Ion Chromatography Coupled with Mass Spectrometry for Metabolomics

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Karl Burgess
Functional Genomics, Joesef Black building, Glasgow University, Glasgow, UK

Introduction

The fields of metabolomics and metabonomics attempt to phenotype and quantify the vast array of metabolites present in biological samples. Reversed-phase liquid chromatography (LC) coupled with mass spectrometry (MS) is a valuable tool in the separation and identification of these compounds. Reversed-phase HPLC techniques cover a wide range of compounds. However, ionic and polar compounds such as organic acids, carbohydrates, nucleotides, and amino acids are difficult to separate or even retain on traditional reversed-phase columns. Ion-exchange chromatography offers a far better separation process for these compounds. The problem with the separation mode in this application relates to the salt eluents employed in ion exchange being incompatible with mass spectrometers. Here, the author describes an ion-exchange system that provides good separation of these polar compounds with on-line desalting to allow MS detection. This technique will allow studies of key metabolites that do not separate well on traditional reversed-phase columns. Some ionic metabolites may be positional isomers adjacent to each other in a synthetic pathway and as such, are isobaric. In such cases, MS is not sufficient to differentiate the two compounds. There is clearly a need for adequate separation techniques for isomeric metabolites.

Ion Chromatography

Ion chromatography (IC) was developed with eluent suppression techniques in 1978 by Small et al.[1] It has grown to be the primary technique for the analysis of small anions and a strong method choice for inorganic cation analysis.

Also, IC is an efficient method for the analysis of small polar compounds such as organic acids and amines[2]—all detected by conductivity. Larger charged biomolecules, such as peptides, nucleotides, and carbohydrates, are successfully separated by IC but typically without the use of suppression techniques.[3, 4] Suppression is routinely linked to conductivity detection of the analyte. In the case of nucleotides and peptides, UV detection is used. Carbohydrates can be detected electrochemically with pulsed amperometic detection (PAD). However, it is eluent suppression that is of interest to MS as it converts some high-salt eluents from ion-exchange chromatography to MS-compatible pure water. Therefore, the ion-exchange separation of these metabolites can be utilized and coupled to suppression to allow detection by MS. The field of IC covers an impressively wide range of compounds with an array of column chemistries and detection techniques utilized in addition to suppressed conductivity.[5] The importance of making these analytes accessible to a metabolomics study using an on-line desalting technique is quite evident.

IC/MS System

Mass spectrometry is a preferred detection method due to its exceptional sensitivity, its ability to resolve coeluting compounds, and the additional information it provides for the identification of compounds. The coupling of ion-exchange chromatography to MS has never really been explored due to the high salt concentrations used in the elution of compounds from the ion-exchange column. Although eluent suppression techniques are available that effectively convert high-conductance sodium or potassium hydroxide eluent back to pure water via an ion-exchange substitution with H^+ ions, IC has remained associated with the analysis of small inorganic ions using conductivity detection.

Figure 1 outlines the chemistry inside an anion-electrolytic suppressor. The cation suppressors work in a similar manner with opposite charges. The suppressor devices use a platinum electrode for the hydrolysis of water, as a source of the H^+ required to keep the charged semipermeable membrane in the H^+ form. As potassium hydroxide (KOH) enters the suppressor, it is converted to water in an exchange reaction on the membranes. The use of membrane screens in the suppressor allows a large surface area for exchange while keeping the dead volume to a minimum. Due to this, the eluent is continuously converted to water and the membranes permanently charged for continuous use. The conductivity of the eluent passed on to the mass spectrometer is monitored by a conductivity detector and is usually below 1.0 $\mu s.cm^{-1}$, demonstrating the purity of the effluent at all times.

As the eluent from the analytical ion-exchange column is transformed to pure water from both anion and cation exchangers in suppressed IC, direct coupling to MS is possible. In addition to this, all the compounds that bind and elute from ion-exchange columns are naturally ionic or highly polar and so ideal candidates for electrospray ionization (ESI). Highly polar and ionic compounds that tend to come straight through reversed-phase columns without separation are more likely to separate well on ion-exchange columns. This allows many classes of compounds—often difficult to measure with reversed-phase MS—to be analyzed by IC-MS.

