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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.

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Supporting Information Available. Descriptions of the husbandry practices and treatment of rats and *Staphylococcus aureus* cultures; uHPLC and EIC traces for standards under different solvent conditions; demonstration of the efficiency of column purification of standards; tabulated quantitative porphyrin profiles for standards, standards injected into biological milieu, and untreated/hexachlorobenzene treated rat livers. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

metabolomic; porphyrin; biomarker; high mass accuracy mass spectrometry; toxicology

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, detected, and in many cases quantified within these biological milieu. High performance liquid chromatography (HPLC) 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 porphyrin-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, 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, turn-key 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 pre-concentration 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 SECTION

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 porphyrin standards were purchased from Frontier Scientific Inc. (Logan, UT). Methanol, water, acetonitrile, DMSO, and formic acid solvents were all HPLC or Trace Metal grade and were purchased from Fisher Scientific. Samples were stored in Supelco slit top 2 mL vials prior to and during analysis (Fisher Scientific). These were stored at -20°C when not in use and kept at 5°C while in the autosampler tray immediately prior to analysis.

Instrumentation

uHPLC separations were carried out on a Dionex Ultimate 3000 uHPLC system using a BDS Hypersil C18 column (150×2.1 mm) with a $2.4\text{ }\mu\text{M}$ particle size (Thermo-Scientific; cat # 28102-152130) which can be run at pressures as high as 8 kPa. This standard reverse-phase column was chosen for its low cost and broad applicability to chemical separations. Experiments described here were carried out at 2–4 kPa. Mass detection and analyte quantification were carried out using a standard microTOF-Q11 electrospray ionization time-of-flight quadrupole mass spectrometer equipped with a heated-electrospray ion source (Bruker). The data were analyzed by Bruker's Compass Data Analysis post processing software.

uHPLC-MS

Separations were achieved by linear gradient elution transitioning from 100% Solvent A (aqueous) to 100% Solvent B (organic) over 20 min, followed by a 3 min run of 100% solvent B and then a return to 100% Solvent A in a final 3 min washing. A flow rate of 0.4 mL/min with a column temperature of 50°C was used. Solvent A: ultrapure water with 0.1 % formic acid; Solvent B: MeOH with 0.1 % formic acid. UV spectra were measured over 390–420 nm using the HyStar software package. The uHPLC was coupled to an electrospray mass analyzer operating in positive ion mode. The spectrometer used a capillary voltage of 4500V and capillary temperature of 180°C . The nebulizing gas was set at 6.0 L/min. The software used for data analysis was Bruker Compass DataAnalysis.

uHPLC (for isomer separation)

The widely-cited method of Lim *et al.* was used with slight modifications [30]. Briefly, separation was achieved by linear gradient elution transitioning from 100% solvent A (aqueous) to 100% solvent B (organic) over 20 min, followed by a 3 min run of 100% solvent B and then a return to 100% solvent A in a final 3 min washing. A flow rate of 0.4 mL/min with a column temperature of 50°C was determined to be optimal for separation using the above method. Solvent A: 1 M ammonium acetate with 9% acetonitrile (pH 5.16,

adjusted with glacial acetic acid); Solvent B: MeOH with 9% acetonitrile. UV spectra were again measured over 390–420 nm using the HyStar software package.

Preparation of porphyrin standard solutions

Stock solutions were prepared in pure acetonitrile (ACN). Solutions were also made in 100 mM potassium phosphate buffer (pH = 6.8) for use in generating standard curves for bacterial lysates. To facilitate their solubilization in organic media, standards were acidified with concentrated HCl, neutralizing negative charges on the carboxylic acid side chains of the porphyrins. All stock solutions were kept in the dark at –20 °C to avoid photo-degradation. Porphyrin standard solutions were diluted in acetonitrile to working concentrations (0.05–10 µM) immediately before each run. An internal standard (2-vinyl-4-hydroxymethyl-deuterioporphyrin IX) was added to each sample to a final concentration of 0.05 µM in case it might be needed for quantification, or to assess run to run variability and instrument drift. Use of the standards for quantifications proved not to be required. Samples were stored in the dark at –20°C.

Husbandry practices and treatment of rats, and preparation of bacterial cell cultures

See Supplementary Information.

Homogenization and extraction of rat livers

Extraction procedures were adapted from Danton *et al.* and from Kennedy and James [31, 32]. According to the Danton procedure: diced 1g sections of liver were mixed with 0.5 mL of 0.01 M HCl and homogenized in 10 mL glass tubes using a Teflon pestle with a Caframo RZR50 homogenizer for 2 minutes. One mL of acetonitrile/dimethyl sulfoxide (4:1, v/v) was added, and the mixture was homogenized for a further 2 min. The sample was centrifuged for 15 min at 6400 – g in 50 mL tubes using a Beckman Coulter, Inc. J2–21 (4 °C) and the supernatant collected. The extraction was repeated twice and the supernatants pooled in a clean tube. The supernatants were then filtered through a 0.4 micron syringe filter (Millipore) to remove particulates. The resulting material is referred to as rat liver extract herein.

