Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1119 (2006) 147–155
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

A systematic investigation of recovery in preparative reverse phase high performance liquid chromatography/mass spectrometry

Xu Zhang, Wendy Picariello, Nicole Hosein, Marc Towle, Wolfgang Goetzinger*

ArQule, Inc., 19 Presidential Way, Woburn, MA 01801, USA

Available online 4 January 2006

Abstract

In this paper we report a systematic recovery study based on reversed phase high performance liquid chromatography (RP-HPLC) separation and mass spectrometric (MS) based fractionation. Factors including a compound's physicochemical properties, column mass loading and presence of impurities were investigated through commercially available compounds. Results suggest that the delay time between MS peak detection and fraction collection, fraction detector's signal-to-noise ratio and compound's base peak width in the chromatogram have the biggest impacts on purification recovery. In an effort to assess sample recovery within our high throughput purification process, re-purification was performed on four compound libraries that were synthesized in-house. Reproducible recoveries (>80%) were achieved in all tests.

Keywords: Recovery; High throughput purification; HPLC; MS

1. Introduction

Automated parallel synthesis is able to generate large numbers of small organic molecules for high throughput screening [1]. Since the reliability from high throughput biological screening is dependent on the quality of the compounds screened, high throughput purification of library compounds has drawn more and more attention over the past decade [2–5]. Over the years, a number of synthetic cleanup approaches have been successfully adopted in a high throughput fashion, such as liquid/liquid extraction [6,7], solid phase extraction [8,9], fluorous extraction [10] and scavenger resins [11,12]. Despite the fact that these approaches are fast and automation-friendly, they do not consistently provide the high purity required in biological screening and can fail in separating impurities that have similar structural properties with the target compounds. Over the years, semi-preparative HPLC has remained the most powerful and widely used technique to purify these crude reaction products, ensuring reliable results from biological screens [3,13,14]. RP-HPLC is particularly well suited to deal with the quantities and polarity of the compounds developed for pharmaceutical applications, allowing even separation of closely related molecules, such as intermediates and side products. Earlier work on RP-HPLC based high throughput purification utilized ultraviolet (UV) response to trigger fraction collection, followed by flow injection MS analysis to identify the fraction of interest [15,16]. UV detectors offer advantages of being economical and rugged. Since flow path modification is not necessary, they are also easy to operate and maintain. However, issues remain with UV-directed fractionation in that the number of fractions from each sample is unpredictable. Software needs to be implemented to track the location of the desired fractions and reformat them into an appropriate plate format for later-stage screening. In addition, since each sample is likely to generate multiple fractions, the capacity of a purification system is limited by collector deck space. Yan et al. developed a solution to this issue. In their Accelerated Retention Window approach, fraction collection is achieved by the combination of a pre-set collection window and analog threshold [13]. This approach addressed the capacity limitation associated with analog-based fractionation by generating only one fraction per sample. It requires, however, reliable analytical characterization of each sample prior to purification, which makes the process lengthier and the data management more complex. Also, without an on-line MS identification, it is difficult to identify inaccurate fraction collection caused by possible instrument failure or system variation.

* Corresponding author. Tel.: +1 781 994 0393; fax: +1 781 994 0677.
E-mail address: wgoetzinger@arqule.com (W. Goetzinger).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.12.038

In 1998, Zeng et al. reported the first MS-based automated purification system [17]. Since then, the advantages of mass-directed purification have been increasingly acknowledged and the technology itself grown more robust and reliable. To this day mass-directed fractionation has been widely adopted in purifying library compounds [18–26]. During the past 3 years at ArQule, we have used RP-HPLC separation combined with mass-directed fraction detection to successfully purify over a million compounds [27].

While the value of purification is widely acknowledged [28], recovery during purification process is often difficult to assess since purification happens after a lengthy synthesis process and accurate information on pure compound yield only becomes available afterwards. Compared to the increasing number of reports on high throughput purification, very little discussion has been seen on purification recovery. We fully recognize the importance in understanding mass-directed purification recovery and have conducted a systematic purification recovery study with commercially available compounds. We investigated factors that might have an impact on purification recovery, including compounds' physicochemical properties, mass loading and presence of resolvable impurities. The relationship between compound purity and recovery in purification was also studied using a close-eluting compound-pair. In an effort to validate our overall purification process, a similar recovery study was conducted on four compound libraries synthesized in-house.

