Global and targeted metabolomics

There are two conceptually different branches to metabolomics research. The first, often referred to as “global metabolomics”, can be defined as follows: “The dynamic, qualitative and quantitative analysis of all small molecules (<1000 Da) in a cell-type, tissue, body fluid or organism”. With the proviso that it is unlikely that it will be possible to analyse every single small molecule, at least in the foreseeable future, the fundamental concept of “global metabolomics” is now realistic and achievable, if not by individual laboratories, but certainly by consortia of groups. In contrast to the “global metabolomic” approach, “targeted metabolomic” efforts are centred on the analysis of individual groups of metabolites.

Whether a “global” or “targeted” approach to metabolomics is taken, the fundamental work-flow is essentially similar. Initially, there will be an extraction step, followed by some form of separation, then analysis by mass spectrometry (MS) [for example, direct-infusion MS, gas chromatography (GC)-MS, liquid chromatography (LC)-MS, each with or with out tandem mass spectrometry (MS/MS)] and the final step is one of data analysis. In this article, only metabolomic approaches based on MS will be discussed, however, nuclear magnetic resonance-based methods, popularised by the Nicholson group at Imperial College, London, also exist.1

The data content in publications describing a “global metabolomic” approach varies greatly, however, a current benchmark for this discipline is provided by a recent publication from Siuzdak’s group at Scripps Institute.2 In an investigation of the optimal method of metabolite extraction from serum, they concluded that protein precipitation with methanol, followed by metabolite extraction with the same solvent after centrifugation, provides the most straightforward and reproducible approach, resulting in over 2000 reproducible metabolite “features”. They defined a “feature” as a peak in a mass spectrum with a signal-to-noise ratio of 10 to 1. They performed their studies on 50 µL of serum, injecting the equivalent of 10 µL on a 2.1 × 100 mm C18 column (3.5 µm particle size) and used a CH3CN/HCO2H/H2O gradient. Analysis was by positive-ion electrospray (ES)-MS at a flow-rate of 250 µL min⁻¹.

By performing protein precipitation and metabolite extractions with a variety of other organic solvents, they were able to produce over 10000 unique metabolite features. LC-MS data were processed using XCMS software, written in-house but freely downloadable (http://metlin.scripps.edu),1 which matches and aligns features between samples allowing for variations in retention time and providing a


comparison of features between samples. In this study, only positive-ion ES was employed, but as the authors point out, application of negative-ion ES and alternative atmospheric pressure ionisation (APCI) modes would allow the detection of molecules unobservable by positive-ion ES. Further, the application of capillary- or nano-LC-MS would further improve dynamic range and allow the identification of additional metabolites.

While the goal of the “global metabolomic” approach is to identify as many features as possible in a sample, the “targeted” approach differs in that the analytical method is designed to highlight metabolites of a particular class in an unbiased fashion. In the area of lipid analysis, this approach has been adopted by the lipidmaps consortium (http://www.lipidmaps.org/) in the USA. Their initial effort was to define a comprehensive classification system for lipids, which are then analysed in six specialised areas, i.e. (i) fatty acids and eicosanoids, (ii) neutral lipids, (iii) glycerophospholipids, (iv) sphingolipids and glycosphingolipids, (v) sterols and (vi) isoprenoids, by a consortium of laboratories.

An alternative and somewhat older term for “targeted metabolomics” is “metabolite profiling”. In the field of the authors’ interest, namely, bile acids, sterols and steroids, “metabolite profiling” can be dated back to the early 1960s and the work of Evan Horning who used GC to profile bile acids and steroids in urine and serum. With the advent of GC-MS, Sjövall’s group in Stockholm refined methods for the analysis of bile acids and steroids and their group-separation methods have since been extensively used in the laboratories of Cedric Shackleton in California and Ken Setchell in Cincinnati. Sjövall’s group-separation strategy is shown in Figure 1 (left) and, by using this procedure in combination with GC-MS, Almé, Sjövall and colleagues were, in 1977, able to identify more than 150 bile acids and alcohols in a urine sample from an infant with cholestatic liver disease. One of the bile alcohols identified at high levels in this work was (24S)-24-hydroxycholesterol 3-sulphate, 24-glucuronide [Figure 1 (right)].