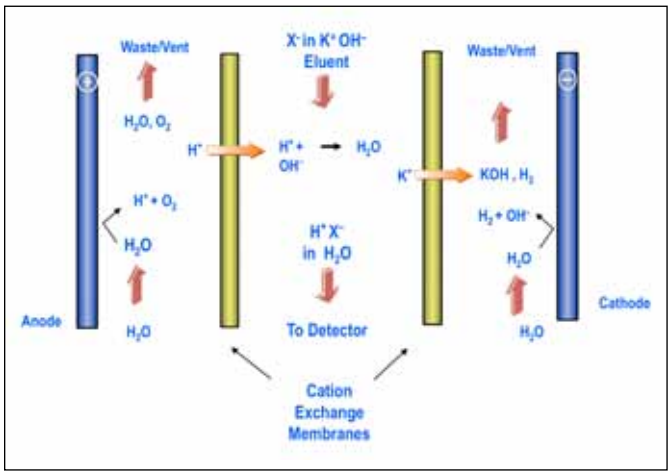

Figure 1. Conversion of a KOH eluent to water using a modern electrolytic anion suppressor. Desalted hydroxide eluents are compatible with MS detection.

Continuous On-Line Desalting

The coupling of IC to MS is now commercially available and the viability of the technique across several analytical areas is being explored. A system consisting of a PEEK™-based pump provides a controlled delivery of ultrapure water to an electronic eluent generator. The KOH gradient produced is used to separate the sample on a high efficiency 2 mm i.d. anion-exchange column. The KOH effluent from the column is converted back to high-purity water on passing through a low-dead-volume, continuous electrolytic suppressor. The separated ionic metabolites are then detected and identified using a high-resolution mass spectrometer. A schematic of a typical IC system coupled to a high-resolution mass spectrometer is shown in Figure 2.

The modern systems employ eluent generators to electrolytically produce KOH in pure form at the head of the column. This is done similar to the suppressor by generating OH^- from water at a platinum electrode. The amount of OH^- produced is directly proportional to the flow rate and the current applied to the electrode. The K^+ is added across a semipermeable membrane as a counterion from a reservoir. As a result, pure and accurate salt gradients can be produced with virtually no delay volume. An electrolytic suppressor is also used postcolumn to create a compatible effluent for conductivity and MS detection. Due to this, the effluent supplied to the mass spectrometer is kept in pure form to reduce the background and increase sensitivity. Organic solvent may be added as a makeup flow to aid the desolvation process in the electrospray interface, but this is often not required.

IC-MS Schematics

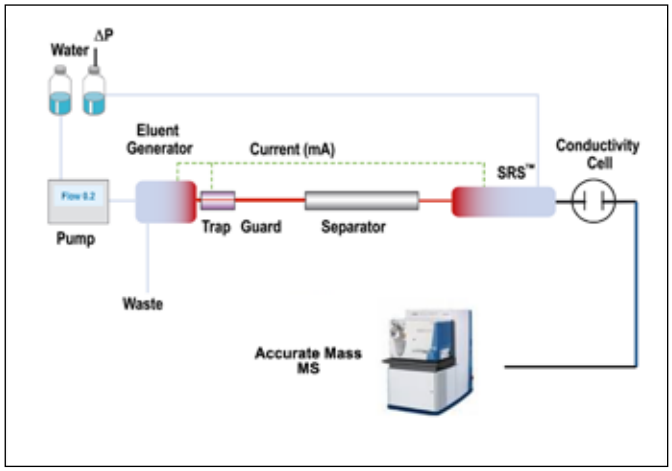

Figure 2. Schematic of a modern IC-MS system.

Practical IC-MS

The coupling of IC to MS is well-documented, such as in the analysis of organic acids in beverages,[6] agricultural chemicals, and water pollutants.[7,8] The use of suppressed IC for carbohydrate analysis with MS detection has also been demonstrated.[9] Carbohydrate analysis by anion-exchange chromatography is usually coupled to electrochemical detection and thus, does not normally utilize the on-line desalting provided by a suppression system. Here, the reduction of the KOH/acetate eluents to volatile acetic acid in the suppression system allows the coupling of an efficient separation to on-line electrospray-MS. Despite key applications now being coupled to MS, few have used this technique in the field of biological metabolites where the sample matrix is complicated and the numbers of potential ionic metabolites are vast. For ionic and polar compounds such as organic acids, carbohydrates, nucleotides, and amino acids that are difficult to separate on traditional reversed-phase columns, ion-exchange chromatography offers a better separation option. An example of nearly all these compounds being separated on an anion-exchange column with a simple KOH gradient, can be seen in Figure 3. An ion-exchange system, which provides good separation of these polar compounds with on-line desalting to allow MS detection, offers a new analytical platform to study metabolism. This technique allows studies of key metabolites that do not separate on traditional reversed-phase columns, and may often be isobaric, preventing easy identification by MS.