Kennedy and James developed a procedure specifically for quantitative isolation of structurally diverse porphyrins from animal tissue [31]. In it, smaller masses (100 mg or 200 mg samples) of liver were homogenized in a manner similar to the above, but in 6 mL 1:1 1M HCl:DMSO. Strong acid specifically promotes extraction of the more hydrophilic porphyrins into the organic phase. The rat liver samples used here were centrifuged at 6400 x g, the supernatants pooled following two successive extractions, and filtered as described above. These extracts were further concentrated and solvent exchanged on syringe-style Waters C18 trifunctionalized columns (WAT036815). This allowed for removal of the high concentrations of Cl[–] added during sample acidification, as well as for some pre-purification and concentration of the samples. The eluates (in ACN) were then either analyzed immediately by uHPLC-MS or stored at –20°C until analysis.

Both methods for preparing extracts were tested using livers from healthy/untreated Sprague-Dawley rats into which a standard stock solution, containing all 12 porphyrin derivatives described above, was injected to yield 1 nmol of each metabolite.

Measurement and application of standard curves

Porphyrin standard curves were generated in ACN and in phosphate buffer. Serial dilutions of a mixture of all 12 porphyrin metabolites were prepared at 0.1–100 μ M of each. Extracted ion chromatograms (EICs) were recorded for each individual standard and the peak areas (proportional to concentration) plotted versus the mass injected onto the column. Linear regression analysis was used to determine the coefficients of ionization (slopes of the standard curves) for each of the individual compounds. Standard curves were generated prior to each set of measurements on a given day. Typically, the coefficients of ionization measured from day to day varied by less than $\pm 10\%$.

RESULTS

Separation of pure standards by uHPLC

HPLC alone has previously been used as a standard method for the identification of porphyrins, including isomers, via their UV/Vis absorbance [32–33]. Typical run times for these measurements are ~60 minutes (excluding washes). Using the same solvent system (1M ammonium acetate/MeOH) and the increased pressure afforded by uHPLC, run times (excluding washes) were reduced to 26 minutes. Separation of 12 porphyrin standards using this standard solvent system and a typical C18 column is shown in Figure 1 (a subset of retention times is given in Table 1), with well-resolved peaks and elution times for all compounds analyzed [32–33]. The high concentration of ammonium acetate increases separation greatly enough to detect structural isomers (See, for example, Figure S1).

However, splitting of several peaks is observed. The exact cause of peak splitting is unknown; however, it was evident only at higher concentrations of standards (though carryover of compounds from one run into the next was not observed) and when there were many compounds present. This suggested that either interactions between the compounds in solution, or competitive binding of analytes on the column, was causing pure standards to elute non-isotropically. Such non-isotropic elution may be observed if the pure standard migrates through the column both alone and loosely complexed with another molecule, or if other analytes present in the same sample render the column matrix non-uniform via their interactions with it. Alternatively, double peaks could be due to protonation state isomers of the pyrrole rings; that is, one pair of tetrapyrrole rings (A/C) may be protonated in one peak versus another pair (B/D) in the second. Either way, if HPLC were to be used as an independent analytical method for a broad variety of porphyrins, the observed peak splitting would complicate compound assignment and quantification, particularly in the presence of background metabolites. Further refinement of the method, likely involving screening a variety of C18 column media, for example, would be essential. Additionally, liver metabolites were expected to contribute substantially to and compete with the UV/Vis absorbance of the analytes, complicating any HPLC-based method. As a consequence,

further optimization of HPLC/uHPLC was abandoned in favor of the development of an MS method.

Separation, identification, and quantification of standards by uHPLC-MS

The high concentration of ammonium acetate required by the above standard HPLC method strongly suppressed ionization of the compounds in the electrospray mass spectrometer, leading to low intensity mass spectral peaks. The extent of ionization suppression is illustrated in Table 1 for six porphyrin standards. The ammonium acetate suppressed ionization so drastically that the more hydrophilic compounds could only be detected at relatively high concentrations. As a consequence, this HPLC method could not be directly coupled to MS in an uncomplicated manner.