This bile alcohol aroused our interest, as 24S-hydroxycholesterol is mainly synthesized in the brain and normally provides only a minor route for overall cholesterol elimination.

**Sterols in the brain: an application of targeted metabolomics**

In humans, although the central nervous system (CNS) accounts for only 2.1% of body weight, it contains 23% of the unesterified cholesterol present in the whole body. Cholesterol cannot cross the blood brain barrier and is synthesised de novo in the brain. In adults, the rate of synthesis of new cholesterol in the CNS exceeds its requirement, and excess cholesterol is removed by metabolism to oxysterols and neurosteroids which can pass the blood brain barrier. In animals, 24S-hydroxycholesterol has been identified as the major oxysterol in the brain and this molecule can pass the blood brain barrier and be transported to the liver, where it can be sulphated or converted to bile acids for excretion. 24S-Hydroxycholesterol is present in the human brain at a level of 5–15 ng mg<sup>−1</sup> (cf. cholesterol 7–8 µg mg<sup>−1</sup>) and other oxysterols have also been identified including 7-hydroxycholesterol (1–3 ng mg<sup>−1</sup>), 22R-hydroxycholesterol (3–6 ng mg<sup>−1</sup> protein, ~45–90 pg mg<sup>−1</sup> brain), 7-hydroxycholesterol (234–745 ng mg<sup>−1</sup> cerebellar cortex<sup>23</sup>) and 7-oxocholesterol (170–640 ng mg<sup>−1</sup> cerebellar cortex<sup>23</sup> 0.5–7.5 mg L<sup>−1</sup> cerebellar cortex<sup>23</sup> 0.3–4.6 µg L<sup>−1</sup> in CSF). Many of these oxysterols, including 24S-hydroxycholesterol (40 ng mg<sup>−1</sup> mouse brain<sup>24</sup> 27-hydroxycholesterol (4–6 ng mg<sup>−1</sup> mouse brain<sup>20</sup> and also 20-hydroxycholesterol (~ 50 pg mg<sup>−1</sup> rat brain<sup>25</sup> have also been identified in rats and mice. While the experimental evidence for the presence and abundance of 24S- and 27-hydroxycholesterols in the brain is clear-cut, some...
doubt exists over the identification and quantification of other oxysterols in the brain. In an attempt to resolve this uncertainty, we have embarked on a “targeted metabolomic” investigation of oxysterols in rat brain.

Rat brain (100 mg) was homogenised in ethanol (1 mL) and, after centrifugation, sterols were further extracted with methanol : dichloromethane (1 : 1, v/v, 1 mL). The ethanol and methanol/dichloromethane extracts were combined, solvents evaporated and extracts re-constituted in hexane:dichloromethane (2 : 8, v/v, 2 mL). This solution was then applied to a Unisil column (8 × 0.8 cm, 200–325 mesh, activated silicic acid) washed with 20 mL of hexane/dichloromethane (2 : 8, v/v) prior to sample application. Following sample application the column was washed with 80 mL of hexane/dichloromethane (2 : 8, v/v, fraction 1) and eluted with 10 mL of ethyl acetate (fraction 2). The eluate from fraction 2 was dried and re-dissolved in 50 µL of isopropanol. For brain samples still found to contain a significant amount of cholesterol in fraction 2, the eluate was dried and re-constituted in 2 mL of hexane/dichloromethane (2 : 8, v/v) and re-applied to an additional Unisil column and chromatographed as before. Oxysterols re-constituted in 50 µL of isopropanol were incubated for 60 min at 37°C with 2 µL of cholesterol oxidase from Streptomyces sp (2 mg mL⁻¹, 44 U mg⁻¹ protein) in 1 mL of buffer (50 mM KH₂PO₄, pH 7). This results in the conversion of 3β-hydroxy-5-ene sterols to 3-oxo-4-ene sterols, which were subsequently derivatised with Girard P (GP) hydrazine [Figure 2 (a)].