In the application presented here, the most commonly used mode is an anion-exchange system to target organic acid-based metabolites, specifically carboxylated, phosphorylated, and even sulphonated species. An added advantage of the high pH KOH eluents used in this system is that compounds such as sugars,which contain OH groups, ionize to form anionic species and therefore, also have interaction on the ion-exchange column. There is also the possibility of using a cation-exchange system to target amine-containing compounds, as demonstrated in the analysis of biogenic amines.[10] The system layout is identical to that outlined in Figure 2, where the columns and suppressor would be cationic versions and the eluent generator would create methane sulphonic acid with a cation-generation cartridge.

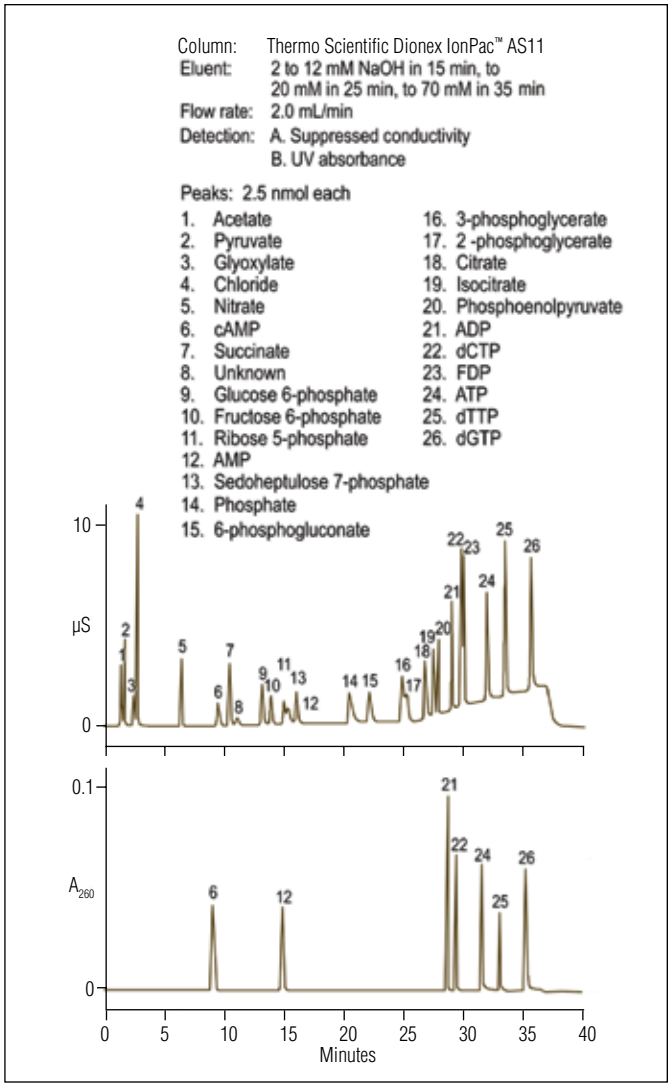

Figure 3. Separation of a series of metabolic intermediates.