A more MS-friendly solvent system was therefore sought for further testing. Separation of the porphyrin standards by uHPLC using a simple, inexpensive, and MS-compatible formic acid/methanol solvent system is shown in Figure S2. Figure S3 shows the mass spectrometric extracted ion chromatograms (EICs) used to identify and quantify (via peak integration) each standard. For either solvent, the elution time of the porphyrin increases with increasing hydrophobicity and decreasing number of carboxylic acid groups (Scheme 1). The latter solvent system has elution times slightly later in the run, ranging from 10 to 22 minutes (Table 2). The separation of the compounds is also less effective in this solvent system than in the ammonium acetate described above. Biliverdin and pentacarboxylic acid fail to resolve well, many of the compounds exhibit broadened and/or split peaks, and there is incomplete separation of the respective uroporphyrin and coproporphyrin isomer pairs. As with some of the analytes in the ammonium acetate solvent, double peaks were seen consistently for some compounds. However, the poorer chromatographic resolution was not problematic for the MS-based analysis, since detection was based on mass accuracy and quantitation was relative to integrated standard curves which exhibited the same peak profiles.

Finally, it is important to note that all standards could be identified and quantified upon direct injection into the mass spectrometer. However, the inclusion of a wide array of compounds in a single injection quickly saturated the detector. There was also some evidence of standard compounds mutually suppressing one another's ionization. Both problems were expected to limit the sensitivity of the method for detecting small amounts of metabolites from liver, and to be exacerbated in the presence of competing background metabolites. Therefore, the chromatography step was deemed useful for applying the method to complex, background-rich biological media. The choice of solvent and column, and the use of uHPLC in place of HPLC, were expected to minimize the expense and time of this step, respectively.

Standard curves and quantification

Standard curves were generated for all porphyrins by plotting the average chromatogram peak area versus concentration. The standard curves were linear over at least 0.1–10 μM , and up to 100 μM for heme and PPIX. Higher concentration ranges were not investigated for the other standards. Slopes for standard curves for compounds dissolved in pure acetonitrile

are reported in Table 2. As stated in the footnote to that table, deviation of least squares R^2 values from 1 were minimal for the linear fits. As evidenced by the differences in slopes and individual integrated EIC peak areas (Tables 1–2), the compounds in this set have intrinsically very different ionization efficiencies (see below), reflecting their diverse physicochemical properties. The lower limits of detection were estimated using a signal-to-noise ratio of 3. By this criterion, detection limits were approximately 50 nM for all porphyrins in solution.

Precision between and within runs and compound stability

Precision with respect to quantitation of standards was determined by measuring standard solutions in triplicate and computing EIC peak area standard deviations. Inside the linear range, measurements of the concentrations of individual standards made via sequential injections into the uHPLC-ESI-qTOF exhibited standard deviations of • 15%. Measurements were also made at different times over a period of week (0 days, 3 days, 7 days) to determine the stability of the compounds. Between injections, standards were stored in the dark at -20°C . Detected quantities of the stored compounds were compared with standards that were freshly made. Significant degradation was not observed in any of the porphyrin standards. No compound carryover effects were observed when a 15-minute wash of the uHPLC column with the organic solvent before and after each analytical series was used.

Porphyrin solubilization and extraction from rat liver

Quantitative porphyrin extraction from a biological/cellular sample is particularly challenging since the metabolites of interest range from highly hydrophilic at the earlier steps of the biosynthetic pathway to highly hydrophobic at the end and following heme degradation (Scheme 1). Porphyrin extraction using the generalized method of Lim *et al.* was tested for generating analytical extracts from solutions of standards injected into untreated rat livers [32]. This method proved less effective at isolating the more polar/hydrophilic heme precursors, giving typical extraction yields of 5–50% (data not shown). The method developed by Kennedy and James for the extraction of porphyrins specifically from tissue, with the modifications described above, was much more effective in the context of liver [31]. Typical porphyrin recoveries were •50% in yield for each standard (Table S1).

Evaluating the effects of matrix suppression and the need for column purification

The porphyrin analytes used here vary widely in polarity and molecular weight; as a consequence, their intrinsic ionization efficiencies were observed to be highly variable, leading to very large differences in measured EIC peak areas for equimolar solutions of different compounds (Figure S3). Referencing to standard curves is therefore clearly essential for quantifying the analytes' actual or relative concentrations.

Suppression of analyte ionization in the mass spectrometer due to constituents of the matrix was also considered. Ionization suppression could limit the accuracy and sensitivity of analyte quantification. The liver medium was anticipated to contain a high concentration of small organic molecules of varied composition. At the same time, analyte extraction conditions intended to protonate the porphyrin propionates could render the extract high in ionic strength, limiting the ionization of the analytes as was already observed in the 1M

ammonium acetate solvent system, Table 1. The rat liver extracts initially prepared by the method of Kennedy and James, for example, contained not only complex background metabolites but 0.5 M Cl^- from the HCl used to acidify and hence neutralize the negative charges on the porphyrins. The final step of the Kennedy and James procedure used syringe-style C18 columns to pre-purify, concentrate, and exchange the sample analytes into a new solvent (prior to HPLC analysis). We therefore examined the potential improvements for the MS method afforded by a column isolation step in which the extraction matrix is exchanged for ACN, an optimal MS solvent.

