Short Communication

Applications of HILIC for targeted and non-targeted LC/MS analyses in drug discovery

RP-HPLC has largely been the analytical method of choice in the pharmaceutical industry for many years because of the poor aqueous solubility and hydrophobicity of most small molecule drug candidates. RP-HPLC coupled to MS has provided an excellent analytical tool for qualitatively and quantitatively determining levels of drug molecules or drug metabolites for studies such as purity assessment and pharmacokinetic profiling. Quantitation of endogenous metabolites is an emerging field in drug discovery, gaining popularity for evaluating pharmacokinetic–pharmacodynamic relationships and targeting activity and efficacy. While RP-HPLC-MS is suitable for a range of applications, many endogenous molecules, especially those found in urine, are small polar compounds that do not retain well by RP-HPLC. This has made hydrophilic interaction LC (HILIC) an attractive alternative and useful approach to polar molecule analysis. Additionally, because HILIC is routinely used with traditional RP organic solvents such as ACN and methanol, it can be easily coupled to MS. This paper will review selected examples from the current literature as well as discuss some new applications from the author’s own laboratory focusing on the applicability of HILIC to quantitative and qualitative profiling of endogenous metabolites in drug discovery.

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1 Introduction

Just as the characterization of drug properties is critical to drug development, evaluation of pharmaceutical targets for therapeutic potential is integral to the drug discovery process. One of the main challenges of evaluating these targets is measurement of endogenous metabolites that help define target activity. Many of the metabolites are polar, water-soluble compounds that can be difficult to retain and separate using RP-HPLC-MS conditions. Hydrophilic interaction LC (HILIC)-MS provides a complementary chromatographic technique for effectively retaining and separating polar endogenous components of interest. It is being utilized more often as the technique has gained popularity.

Prior to choosing a suitable analytical technique (i.e. RP versus HILIC) the endogenous components of interest must first be identified; there are several approaches to this identification. Early phase programs often must first establish a pharmacodynamic (PD) marker(s) of on-target efficacy in order for the program to move forward. Although monitoring a single PD marker of interest is sometimes required, it is also often the case that monitoring changes in a series or class of components is desired. Depending on the biological or metabolic pathway(s) altered upon perturbation of the selected target, investigators may be interested in monitoring the levels of a particular analyte, a group of analytes, or multiple components within each sample. This concept is often referred to as metabonomics or metabolomics, although the definitions of these terms tend to be similar and often overlap [1–4]. Further, other terms such as metabolic fingerprinting and metabolite profiling are often used in conjunction with metabonomics and metabolomics [4]. To avoid confusion, for the remainder of this paper we will use the term endogenous metabolite profiling.

Endogenous metabolite profiling, in the broadest sense, is the capture of all changing components within a system’s metabolome. This type of global, or non-targeted, profiling can be used to assess changes in a system that correspond to disease state, toxicity, and/or changes in metabolic pathways. Such broad investigation into the altered pathways or metabolic states of a system, whether it be an intact organism or a cell culture/in vitro system, can provide vital guidance to researchers by highlighting important areas in the regulatory pathways that might help the therapeutic intervention. Richardson et al. tracked changes in mammary tumor cell metabolism by monitoring carbon flux following
introduction of labeled glucose [5]. Identifying the pathways affected by tumor cell progression provided important information regarding how tumor cell metabolic changes related to tumor progression. The individual steps of tumor progression could then be further evaluated for their importance in cell survival and thus for their potential as a target of therapeutic intervention. In a similar approach Munger et al. incorporated labeled nutrients into mammalian cells in order to monitor downstream metabolites altered by viral infection [6]. The metabolic pathways up-regulated and essential for viral replication were identified and therefore provided an important insight into the antiviral therapy. Serum metabolite profiling in a mouse model for inflammatory bowel disease led to the identification of two lyso-phosphatidylcholines elevated upon inflammation. Elevation of the lyso-phosphatidylcholines led to the identification of SCD1 (stearoyl-CoA desaturase) as a potential target for the prevention of inflammation [7].

An alternative approach is to narrow the characterization of a metabolome to specific components or classes of components within the system. Such an approach can be described as semi-targeted or targeted endogenous metabolite profiling. This type of profiling is common in drug discovery because often the biological targets of interest are fairly well characterized in terms of where they fit into a metabolic pathway. A discovery target for obesity, for example, could likely be a component of the fatty acid synthesis pathway. In this particular case it may already be known that lipid levels will change upon inhibition of the target, but would not necessarily inform which specific lipid(s). Thus, a semi-targeted approach, focusing on changes in, for example, fatty acids and phospholipids would be very informative. Identification of one or two specific lipids that could be easily measured and used as biomarkers for target efficacy would greatly facilitate the development of quantitative assays for a comprehensive characterization of the target and subsequently the identification of small molecules that can alter these targets.