Metabolomics/Metabonomics Analysis

The study of metabolic pathways using LC-MS methodology is still in a development stage. Chromatography methods are being improved, along with mass spectrometry, metabolite databases, search engines, and pathway analysis software. All these need to be put together to allow biological samples to be analysed in detail, then suggested conclusions can be given regarding the state of the metabolic pathways under study. Different chromatography conditions, such as high performance reversed-phase and hydrophilic interaction chromatography (HILIC), have been compared to increase the coverage of the metabolites seen in biological samples.[11]

Metabolomics presents the problem of cataloging and quantification of the vast number of small-molecule components of biological fluids and cell extracts.[12] Metabonomics is also faced with a greater challenge of achieving this quantification in response to stimuli or genetic modification.[13] Control and test samples must be carefully prepared, separated, and identified for changes in a few compounds within an extremely large group of unaltered metabolites. Separation of all these components to allow reasonable quantification and identification will require more than one chromatography technique. Gas chromatography coupled with MS can be used for the volatile compounds with high resolution. Liquid chromatography opens up a wider range of analytes and the advent of high-resolution systems adds more power to the resolving capabilities in reversed-phase mode. The proposed use of ion-exchange chromatography permits the analysis of components that are not retained on reversed-phase columns, and targets a different class of compounds than HILIC.

The use of orthogonal separation modes before MS identification increases the coverage of the metabolic classes present in these mixtures. Overall, C18 chromatography covers hydrophobic compounds, HILIC mode selects hydrophilic components, and ion-exchange systems target ionic compounds.

Identification and Classification of Metabolites

Currently, the identification of metabolite information obtained from complex LC-MS data is performed by accurate mass alone. XCMS, MZmine, SIEVE™, MassTRIX, and MetExplore are examples of databases that can be used. An important way to improve the reliability of identification is by the incorporation of isotope pattern and fragmentation data. Retention can also be applied to significantly improve identification confidence, but is severely limited due to the requirement for standardized LC gradients and instrumentation, and restriction to metabolites with available standards.

Identification of individual metabolites of interest is significantly more robust, as standards may be available and more rigorous analysis may be performed. For these reasons, the process by which metabolomic experiments are performed consists of collecting quantitative data, including multiple replicates, performing label-free quantification to analyze differences or trends, and producing a short list of metabolites of interest (which are then identified by accurate mass).

Label-free quantification can be performed by the use of MZmine, XCMS, SIEVE, and profile analysis software. Most software also includes clustering analysis, for example principal component analysis (PCA) and multiple statistical tools. Identification of metabolites is typically performed against a large metabolite database, such as the human metabolome database (HMDB), Kyoto encyclopaedia of genes and genomes (KEGG), or ChemSpider. Although the use of a database provides some identification bias, the broad specificity of these databases means that the majority of metabolites will be identified, and if a compound of interest is observed but not identified, more rigorous methods must be employed to determine its structure.

In addition, metabolites are commonly placed in a systems context with the use of pathway analysis software such as MassTRIX or MetExplore. Both applications attempt to match observed masses to components of known biological pathways (from KEGG and BioCyc respectively), providing a schematic of the relationships between metabolic intermediates. Furthermore, the MetaNetter software infers chemical relationships between intermediates with the use of chemical transformation rules. Although many relationships may be the consequence of sample manipulation, this methodology is invaluable in elucidating networks from poorly characterized organisms and in predicting previously undiscovered metabolic pathways.

Benefits of the Inclusion of IC-MS in Metabolite Studies

Reversed-phase, HILIC, and anion-exchange chromatography were used with high-resolution MS to investigate the metabolic profile of biological samples. The classes of compounds identified with each technique were compared to qualify the advantages of IC in a metabolomics environment.

Investigation of urine analysis shows an expected overlap between different analytical methods in terms of compounds identified (Figure 4). It also demonstrates a significant decrease in the coverage of metabolites identified if any of the three techniques are dropped.

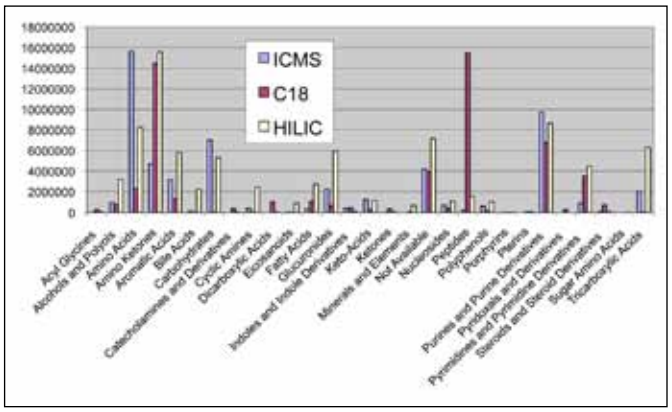

Figure 4. Classes of compounds identified by ion-exchange, reversed-phase, and HILIC chromatographies.

