

HYPHENATED LIQUID CHROMATOGRAPHY TECHNIQUES IN CLINICAL METABOLOMICS: PRINCIPLES AND BIOMEDICAL APPLICATIONS

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ABSTRACT

Clinical metabolomics refers to the rapid, broad-spectrum detection of all endogenous and environmental small-molecule metabolites in a biological system and their determination in complex biological samples. Precision medicine, biomarker discovery and personalised medicine are being advanced by this progress, much of which rests on hyphenated liquid chromatography systems, especially UHPLC-MS/MS and HRMS. Even though other methods are being developed, these remain the major platforms used to characterise the human metabolome. This review takes account of the basic principles, stationary-phase chemistries (reversed-phase and HILIC) and the ionisation interfaces that couple chromatography to mass spectrometry, together with the process safeguards clinical laboratories use to limit error from sample collection to finished analysis. This paper then looks at where these tools already impact patient care: the tracking of metabolic reprogramming in cancer; revealing disordered lipid metabolism in cardiometabolic disease; and enabling rapid newborn screening. The review concludes by examining the hurdles that remain, including cross-laboratory standardisation and what is required to advance this technology from the research bench to routine clinical use.

KEYWORDS: Biomarker discovery; clinical metabolomics; data processing; HRMS; hyphenated methods; metabolic reprogramming; UHPLC.

1. INTRODUCTION

The metabolome, the entire collection of small molecules present in a cell, tissue or biofluid at a given moment, is the 'omic' level that sits closest to what is actually happening in the body. Genomics examines the complete set of DNA in an organism, transcriptomics the RNA transcribed from it, and proteomics the proteins present in a cell, tissue or organism, while metabolomics is the large-scale study of small molecules such as sugars, amino acids and lipids across cells, biofluids or tissues. Clinical metabolomics has expanded considerably over the past two decades, for good reason: a shift in just a handful of circulating metabolites can signal disease

before symptoms appear, indicate whether a treatment is working, or reveal a toxic exposure that no other test would detect.^[2]

Nonetheless, this promise cannot be realised without the analytical technology to make it happen. Biofluids including plasma, serum, urine and cerebrospinal fluid contain an astonishingly wide variety of metabolites with respect to polarities, sizes and concentrations, such as millimolar glucose and picomolar signalling lipids. No single platform encompasses the entire spectrum. NMR spectroscopy is reproducible and requires little preparation, but is not very sensitive. GC-MS methods

are effective mainly for substances that can be volatilised and are not too heavy. Liquid chromatography coupled with tandem mass spectrometry has become the workhorse of the field largely because it can be tailored, through column chemistry, mobile phase and ionisation mode, to capture nearly every class of metabolite.^[1]

This review covers the use of hyphenated LC platforms in clinical metabolomics today: the chemistry and instrumentation underlying their operation, the workflows laboratories have developed to ensure reliable results, the impact these tools are already having on patient care, and the challenges that remain before they can be used routinely in diagnostic testing.

2. BASIC PRINCIPLES OF HYPHENATED LC SYSTEMS

2.1 Ultra-High Performance Liquid Chromatography (UHPLC)

Columns packed with particles sized 3-5 µm are the basis of conventional HPLC. In a single injection, separating and quantifying hundreds or thousands of metabolites is much more effective using ultra-high-performance liquid chromatography (UHPLC) than with standard HPLC. Clinical metabolomics relies notably on speed and the ability to separate structurally similar metabolites (isomers, isobars, etc.). Thus, conventional HPLC has been substantially replaced by UHPLC in the clinical metabolomics field.^[6]

2.2 Chemistry of the Stationary Phase

Reverse-phase (RP) chromatography is still the default for moderate to strongly hydrophobic metabolites, lipids, steroids, pharmaceutical metabolites and non-polar amino acid derivatives. C18 columns retain analytes here via hydrophobic interactions; the analytes are then eluted as the mobile phase becomes more organic.

HILIC succeeds where RP fails. Metabolites such as sugars, amino acids, nucleotides and organic acids are very polar and therefore poorly retained on a C18 column. As a result, they elute close to the solvent front, where ion suppression occurs and they are poorly separated from the background matrix, rendering quantification unreliable. HILIC uses polar stationary phases (e.g. bare silica, amide, zwitterionic sulfobetaine) and a mostly organic mobile phase that keeps polar analytes within a water-rich layer on the stationary-phase surface. HILIC is important for comprehensive coverage because many key pathways, such as glycolysis, the TCA cycle and nucleotide metabolism, involve highly polar intermediates. Most clinical workflows now run RP and HILIC side by side, or in sequence, to make sure nothing important is missed.^[11]

2.3 Atmospheric Pressure Ionisation Interfaces

All LC-MS results are first determined by the interface used and which metabolites can be observed at all. The field is dominated by three ionisation techniques.