Whether applying a non-targeted or targeted approach, the number of endogenous metabolites is vast. The composition of a system or pathways metabolome will likely include analytes of very different physicochemical properties. It is currently impossible to apply only one analytical technique to the measurement of all components in a system, as all analytical approaches have their own set of strengths and limitation. For example, NMR offers structural confirmation and ease of sample preparation but suffers from low sensitivity and the inability to distinguish individual molecules within complex classes such as cholesterol esters or phospholipids [8]. GC/MS provides good sensitivity but often requires sample derivatization and extensive sample preparation. LC/MS (and ultra-high pressure LC/MS) has become increasingly important to the field of endogenous metabolite profiling because the technique is both sensitive and the sample preparation can be, in some cases, minimal. However, as mentioned above, one potential limitation is the inability of RP-HPLC to retain polar endogenous compounds of interest. For this reason, HILIC-MS has emerged as an important and complementary addition to the existing tools available for endogenous metabolite profiling and more specifically quantitation of endogenous compounds.

2 Materials and methods

2.1 Instrumentation

Non-targeted LC/MS experiments were performed on a ThermoFisher (San Jose, CA, USA) Orbitrap Discovery mass spectrometer interfaced to a ThermoFisher Accela UPLC pump and a Leap (Carborro, NC, USA) CTC Pal autosampler. Full scan accurate mass data was acquired at a resolution of 15 000 in either positive or negative ionization modes.

Targeted LC/MS experiments were performed on a ThermoFisher LTQ ion trap equipped with a ThermoFisher Surveyor pump and a CTC Pal autosampler.

2.2 Non-targeted LC/MS

HILIC-MS of protein precipitated human plasma (200 µL of plasma precipitated with 800 µL of cold methanol, dried, and reconstituted) or human urine (200 µL urine diluted with 800 µL cold methanol) was performed on four HILIC columns: Luna 3 µm NH2 100 × 2 mm, Luna 3 µm HILIC 100 × 2 mm, Acquity UPLC BEH HILIC 1.7 µm 100 × 2.1 mm, and the Sielc Obelisc N 5 µm 2.1 × 150 mm column. Gradient conditions using a mobile phase of aqueous 10 mM ammonium acetate and 10 mM ammonium acetate in 98:2 ACN/water were as follows: 100–80% B in 3 min, 80–50% B in 9.5 min, re-equilibrate for a total run time of 14 min. The flow rate was 0.5 mL/min and the column temperature was 65°C. RP-HPLC of plasma and urine was performed on an Acquity UPLC BEH C18 column using a 15 min gradient of 0.1% formic acid in 98:2 water/ACN and 0.1% formic acid in 2:98 water/ACN.

2.3 Targeted LC/MS

1,5-Anhydroglucitol (1,5-AG) and methyl-glucoside (AMG) were analyzed as previously described [9].

3 Results and discussion

3.1 HILIC for non-targeted analysis in drug discovery

Non-targeted endogenous metabolite profiling seeks to provide a comprehensive list of metabolites within a system that is based on the total number of components measured. Metabolite components are typically chosen from an initially
complex field of chromatographic and spectral peaks by applying filters that subtract out background peaks and recognize and group derivatives or adducts of the same metabolite. The goal is not to structurally identify all components that are measured, but to create the most complete metabolome fingerprint possible and to identify which components might be of interest based on their decrease/increase in comparison to a control sample. Because virtually any biological system will consist of both polar and non-polar entities, the chances of capturing a complete representation of each using only one analytical technique is unlikely. Screening a metabolome using both RP-HPLC-MS and HILIC-MS should significantly increase the likelihood of capturing a representative sampling from both the non-polar and polar end of the metabolome spectrum. This was illustrated by Gika et al. who profiled the urine of leptin-deficient and lean rats using both RP-HPLC-MS and HILIC-MS [10]. Though the two methods produced some of the same conclusions (differences between the groups were more related to age than to strain) HILIC-MS identified two ions that differentiated the two rat strains. These two ions were different from the ion identified by RP-HPLC-MS, thus illustrating the complementary advantage that HILIC-MS can provide. Another example of using both HILIC-MS and RP-HPLC-MS for complementary profiling was shown by Cubbon et al., who compared the profile of healthy human urine using both techniques [11]. As seen with the previous study, although HILIC-MS provided similar classification data compared with RP-HPLC, different compounds from each technique contributed to the classifications, again highlighting how pairing the two methods can enhance the information collected on a system. A disadvantage of using the two techniques is the need for two separate injections of the same sample, which typically requires two separate systems if the samples are to be analyzed at the same time, or a significant delay in the analysis for one of the techniques, which could potentially lead to sample degradation, especially in studies with many samples. A possible solution involves implementing column-switching between RP and HILIC columns so that only one injection is needed for both analyses. This was demonstrated for profiling the urinary metabolome of rats where polar components were retained and separated on the first HILIC column and the unretained components were then transferred to an RP column for separation [12, 13]. Again, the HILIC column identified potential biomarkers that would have been lost using only the RP column, and running the two columns in sequence resulted in substantial time savings.