2. Experimental

2.1. Instrumentation

The HPLC/MS purification system consists of a Shimadzu (Columbia, MD) preparative HPLC system, a Waters ZQ (Milford, MA) single quadrupole mass spectrometer and two Gilson 215 liquid handlers (Madison, WI) for injection and collection. The Shimadzu HPLC system includes two LC-8A solvent delivery pumps, one LC-10Ai inert HPLC pump for mobile phase additives, one LC-10ADvp pump as online dilution pump [29], one LC-10ADvp pump as make-up flow pump for the MS, two SPD-10Avp UV detectors and a SCL-10Avp system controller to control the HPLC gradient. Sample injection and collection are controlled by MassLynx 4.0 SP2 and FractionLynx 4.0^{TM}. HPLC grade acetonitrile by J.T. Baker (Phillipsburg, NJ) is delivered by a custom built solvent delivery system. Water is purified and delivered by Millipore's Milli-Q system (Bedford, MA).

2.2. HPLC and MS conditions

All preparative HPLC separations at low pH were carried out using 20 mm × 50 mm, 10 μm Maccel 120-10-C18 SH column (The Nest Group, Inc. Southborough, MA). Separations at high pH were conducted using 19 mm × 50 mm, 10 μm XTerra® Prep MS C_{18} OBD^{TM} column (Waters, Milford, MA). We used a flow rate of 40 mL/min with a gradient of 5–95% acetonitrile in water in 3.5 min with a cycle time of 5 min. The online dilution pump delivers acetonitrile at 4 mL/min. A capillary splitter was used to divert flow after the UV detector to the MS, which was diluted by 2 mL/min of make-up flow before entering the MS. The make-up flow consists of 90% methanol and 10% water with 0.1% formic acid. The first UV detector (referred to as prep UV) was placed immediately after the column. A second UV detector (referred to as waste UV) was used after the fraction collector to monitor the collection. Both UV detectors are set to 254 nm with the fastest response rate. The MS scans from 190 to 800 amu at a span of 0.5 s with 0.1 s of inter-scan delay.

2.3. Fraction collection, weighing and evaporation

Crude samples were injected from two-dram vials plated in custom-made 24-well aluminum blocks. Fraction detection peak type was set to "preparative". Minimum intensity threshold (MIT) only was used in setting the threshold. Purified fractions were collected into culture tubes that hold up to 14 mL of solvent. These fraction collection tubes were plated in custom made 24-well collection racks and tared before purification. After purification, fractions were dried with Mega 980 Evaporators from Genevac, Inc. (Valley Cottage, NY) before the collection tubes were re-weighed. Weighing was performed using balances (SAG 285, $d = 0.00001$ g, Mettler Toledo, Columbus, OH) controlled by an automated Bohdan weigher (Mundelein, IL). Sample location and quantity information were stored into a company database through bar code on the collection racks. An analytical balance (PG503-S Delta Range®, $d = 0.001$ g, Mettler Toledo, Inc., Columbus, OH) was used in making stock solutions of commercially available test compounds.

2.4. Chemicals

HPLC grade trifluoroacetic acid (TFA) was purchased from J.T. Baker. Formic acid (88% A.C.S. reagent) and ammonium hydroxide (A.C.S. reagent) were purchased from Aldrich (Milwaukee, WI). HPLC-grade methylsulfoxide (DMSO) and methanol were purchased from EM Science (Gibbstown, NJ). Acetazolamide, arbutin, atenolol, chlorogenic acid, flavone, chlorthalidone, cortisone, nabumetone, metergoline, trimethoprim and trimipramine were purchased from Sigma Chemical Co. (St. Louis, MO). Quinine was purchased form Aldrich. Compounds used for the in-house recovery study were produced using our automated high throughput synthesis platform and processes [23].

2.5. Recovery test procedure

2.5.1. Recovery test at different mass loadings

Stock solutions of acetazolamide, atenolol, chlorogenic acid, chlorthalidone, cortisone, metergoline, nabumetone, quinine, trimethoprim and trimipramine were prepared in DMSO at concentrations of 10, 20, 40, 60, 80, and 100 mM. Stock solutions of arbutin were prepared in MeOH at the same concentrations. Injection volume was 1.5 mL. Recoveries were calculated based on dry weights of the purified compounds. Choices of mobile phase additive and ionization method were determined