Electrospray ionisation (ESI) is the method of choice. It is a soft ionisation process that works well for polar and moderately polar metabolites, and it can be run in either positive mode (which favours amines and basic compounds) or negative mode (which favours acids, phenols and many lipids).^[1]

Because of this, most workflows collect data in both polarities instead of just one. Atmospheric pressure chemical ionisation (APCI) is suitable for less polar, more volatile compounds that ionise poorly under ESI, and it is still widely used for various classes of steroids and lipids. Atmospheric pressure photoionisation (APPI) extends coverage further into highly nonpolar compounds; however, it has not been adopted to the same extent as ESI or APCI.

2.4 Tandem Mass Spectrometry and High-Resolution Mass Spectrometry

Triple quadrupole instruments operated in SRM or MRM mode remain the gold standard for focused quantitative work. Neonatal screening panels for amino acids and acylcarnitines are the classic example, owing to their sensitivity, wide linear range and quantitative reliability.^[5]

Whether run on QTOF or Orbitrap analysers, high-resolution mass spectrometry (HRMS) offers accurate mass measurement with typically under 5 ppm error and high resolving power for untargeted, hypothesis-free profiling. A particularly useful feature of HRMS is retrospective data mining: because a full spectrum is acquired rather than a pre-selected list of transitions, a single run performed today for one purpose can be revisited months later to search for an entirely different metabolite, without a new injection. Triple quadrupole (QqQ) instruments remain standard for established quantitative clinical assays, although they lack this retrospective capacity for biomarker identification efforts.^[7]

3. CLINICAL METABOLOMICS WORKFLOWS

3.1 Preanalytical Variability

Because metabolites are acted on by active enzymes that continue reacting even after leaving the organism, they can change almost instantly; haemolysis further complicates this issue. Clinical metabolomics often tries to identify small disease-relevant differences, so it is crucial to tightly control preanalytical factors such as fasting status, time of day, anticoagulant choice, centrifugation speed, storage temperature and freeze-thaw cycles.^[17] When preanalytical noise is the cause of the signal rather than biology, its clinical utility is nil. As a result, laboratories increasingly follow documented SOPs for collection, prompt processing and defined storage, well before chromatography commences.^[16]

3.2 Sample Preparation and Extraction

Due to their high protein and salt content, biofluids cannot be applied directly to columns. In protein

precipitation, cold organic solvents (for example, methanol or acetonitrile) are added to the biological sample; this is generally a fast procedure and widely applied for untargeted profiling. Liquid-liquid extraction (LLE) uses phase separation to enrich nonpolar, lipid-class metabolites. Another useful strategy for sample preparation is solid-phase extraction (SPE), a more selective and reproducible clean-up procedure that is better suited to targeted quantitative assays and is also applied to remove ion-suppressing matrix components. To enhance productivity and reduce run-to-run mismatch, clinical laboratories are increasingly automating these steps with liquid-handling robotics.^[15]

3.3 Data Collection Strategies

The choice of strategy guides clinical metabolomics workflows. Targeted metabolomics quantifies a pre-defined panel using calibration curves and isotope-labelled internal standards, and is precise and reproducible enough for diagnostic use.^[3] Untargeted metabolomics instead aims to detect as many metabolites

as possible without prior assumptions, generating hypotheses about metabolites that differ between patient groups. Artificial intelligence is increasingly useful for biomarker discovery, but any candidate identified still needs substantial downstream verification before clinical use.^[8]

3.4 Preprocessing of Data and Chemometrics

Before biological interpretation can be established, raw LC-MS data must undergo peak detection and normalisation. Once a clean data matrix is obtained, further data analysis techniques can be applied – PCA for unsupervised pattern recognition, PLS-DA for supervised classification, and, increasingly, machine-learning algorithms for identifying metabolite patterns that distinguish disease from health.^[4] Metabolite identification remains one of the bottlenecks of the field: comparison of observed spectra with relevant databases and spectral libraries leaves a large proportion of features unexplained in untargeted studies^[19] (Fig. 1).

