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Mass-spectrometric profiling of porphyrins in complex biological samples with fundamental, toxicological, and pharmacological applications

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ABSTRACT

Rapid, high-throughput, and quantitative evaluations of biological metabolites in complex milieu are increasingly required for biochemical, toxicological, pharmacological, and environmental analyses. They are also essential for the development, testing, and improvement of new commercial chemical products. We demonstrate the application of ultra-high performance liquid chromatography-mass spectrometry (uHPLC-MS), employing an electrospray ionization source and a high accuracy quadrupole time-of-flight mass analyzer, for the identification and quantification of a series of porphyrin derivatives in liver: a matrix of particular relevance in toxicological or pharmacological testing. Exact mass is used to identify and quantify the metabolites. Chromatography enhances sensitivity and alleviates potential saturation issues by fanning out the contents of a complex sample before their injection into the spectrometer, but is not strictly necessary for the analysis. Extraction and sample treatment procedures are evaluated and matrix effects discussed. Using this method, the known mechanism of action of a well-characterized porphyrinogenic agent was verified in liver extracts from treated rats. The method was also validated for use with bacterial cells. This exact-mass method uses workhorse instruments available in many laboratories, providing a highly flexible alternative to existing HPLC- and MS/MS-based approaches for the simultaneous analysis of multiple compounds in biological media.

The cyclic tetrapyrroles are organic compounds that are ubiquitous in biology, forming the core of molecules as diverse as light-harvesting chlorophylls, cobalamines (including vitamin B12), and porphyrins. The latter, when bound to iron, are known as hemes. Interruptions of one or more of the eight steps of the heme biosynthetic pathway can give rise to a diverse group of diseases known as porphyrias [1,2]. These can result from inborn errors in metabolism; additionally, an extensive array of xenobiotic agents chemically induce porphyrias via the selective inhibition of enzymes in the pathway. Examples include heavy metals such as lead, arsenic, aluminum, iron, and mercury [3–8]; aromatic compounds including benzene, dioxins, pyridine, and polychlorinated biphenyls [4,9–12]; and a variety of pharmaceuticals, agrochemicals, and complex natural products [13–16]. Additionally, many xenobiotics are known to be metabolized by mammalian cytochrome P450s [17], generating modified porphyrin products which themselves interfere with heme biosynthesis. Some of these xenobiotics are bulk chemicals which have become widespread environmental contaminants, including hexachlorobenzene [16,18–21].

Having the ability to quickly assess whether a chemical agent interferes with heme metabolism is important for the pharmaceutical and chemical industries as well as many research labs. Blockage of any step in the heme biosynthetic pathway, whether by xenobiotic exposure or genetic abnormality, causes an accumulation of heme intermediates (Scheme 1) [9]. Because of regulatory and feedback mechanisms among the steps for heme synthesis, degradation, and trafficking, the disease/exposure biomarker may consist of not just one compound, but a pattern of various substituted tetrapyrroles which selectively accumulate in the tissues, blood, urine, or feces [10–12]. These molecules must be separated,

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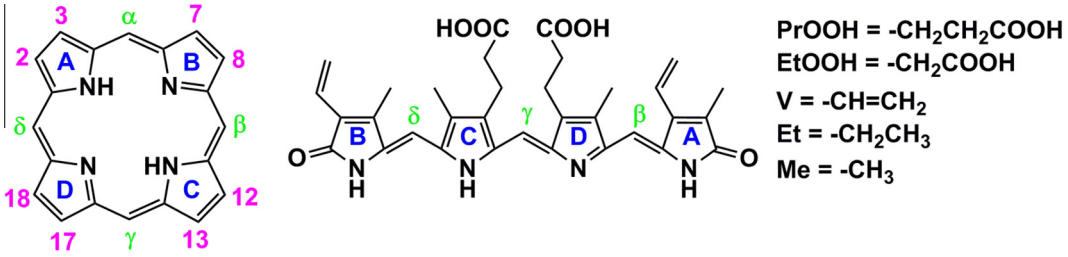

Scheme 1. Porphyrin compounds analyzed in this study and described in the text. The cyclic porphyrins share the scaffold on the left and include: uroporphyrin III (3, 8, 13, 17 = PrOOH; 2, 7, 12, 18 = EtOOH); uroporphyrin I (3, 8, 13, 18 = PrOOH; 2, 7, 12, 17 = EtOOH); heptacarboxylporphyrin I (3, 8, 13, 18 = PrOOH; 2, 7, 12 = EtOOH; 17 = Me); pentacarboxylporphyrin I (3, 8, 13, 18 = PrOOH; 12 = EtOOH; 2, 7, 17 = Me); coproporphyrin III (3, 8, 13, 17 = PrOOH; 2, 7, 12, 18 = EtOOH);coproporphyrin I (3, 8, 13, 18 = PrOOH; 2, 7, 12, 17 = Me); mesoporphyrin IX (13, 17 = PrOOH; 2, 7, 12, 18 = Me; 3, 8 = Et); protoporphyrin IX (13, 17 = PrOOH; 2, 7, 12, 18 = Me; 3, 8 = V); hemin, Fe-protoporphyrin IX; *N*-methyl protoporphyrin IX. The linear heme breakdown product, biliverdin, is shown on the right. Note that several of these porphyrins are the oxidation products of natural heme porphyrinogen intermediates that spontaneously oxidize in air. Some are unnatural derivatives formed during xenobiotic metabolism (e.g., *N*-methyl protoporphyrin IX).

