SPE polymeric sorbents allow the loadability of crude extracts to be enhanced to obtain an optimum yield of the hydrophilic metabolite with high purity using the minimum elution and washing steps. We applied this methodology to speed up the isolation of two hydrophilic bioactive marine natural products, the siderophore vanchrobactin (**1**), as shown in Fig. 1, responsible for the iron uptake of the pathogen bacteria *Vibrio anguillarum* serotypes O2 and O3 [12], and the allelopathic clionapyrrolidine A (**2**) (Fig. 1) from the excavating sponge *Cliona tenius* that kills coral tissue upon contact [13]. Although SPE polymeric hydrophilic–lipophilic balance (HLB) and mixed-mode strong cation exchange (MCX) Oasis® cartridges have been widely used in pre-concentration (enrichments) processes or clean-up procedures at the analytical level [14–19], this is the first time that they have been applied to natural crude extracts for the isolation of hydrophilic compounds at a semipreparative level.

2. Experimental

2.1. Apparatus

Chromatographic separation was carried out on an Agilent 1100 liquid chromatography system equipped with a solvent degasser, quaternary pump, auto sampler, column compartment and a diode array detector (Agilent Technologies, Waldbronn, Germany). The UV wavelength was set up at 214 nm, band width 8 nm. Electrospray mass spectrometry measurements were performed on an MSD ion trap mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) interfaced to the HPLC system. MS measurements were acquired in positive ionization mode over the mass range of 100–700 amu. NMR spectra were measured on a Bruker Avance DRX500 MHz in D_2O. Spectra were referenced to the residual solvent peak.

SPE cartridges were Oasis® HLB and MCX from Waters (Milford, MA, USA) and Strata silica from Phenomenex (Torrance, CA, USA). SPE steps were conducted on an IST VacMaster SPE manifold of 10 positions. The HPLC columns were Atlantis dC18 100 mm × 4.6 mm, 5 μm and Atlantis Hilic 4.6 mm × 50 mm 3 μm from Waters (Milford, MA, USA), Chromolith SpeedRod 50 mm × 4.6 mm from Merck (Darmstadt, Germany), Discovery F5 50 mm × 4.6 mm, 3 μm and 100 mm × 10 mm, 5 μm from Supelco (Bellefonte, PA, USA). The HPLC mobile phases were water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid (FA) or 0.05% triflouroacetic acid (TFA). The gradient programs employed are described in each chromatogram.

2.2. Reagents

Acetonitrile and methanol HPLC grade were obtained from Lab Scand (Dublin, Ireland), formic acid and trifluoroacetic acid, were from Sigma–Aldrich (Steinheim, Germany), ammonia 7 N solution in methanol (CH_3OH:7 N NH_3) was from Acros (Geel, Belgium) and water was purified in house with a Milli-Q plus system from Millipore (Bedford, MA, USA).

2.3. Separation procedure

2.3.1. V. anguillarum serotype O2

Two litres of cell-free culture broth was lyophilized to give 3 g of material, which was dissolved in water (4.5 mL) and distributed in three HLB cartridges (35 cm^3, 6 g), loading 1.5 mL in each one. The mobile phase employed with the HLB cartridges was water (solvent A) and acetonitrile (solvent B), each containing 0.1% TFA (v/v). The cartridges were conditioned and equilibrated in parallel with 60 mL of solvent B and 60 mL of solvent A. After washing the cartridges with 60 mL of solvent A, the siderophore was eluted with 30 mL of a mixture of solvents A:B 1:1 and was detected by RP-HPLC/(+)-ESI-MS at m/z 398 ($[M+H]^+$). Each of the three fractions without drying was directly loaded into three MCX cartridges in parallel (20 cm^3, 1 g, conditioned with 10 mL of CH_3OH:TFA 0.1%, v/v) and then washed with 10 mL of solvent A, 10 mL of CH_3OH, and 20 mL of CH_3OH:7 N NH_3. RP-HPLC/(+)-ESI-MS showed that the fraction eluted with the latter mixture (2.4 mg after evaporation to dryness) contained siderophore **1** as the major component with a relative UV purity 76%. Final purification by HPLC using an Atlantis dC18 column with a mobile phase consisting of a 6 min gradient from 0 to 50% CH_3CN/TFA 0.05% (v/v) at a flow rate of 1 mL/min gave 1.2 mg of vanchrobactin (**1**) with purity greater than 95%.

2.3.2. V. anguillarum serotype O3

The lyophilized material (2.15 g) obtained from 2 L of cell-free culture of this organism was also subjected to the above mentioned protocol. Fractionation steps by HLB and MCX were monitored by RP-HPLC/(+)-ESI-MS. Thus, 17 mg of the MCX fraction containing siderophore **1** were finally purified by HPLC to yield 0.7 mg of vanchrobactin (**1**) with purity greater than 90%.