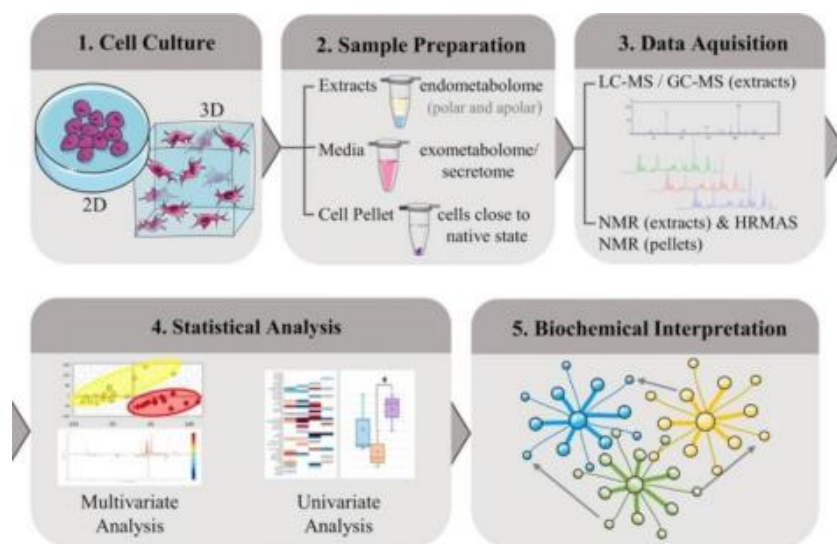


Figure 1: Workflow of clinical metabolomics.

4. BIOMEDICAL APPLICATIONS

4.1 Oncology: Monitoring of Metabolic Reprogramming

To achieve increased rates of proliferation, cancer cells reprogram their metabolism, for example through the Warburg effect (a shift toward aerobic glycolysis), altered glutamine use, and altered lipid biosynthesis. Hyphenated LC-MS systems have characterised these changes in tumour tissue, plasma and urine, providing biomarker candidates for early detection, subtyping and treatment-response monitoring. Because these metabolic changes occur before the clinical manifestation of disease progression, metabolomic analysis has great potential for early detection of recurrence.^[10]

4.2 Lipidomics in Cardiometabolic Disorders

Obesity and metabolic syndrome cause disturbed lipid metabolism and fatty-acid oxidation. Using LC-MS-based lipidomics, particular lipid species – especially

ceramides, phosphatidylcholines and lysophospholipids – have been found to relate to cardiovascular disease more strongly than traditional measures such as total cholesterol and triglycerides. The integration of HILIC and RP separations with HRMS enables investigators to move toward monitoring individual lipid species rather than just bulk lipid-class measurement, potentially enhancing risk stratification for atherosclerosis, type 2 diabetes and metabolic syndrome.^[13]

4.3 Newborn Screening

Newborn screening is arguably the most mature clinical application of metabolomics. By tandem MS testing of dried blood spots, a single small blood sample, generally taken within the first few days of life, can flag dozens of inherited metabolic disorders – including phenylketonuria, maple syrup urine disease, and various fatty-acid oxidation disorders – by virtue of its amino acid and acylcarnitine profile. This multiplexed, high-

throughput approach is now the standard of care in many countries, demonstrating that hyphenated LC-MS metabolomics can work reliably in a regulated clinical environment, as opposed to a research laboratory.^[14]

5. CHALLENGES AND FUTURE PERSPECTIVE

5.1 Inter-Laboratory Standardisation

The analytical power of clinical metabolomics is great, but reproducibility across laboratories remains an issue. Different equipment platforms, column chemistries, sample preparation procedures and data pipelines mean that a metabolomic signature generated in one laboratory often does not easily transfer to another. Considerable work is under way to create standardised reference materials, harmonised SOPs, and common data-reporting criteria; however, achieving consistent reproducibility across platforms and laboratories is not yet solved.^[18]

5.2 Regulatory Compliance

For a metabolomic biomarker or panel to become an approved clinical test, it must undergo rigorous analytical and clinical validation, good laboratory and manufacturing practice, and regulatory sign-off. Multi-analyte, LC-MS-based assays carry a heavier validation burden than single-analyte immunoassays, so hyphenated LC-MS metabolomic assays need validation frameworks suited to that added complexity.^[9]

5.3 Future Perspectives

The field is likely to see a range of further developments. Ion mobility spectrometry (IMS) as a second separation dimension prior to mass analysis could help resolve isomeric metabolites that cannot be fully separated by UHPLC and HRMS alone. Artificial intelligence and machine learning are being used increasingly in data processing, feature annotation and biomarker-pattern recognition, which could help ease the identification bottleneck that currently limits untargeted studies.^[20] Metabolomic testing may one day take place outside the laboratory, thanks to portable LC-MS devices. Together, these developments show that hyphenated LC metabolomics is shifting from a research tool to a clinical diagnostic tool.

6. CONCLUSION

The use of hyphenated LC techniques, particularly UHPLC-MS/MS and HRMS, is establishing clinical metabolomics as a discipline with real diagnostic prospects, as evidenced by newborn screening programmes already in place. The chemistry has developed at every stage, from complementary RP and HILIC separations through ever more sophisticated ionisation and mass analysis, to the point that it can now provide comprehensive metabolome coverage on a clinically relevant timescale. Further development of analytical capacity is no longer the biggest obstacle; most of the remaining issues concern standardisation, validation and regulatory matters. Addressing these, hyphenated LC metabolomics is well positioned to become routine in precision medicine, informing

diagnosis, prognosis and treatment decision-making in oncology, cardiometabolic disease and well beyond.

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