We have also investigated HILIC-MS as an additional tool for non-targeted metabolite profiling. The advantage that HILIC-MS provides is evident when looking at protein-precipitated human plasma samples. Many endogenous metabolites found in human plasma are not retained with the RP-HPLC-MS (high resolution accurate mass) method that we typically use. For example, Fig. 1A shows the RP-HPLC-MS chromatogram of a human plasma sample. An extracted ion chromatogram created for a select group of polar metabolites (betaine/valine, proline, threonine, histidine, and carnitine) demonstrates that all compounds elute in the void volume. The same human plasma was further analyzed using several different HILIC columns with varying characteristics (Phenomenex Luna NH2 and Hilic columns, the Waters BEH Hilic and the Sielc Obelisc N) under generic gradient and mobile phase conditions. All four HILIC columns retained betaine, valine, proline, threonine, histidine, and carnitine (Fig. 1B–E). Because of the generic gradient conditions the different columns provided varying degrees of chromatographic resolution and retention (any additional peaks present in the chromatograms presumably represent isobaric interferences from the plasma matrix). Naturally, conditions could be optimized for each column to achieve ideal results. A similar experiment with diluted human urine was performed where the retention of several select urinary metabolites (creatinine, glutamine, threonine, histidine, carnitine) was monitored using both RP-HPLC-MS and HILIC-MS (data not shown). Similar results were obtained, where urinary metabolites eluted in the void volume of the RP-HPLC column but were well retained on the HILIC column. These examples illustrate the value that HILIC-MS can add to endogenous metabolite profiling. However, as with RP-HPLC-MS, HILIC-MS has limitations, as illustrated in Fig. 2A–C, which shows a human plasma sample analyzed in negative ionization mode. Several common free fatty acids (which are well-retained by RP-HPLC) elute in the void volume of the HILIC columns (2A = Luna HILIC, 2B = BEH HILIC, 2C = Obelisc N). Thus, similar to RP-HPLC-MS, HILIC-MS does not offer an all-encompassing analytical technique that captures all endogenous metabolites. Rather, HILIC-MS, when used in conjunction with RP-HPLC-MS, resolves a complementary series of metabolites, which result in a more complete characterization of a metabolome. Thus the two techniques are most useful when used in combination for metabolite profiling.

### 3.2 HILIC for targeted analysis in drug discovery

A significant amount of supporting data must be accumulated to build a case that a target is reasonably suitable for pharmaceutical intervention. Often the evaluation process begins with the development of a functional model system for target expression, which could be based on in vitro cell-based assays or in vivo (animal model) methods. The assays are often evaluated based on the measurement of a particular component or a set of components that provide a PD readout of target interaction. The components of interest can vary greatly in their chemical and physical properties depending on the nature of the target and the metabolic pathways involved. The ability of HILIC to separate polar analytes that would typically elute in the void volume of a RP-HPLC method makes it a valuable...
addition to the analytical tool set available for drug discovery support.

Targeted profiling of biological systems involves measurement of pre-determined analytes known to be of interest in the biological system. Some investigators have chosen to use both RP-HPLC-MS and HILIC-MS for targeted sample analysis. For example, Lu and colleagues have applied two separate LC/MS systems using RP-HPLC-MS (negative ionization mode) and HILIC-MS (positive ionization mode) for profiling of endogenous metabolites. In their studies HILIC-MS effectively separated several classes of cellular metabolites, including nucleosides, nucleotides, amino acids, carboxylic acids, and folates while RP-HPLC-MS separated negatively charged metabolites such as glycerol-3-phosphate, orotate, malate, and UDP-D-glucose [14, 15]. Using HILIC-MS alone, isocratic separation of various nucleosides and carbohydrates and gradient elution of peptides was achieved on a HILIC monolithic silica capillary column [16]. Changes in liver metabolism in cancerous versus non-cancerous tissue was monitored using HILIC-MS-MS for the detection of acetylcholine, choline, and other metabolites important for liver function [17]. Acetylcholine was also used for determining changes in signal cascades in cultured cells using HILIC-MS-MS [18]. Methylmalonic acid, a marker of vitamin B12 deficiency, was monitored by HILIC-MS following protein precipitation of human plasma [19]. Phase II metabolic conjugates of estrogens were monitored in the urine of breast cancer patients by HILIC-MS-MS [20]. The above studies are select examples of the contributions that HILIC has made to the targeted metabonomic arena of drug discovery/development. The sections below briefly discuss in-house applications of HILIC-MS to targeted metabolite or biomarker analyses.