detected, and in many cases quantified within these biological milieu. High performance liquid chromatography (HPLC)[1] is the longstanding technique of choice for separating porphyrin precursors and breakdown products of heme [22–24]. It remains a staple technique in many toxicology, pharmacology, and research labs because it is flexible, ubiquitous, and relatively inexpensive. However, conventional UV/visible detection for porphryrin-related compounds is complicated by their varying and overlapping absorbance maxima and extinction coefficients. At the same time, the presence of an array of background metabolites which also absorb in the UV/vis range and are impossible to chromatographically resolve from the analytes is especially problematic in blood or tissue samples, complicating analyte identification and reliable quantification. Finally, conventional HPLC can suffer from high levels of run to run variability, necessitating the use of internal or external standards for compound identification and a significant investment of time/funds for optimizing the choice of column, solvent, and run conditions.

Mass spectrometry, now nearly as ubiquitous a tool as HPLC, offers an attractive alternative method for both compound identification and quantification. Many existing mass spectrometry-based studies on porphyrins have focused on identifying specific analytes or even particular isomers of analytes for diagnostic purposes, with an emphasis on tandem MS/MS [4,22–24]. Because they are highly refined to detect their target analytes, these methods can be exquisitely sensitive. Accordingly, they have been applied most extensively to the clinical monitoring of porphyrias where the biomarkers of disease are very well known [2,4,25–29]. However, of particular importance to the toxicology, pharmacology, and research labs are highly flexible methods. These should ideally use widely available workhorse instruments; provide reasonably rapid throughput with little or no method optimization; and allow for metabolite screening in cases where both the hypothetical drug/toxin target, and the array of background metabolites, may be unpredictable and where the background metabolites may far exceed the target analytes.

As an alternative to both existing HPLC and highly refined MS/MS diagnostics, we therefore examined ultrahigh resolution (±0.005 mass unit) electrospray ionization quadrupole time of flight (ESI-qTOF) mass spectrometry for rapid porphyrin profiling within biological media. Liver was chosen as the target medium, because of its importance in toxicology and pharmacology studies and its relatively greater complexity than blood or urine. We aimed to concurrently quantify a large set of diagnostic compounds having a broad range of solubilities and physical properties, against the background of diverse small molecules found in liver, using their exact masses obtainable from high resolution mass spectra. We reasoned that, if successful, this approach would obviate the need for chromatographic resolution, the analytical step generally requiring the greatest amount of optimization, as long as the compounds of interest differed in mass. A chromatography step was nonetheless evaluated for its capacity to relieve sample congestion and saturation at the ESI source and MS detector, using a solvent system compatible with MS analysis.

A complete, turnkey method is described here, starting from the evaluation of extraction methods for broad sets of porphyrin standards dissolved either in pure solvents or in a biological (rat liver) matrix. The stability, differential ionization, and solubility properties of the target porphyrins, the effects of the biological sample matrix, the effects of preconcentration of the analytes on disposable columns, and the limits of detection of the analytes are all described. The complete method was then applied to biological samples prepared directly from the whole livers of hexachlorobenzene (HCB) treated Sprague-Dawley rats. The results show correct identification of the step of heme biosynthesis interrupted by this toxin, obtainable from a very small mass (0.2 g) of a liver sample, using exact mass ultra HPLC-MS (uHPLC-MS). The same method was also validated for use on fractionated bacterial cell pellets. Bacteria were hypothetically understood to offer a less complex background matrix than liver, and one that might more closely approximate blood or urine. At the same time, tetrapyrrole biosynthesis, degradation, metabolic regulation, and homeostasis in bacteria are of interest for nutritional and pathogenesis research and the development of microbial biofuel cells. The method is immediately amenable to medium to high-throughput applications in research and application-oriented laboratory settings, requiring analytical equipment that is rapidly becoming standard in both environments.

Experimental

Materials and reagents

The porphyrin standards used were uroporphyrin I and III, heptacarboxyl porphyrin I, hexacarboxyl porphyrin I, pentacarboxyl porphyrin I, coproporphyrin I and III, mesoporphyrin IX, protoporphyrin IX, *N*-methyl protoporphyrin IX, and hemin, as well as the heme breakdown product biliverdin. 2-vinyl-4-hydroxymethyl-deuteroporphyrin IX was used as an internal reference. All

[1] *Abbreviations used*: ACN, acetonitrile; EICs, extracted ion chromatograms; ESI-qTOF, electrospray ionization quadrupole time of flight; HCB, hexachlorobenzene; HPLC, high-performance liquid chromatography; uHPLC-MS, ultrahigh-performance liquid chromatography–mass spectrometry.