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Chemical and technical challenges in the analysis of central carbon metabolites by liquid-chromatography mass spectrometry☆

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ABSTRACT

This review deals with chemical and technical challenges in the analysis of small-molecule metabolites involved in central carbon and energy metabolism via liquid-chromatography mass-spectrometry (LC–MS). The covered analytes belong to the prominent pathways in biochemical carbon oxidation such as glycolysis or the tricarboxylic acid cycle and, for the most part, share unfavorable properties such as a high polarity, chemical instability or metal-affinity. The topic is introduced by selected examples on successful applications of metabolomics in the clinic. In the core part of the paper, the structural features of important analyte classes such as nucleotides, coenzyme A thioesters or carboxylic acids are linked to "problematic hotspots" along the analytical chain (sample preparation and—storage, separation and detection). We discuss these hotspots from a chemical point of view, covering issues such as analyte degradation or interactions with metals and other matrix components. Based on this understanding we propose solutions wherever available. A major notion derived from these considerations is that comprehensive carbon metabolomics inevitably requires multiple, complementary analytical approaches covering different chemical classes of metabolites.

1. Introduction

1.1. Metabolomics

Concentrations of metabolites in vivo are a reflection of whole body metabolism. Metabolites are intermediates of biochemical reactions and they are the substrates and products of enzymatic reactions. As a consequence, comprehensive metabolic profiles might be used as a kind of "signature" of the perturbations of metabolism during disease processes.

Profiling strongly ionized, water-soluble small molecules in bodily fluids of patients started in the field of inborn errors in metabolism (for a review see [1]). The approach is based on the simultaneous detection of metabolites belonging to the same class of compounds such as amino acids, organic acids, monosaccharides, acylcarnitines, fatty acids, sterols, etc. This approach, however, is rather limited in comparison with the more general aims of metabolomics.

Metabolomics aims at identifying responses of the metabolic network and its disturbances associated with health and disease as a consequence of naturally occurring genetic variability, type of diet and behavior. To this purpose, high-end analytical techniques like ultra high-performance liquid chromatography (UHPLC) coupled to multiple stage mass spectrometry (MS^n) are applied for comprehensive analysis of bodily fluids. Chemometrics, multivariate

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; BCAA, branched-chain amino acids; CoASH, coenzyme A; CVD, cardiovascular disease; DHA, dihydroxyacetone; DHAP, dihydroxyacetone-phosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; FAIMS, high-field asymmetric waveform ion mobility spectrometry; GA3P, glyceraldehyde-3-phosphate; GA, glyceraldehyde; GC, gas chromatography; GWAS, genome-wide association studies; HAPI, heredity and phenotype intervention; HILIC, hydrophilic interaction chromatography; HPLC, high-performance liquid chromatography; IMS, ion mobility spectrometry; MS, mass spectrometry; MS^n, multistage mass spectrometry; MS/MS, tandem mass spectrometry; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; OGTT, oral glucose tolerance test; PGC, porous graphitic carbon; SPE, solid-phase extraction; SNP, single-nucleotide polymorphism; SRM, single reaction monitoring; TCA, tricarboxylic acid; TCEP, tris(2-carboxyethyl)phosphine; TMAO, trimethylamine *N*-oxide; UHPLC, ultra high-performance liquid chromatography.

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analysis, pattern-recognition and other bio-informatics tools are needed to deconvolute the huge data sets and identify possible affected metabolic pathways. Metabolomics research helps to generate new hypotheses that must be further studied in defined biological model systems and ultimately translated to humans. Metabolomics in the context of a systems biology approach is a tool to gain a better understanding how perturbation of metabolic processes affects the entire biological system [2].

In this review, we would like to illustrate the importance of profiling highly ionized water-soluble small molecules to reveal affected metabolic pathways in central metabolism. On this basis, we will point out some of the unresolved challenges in the field of liquid chromatography (LC)–mass spectrometry (MS) based metabolomics approaches, indicating solution approaches wherever available. In doing so, a special focus is put on relating the chemical properties of the concerned analytes to problems occurring at different stages of the analytical chain—from the pre-analytical phase to detection.

1.2. Examples of success stories in clinical metabolomics

In understanding the peculiarities of cancer metabolism, metabolomic studies have proven to be essential. In most gliomas and secondary glioblastomas in humans the enzyme cytosolic isocitrate dehydrogenase 1 (IDH1) is mutated at p.R132H. With a metabolic approach, Dang et al. were able to show that the R132H mutation in the sequence of cytosolic isocitrate dehydrogenase 1 induced a new biochemical reaction converting the TCA cycle intermediate 2-oxoglutaric acid into (*R*-)-2-hydroxyglutarate (2-OHG) [3]. Moreover, they showed in a series of human malignant gliomas harboring the p.R132H mutation in IDH1 that 2-OHG levels were increased. Changes in the levels of other tricarboxylic acid (TCA) cycle intermediates did not associate with the presence of mutated IDH1. Subsequently the hypothesis that 2-hydroxyglutaric acid drives tumor formation was corroborated in a series of wet experiments in the TF-1 human erythroleukemia cell line. Incubations with trifluoromethyl-benzyl-2-hydroxygluratic acid, a membrane-permeable form of 2-hydroxyglutarate, reversibly induced leukemogenesis. Metabolomics has also been used to trace altered metabolic pathways in tumor cells. When stable isotope labeled metabolites were added to cells and the label incorporation was measured in highly polar small molecular intermediates of central carbon metabolism, the importance of reductive decarboxylation of glutamine in the de novo synthesis of lipid could be shown [4–7].