The use of ion-exchange chromatography, as demonstrated here, brings visibility to several classes of compounds such as amino acids, aromatic acids, keto acids, carboxylic acids, nucleosides, purines, carbohydrates, and glucuronides. These compounds are members of several important biological pathways. High coverage of intermediates involved in synthetic pathways will lead to a better understanding of the control mechanisms and disease states. IC-MS data has been extremely useful in the identification and quantification of components of the glycolytic pathways. This is expected with the intermediates containing carboxylic acid and phosphate groups. These negatively charged metabolites separate well on ion-exchange columns and are already charged for electrospray analysis. Similar improvements were seen in the identification coverage of amino acid synthesis pathways. With this combined approach, 379 of the 652 intermediates of several pathways were reliably identified, with minimum effort in optimization.

Studies using cell extracts from *Trypanosoma brucei* have significantly increased the coverage of several biological pathways, including oxidative phosphorylation, nucleotide, and amino acid metabolism (Figure 5). In the tryptophan pathway, 24 out of 80 components were identified by IC-MS alone.

IC-MS and HILIC

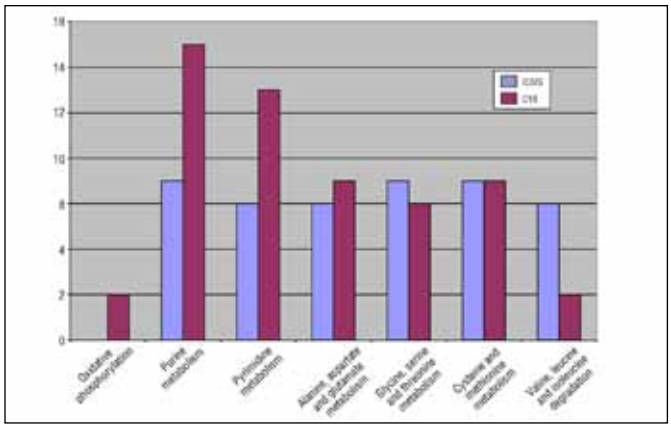

Figure 5. Pathways identified in Trypanosoma brucei.

Future Developments

The studies reported here only include anion-exchange chromatography, thus overlooking cationic metabolites. There must be an effort to examine the contribution of cation-exchange chromatography on increased coverage of biological pathways. Full coverage may not be achieved in some biological pathways due to the extremely short half-life and turnover of some intermediates. A significant increase in the coverage of particular pathways can be expected and predicted from the type of compounds present in the pathway. Changes in the metabolic phenotype in response to clinical conditions and drug treatment can be seen more easily with IC. Studies of biological markers using HILIC and reversed-phase chromatography proved that the markers identified using the two techniques were different in each case.[11] This indicates that important biological markers for certain disease states are found more easily with a targeted approach to analysis–specifically, by isolating the metabolite class most likely to be affected and using the most appropriate separation technique for these compounds.

Systems biology approaches will also benefit from the increased coverage of metabolite classes. Changes in the proteome or genome should be verifiable by a targeted approach to find the expected differences in the metabolome. In addition, the metabolome is often an amplified response to small changes in the proteome and so, theoretically easier to see. A relatively minor change in the proteome can lead to a dramatic change in the activity of the rate limiting enzymes in a biological pathway. The proteome change can be difficult, if not impossible, to see; however, the change imparted at the metabolite level can be dramatic.

A directed approach to biomarker discovery and metabolic phenotyping, with intelligent choices to give the correct coverage of metabolites, can also remove metabolite classes that interfere with the analysis and are of little interest. Many hydrophobic metabolites are not retained on an ion-exchange column and therefore, can be removed from a study of the charged species in pathways such as amino acid metabolism or the glycolytic pathway.

High-resolution, reversed-phase, HILIC, and ion-exchange chromatographies provide excellent depth and breadth of analysis. New developments in capillary IC use smaller column technology, thereby increasing sensitivity and allowing the analysis of much smaller sample volumes.[14] Mass spectrometers are increasingly more sensitive, faster, and provide higher resolution for separation, detection, and identification in metabolomics.

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