### 3.2.1 Targeted analysis: Determination of 1,5-AG, a potential marker of glycemic index, in human urine

1,5-AG is a circulating polyol that is derived primarily from the diet. Plasma levels of 1,5-AG have been shown to

Figure 1. A comparison of extracted ion chromatograms (positive ionization mode) of betaine (m/z 118.0868), valine (m/z 118.0868), proline (m/z 116.0711), threonine (m/z 120.0660), histidine (m/z 156.0773), and carnitine (m/z 162.1130) in human plasma using four different HILIC columns. Human plasma was treated by protein precipitation and the samples were analyzed as described under Section 2. (Panel A: RP-HPLC on BEH C18, Panel B: Luna NH2, Panel C: Luna HILIC, Panel D: BEH HILIC, Panel E Sielc Obelisc N.).
Figure 2. A comparison of extracted ion chromatograms (negative ionization mode) of stearic acid (C18:0, m/z 283.2637), oleic acid (C18:1, m/z 281.2481), palmitic acid (C16:0, m/z 255.2324), palmitoleic acid (C16:1, m/z 253.2168) and arachidonic acid (C20:4, m/z 303.2324) in human plasma using three different HILIC columns. Human plasma was treated by protein precipitation and the samples were analyzed as described under Materials and methods. (Panel A: Luna HILIC, Panel B: BEH HILIC, Panel C: Sielc Obelisc N).

Figure 3. Representative LC/MS/MS chromatograms of a human urine sample. The top panel shows the extracted ion chromatogram for 1,5-AG in urine (m/z 223 → 163 → 101,113); the bottom panel shows the extracted ion chromatogram for the internal standard methyl-β-D-glucoside in urine (m/z 253 → 193 → 101,113). Pooled human control urine was treated and analyzed as described previously [9].
correlate with plasma glucose levels, thus making 1,5-AG a potential biomarker in the diabetes field [21–28]. We investigated the potential for 1,5-AG to serve as a urinary marker for glycemic control. 1,5-AG, which is similar in structure to glucose, is highly polar and was not retained on traditional RP columns. We found that 1,5-AG was adequately retained using a Phenomenex Luna NH2 column (Fig. 3). By using a structural analog of 1,5-AG as an internal standard (AMG) the levels of 1,5-AG were quantitated in human urine samples using a simple dilution sample preparation approach. The method, which utilized HILIC-MS3 on an ion trap mass spectrometer, proved to be sensitive and sufficiently rugged to analyze more than 200 urine samples from diabetic patients. An important outcome of this study was the demonstration that 1,5-AG urine levels were not subject to diurnal variation. The lack of diurnal variation (in contrast to urinary glucose levels) suggests that 1,5-AG could be used as potential spot-check of glycemic control, eliminating the costly and difficult 24 h urine collection required for glucose monitoring. A summary and details of this work is published elsewhere [9].

3.2.2 Targeted analysis: Determination of AMG in a cell-based assay

The work described above with 1,5-AG utilized AMG as an internal standard. The HILIC-MS3 method used for 1,5-AG was adapted for the determination of AMG in a cell culture-based assay for unrelated research work. The study required the monitoring of AMG uptake in a recombinant CHO cell line expressing SGLT1, a Na-glucose co-transporter in human. Again, the HILIC-MS3 method provided a sensitive and robust technique for monitoring AMG, a polar analyte. The effect of phlorizin, a well-known SGLT inhibitor on sodium dependant AMG uptake, was successfully characterized and led to the characterization of the kinetics using this method (Fig. 5).

4 Concluding remarks

Non-targeted and targeted metabolite profiling is an important aspect of the drug discovery process. Creating a metabolic fingerprint that is an accurate representation of an in vivo or an in vitro metabolome remains a challenging endeavor. Part of this challenge is the capture of components that have different chemical properties in a given system. To date, an all-inclusive analytical technique capable of completely capturing all classes of metabolites within a system is not available. RP-HPLC-MS has become an
important tool in many areas of the drug development process because of its flexibility and sensitivity. Despite its advantages polar metabolites are sometimes overlooked because of lack of retention. HILIC-MS has emerged as an important and complementary technique to RP-HPLC-MS, especially in the emerging area of endogenous metabolite profiling. Combining data obtained using both RP-HPLC-MS and HILIC-MS results in a more complete representation of a system’s metabolome than either technique alone would have provided. As the drug discovery process becomes more competitive and resource constrained, it is essential that the characterization of drug targets becomes more efficient. HILIC-MS is emerging as an important additional tool available to researchers which is helping to generate more information to propel and to guide drug discovery efforts forward.

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5 References