An emerging area of the application of metabolomics lies in the combination with genome-wide association studies (GWAS). These combined studies focus more on the effect a single nucleotide polymorphism has on the functioning of the underlying metabolic process [9,10]. In this emerging field it is becoming clear that knowledge of the genetic basis of human metabolic individuality is a key ingredient of personalized medicine (for a review see [11]). Using LC-electrospray ionization (ESI)–tandem mass spectrometry (MS/MS) with the Biocrates AbsoluteIDQ targeted metabolomics technology in cohorts of apparently healthy individuals, Illig et al. [10] observed the association of metabolite concentration ratios of acylcarnitines with single-nucleotide polymorphisms (SNPs) in the genes ACADS, ACADM, ACADL, SCD1 and ETFDH. Acylcarnitines in plasma are considered a proxy of intracellular acyl-coenzyme A thioesters (acyl-CoA), which are intermediates in fatty acid oxidation and synthesis. ACADS, ACADM, ACADL and ETFDH are the genes encoding enzymes involved in the first FAD-dependent dehydrogenase step in fatty acid oxidation and SCD1 is the gene encoding the enzyme, which desaturates long-chain fatty acids during elongation.

In the field of pharmacology the combination of metabolomics and genomics can be exemplified by a very recent study conducted by Yerges-Armstrong et al. [12]. They studied the metabolic perturbations, which caused resistance to aspirin therapy in cardiovascular disease. Aspirin is a well-established antiplatelet agent but the mechanism of aspirin resistance remains poorly understood. They conducted metabolomics and genomic studies in cohorts of poor and good responders to aspirin treatment in the heredity and phenotype intervention (HAPI) heart study. The studies revealed a clear difference in the response of metabolites participating in the purine pathway between cohorts of poor and good responders after a dose of aspirin. Combining this with data from a GWAS study the authors were able to show that differences in intracellular purine metabolism induced by SNPs in enzymes such as adenylate kinase (ADK) contribute to the observed difference between good and poor responders.

In this short discussion of applications of metabolomics in the clinical field we have focused on the importance of analyzing water-soluble and highly ionized small molecules. Their pattern contains important information of how metabolic processes active in central carbon metabolism operate and deviate from normal during health and disease. For more general reviews on applications of mass spectrometry in metabolomics and an extension to the field of drug-metabolism the reader may refer to [13,14].

2. Chemical and technical challenges

2.1. A first challenge: Chemical degradation during the pre-analytical phase

The "pre-analytical phase", comprising sampling, sample preparation and storage, is a key factor in metabolomics workflows. It affects the observed spectrum of metabolites both quantitatively and qualitatively. In a clinical context it has been estimated that the pre-analytical phase accounts for 60–80% of all testing errors [15]. A variety of factors affects metabolite yield after sampling, sample preparation and storage and can thus cause errors in the pre-analytical phase:

(i) *Physicochemical* analyte properties determining extraction efficiency, such as solubility, volatility or adsorption behavior.
(ii) *Biological/biochemical* processes causing post-sampling changes in metabolite profiles. These can include residual endogenous enzyme activity but also the action of contaminating microorganisms [16].
(iii) *Chemical* degradation processes taking place without enzyme catalysis such as decarboxylation of oxoacids, hydrolysis of thioesters and pyrophosphates, oxidation of lipids and reducing agents (nicotinamide adenine dinucleotide, β-NADH, and nicotinamide adenine dinucleotide phosphate, β-NADPH) or isomerization reactions.

Losses during sampling and sample preparation, which are due to physicochemical analyte properties, are difficult if not impossible to avoid, particularly when considering a multitude of analytes with diverse chemical features in multi-methods or global metabolic screening. However, such losses can usually also be quantified and corrected for in the frame of method validation (i.e. via recovery correction). This is not possible for metabolite degradation due to residual enzyme activity or bacterial contamination, since these processes strongly depend on the individual sample. Shurubor et al. showed, for example, that the stability of certain metabolites in blood plasma samples depends on the individual blood donor [17]. The rapid, comprehensive quenching of enzymatic activity in the sample is thus of paramount importance if an accurate picture of the biological situation is to be obtained.