This strategy of oxidation with cholesterol oxidase, followed by derivatisation with GP hydrazine, results in an improvement of ES current of at least a factor of 100 and it also adds specificity to the assay in that only compounds with a 3β-hydroxy group and a planar...
A/B-ring junction (i.e. 5,6-ene or 5α-H configuration) are susceptible to cholesterol oxidase activity.

Derivatised oxysterols were analysed by LC-ES-MS on either a capillary-LC system (180µm × 150mm, 3 µm particle C18 column, 800 nL min⁻¹) interfaced to an LCQ mass spectrometer, or on a conventional-LC system (50×2.1 mm, 1.9 µm particle C18 column, 200 µL min⁻¹) interfaced to an LTQ-Orbitrap mass spectrometer. Initially, LC-MS spectra were recorded over the m/z range 300–600, which covers the [M⁺] ions of all derivatised oxysterols likely to be present in the brain. Reconstructed ion chromatograms (RICs) for all possible derivatised oxysterols in this m/z range were then generated. RICs from MS scans on the Orbitrap are made with a 5 ppm m/z tolerance and give chromatograms with minimum noise [Figure 2(b)]. MS³ spectra of oxysterol GP hydrazones [M⁺] ions are dominated by [M–79.04] fragment-ions and MS¹ spectra recorded on these ions ([M⁺]→[M–79.04]→) contain a wealth of structural information (Figure 3). The next step is to make a second injection and record MS² spectra on the oxysterol GP hydrazones indicated to be present in the RICs from the first MS run.

Using this methodology, the oxysterols identified in rat brain were 24S-hydroxycholesterol, 24-oxocholesterol, 24,25-, 24,27-, 25,27-, 7α,25- and 7α,27-dihydroxycholesterols (Figure 4).

In addition to acting as transport forms of cholesterol, oxysterols have biological activity acting as ligands for the liver X receptors and interacting with other transcription factors such as sterol regulatory-element binding proteins (SREBPs) and the possibility exists that the dihydroxy-cholestanes identified above have this as their biological function. Oxysterols have also been implicated with disease processes, particularly atherosclerosis and Alzheimers’ disease (AD). It has recently been suggested that ozone formed as a result of inflammation reacts with cholesterol to form the oxysterol 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (5,6-seco-sterol) and its aldol, 3,5-dihydroxy-3B-norcholestane-6-carboxyaldehyde and that these sterols can initiate amyloidogenesis of proteins and disease. Although, the identification 5,6-seco-sterol and its aldol in vivo has been regarded with considerable scepticism, we have been able to confirm the presence of 5,6-seco-sterol and its aldol in rat brain, by generating a RIC for Figure 4(b).
the [M]+ ion of their GP derivatives at m/z 552.42 [Figure 2(b), lower panel] and recording MS2 and MS3 spectra. The data for the GP derivatives of the endogenous 5,6-seco-sterol and its aldol, match perfectly with data from reference standards.33 However, it is not clear whether these oxysterols are formed as a result of the reaction of cholesterol with ozone or other reactive oxygen species.

Past heroes and future directions

Siuzdak’s group at Scripps have demonstrated the possibilities for “global metabolomics”.2,3 Future developments to allow deeper mining of the metabolome will no doubt revolve around improvements in chromatographic inlets and sensitive MS/MS instruments with high mass accuracy and high resolution capability. Chromatographic improvements can be made by incorporating multiple dimensions of separation (for example, cation-exchange with reversed-phase, or straight- with reversed-phase), miniaturised columns, and columns with reduced size particles.4 Already, such systems exist, but are not yet in widespread use. Although “metabolomics” is a new term, the features of mass spectrometry required for an optimal investigation have changed little from the heyday of organic mass spectrometry in the 1960s and 70s, and the teachings of Beynon,35 McLafferty36 and Biemann37 are still as relevant today as 40 years ago. The widest range and most secure identification of metabolites will be made on instruments operated at high resolution, making use of exact mass measurement of precursor and fragment ions.

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References