Figures S4 (uHPLC trace) and Figure S5 (EICs) show representative analyses for porphyrin standards suspended in the low ionic strength rat liver extract prepared by the method of Lim (i.e., samples acidified in 0.01 M HCl). These samples clearly illustrate the effects of interfering biological metabolites and in particular a significant amount of background metabolites detectable in the UV/vis uHPLC trace. Figure 2 shows similarly prepared samples following extraction and column purification by the full method of Kennedy and James, in which the samples are first strongly acidified (0.5 M HCl) during the extraction, and then solvent exchanged into ACN via a syringe-style C18 column. Recovery of porphyrins following elution appeared to be near 100% (Figure S6). Matrix suppression in the mass spectrometer is still noticeable (Figure 2). Its extent varies from compound to compound, but is reduced in magnitude relative to Figure S5 (no column purification). These results indicate that column isolation and elution are important both for maximizing extraction yields from liver (since they allow for the more strongly acidic extraction conditions) and minimizing subsequent suppression of analyte ionization in the mass spectrometer (due possibly to removal of Cl^-). The yields observed here were less than those reported by Kennedy and James (> 90%), potentially because the compounds in the present study were not dried and acidified following column isolation. Acidification helps to solubilize the porphyrins, particularly the more hydrophilic ones, in ACN. Acidification was avoided here because high concentrations of HCl would result in ionization suppression in the mass spectrometer, not an issue for Kennedy and James since HPLC was their analytical method. Thus, we expect the lower apparent yields reported in Table S2 are due to failure of the more hydrophilic porphyrins to solubilize completely in ACN, a phenomenon observed routinely in this work.

Application to hexachlorobenzene-treated rats

Following optimization of the method against standards dissolved in liver matrix, 200 mg samples from the livers of corn oil-treated rats (negative control samples) were extracted and analyzed to establish baseline levels of metabolites for untreated animals. The livers of hexachlorobenzene-treated rats were similarly analyzed. As seen in Figure 3, uroporphyrin and heptacarboxyl porphyrin are present in high concentrations in the 10 treated animals, in agreement with expectation based on prior literature. The concentrations of the various porphyrins in untreated rat livers (corn oil/vehicle only treated) and hexachlorobenzene-treated livers can be seen in Tables S3 and S4 respectively. Uroporphyrin and heptacarboxyl porphyrin concentrations were detected at ranges of 11–82 nmoles/g-liver and 4.3–36 nmoles/g-liver, respectively, in the treated animals. The observed range of responses to hexachlorobenzene treatment could be due to the animal-to-animal variability intrinsic to the

outbred Sprague-Dawley strain rat population. None of these compounds were detected in the negative control. In addition, hexacarboxyl porphyrin and coproporphyrin can be seen in most of the hexachlorobenzene treated animals at levels less than 2.0 nmoles/g-liver. Notably, heme concentrations increased roughly 2-fold in the hexachlorobenzene treated animals relative to the controls. The reasons for the increases are unclear, but may be due to upregulation of heme-containing cytochrome P450s in the presence of relatively large doses of the xenobiotic compound.

Application to bacterial cell lysates

In addition to tissue samples, bacterial cell lysates were investigated. The weakly acidic extraction method (0.01 M HCl), described above, successfully isolated porphyrins from both cell wall and spheroplast (inner membrane/cytoplasmic) fractions of *Staphylococcus aureus*. Because strong acid was not employed, the syringe-style C18 column clean up step was potentially unnecessary. Matrix effects on the ionization of added standards were minimal in the bacterial extract. Heme was shown to be the major metabolite in the samples (data not shown) at approximately 7500 heme molecules/cell in the cell wall fraction and 40,000 heme molecules/cell in the spheroplast fraction.

DISCUSSION

The accumulation of the porphyrin precursors of heme in tissues can serve as biomarkers of disease and particular xenobiotic exposures. The most widely used methods for rapidly identifying heme and its precursors from biological samples are HPLC-based and consequently can suffer from significant interference from background biological metabolites. These methods, moreover, may require significant column- and analyte-specific optimization in order to render them quantitative. Sophisticated MS/MS methods, on the other hand, have been developed for highly sensitive monitoring of specific porphyrias [4, 22, 22–24], but may not be generally accessible or flexible enough for toxicological, pharmacological, or basic research applications. High resolution (± 0.005 mass units) electrospray ionization time of flight mass spectrometric methods allow for the definitive identification of compounds of interest, even against a complex background, via their exact masses. Moreover, when used with standard curves, ESI-qTOF-MS can be highly quantitative, which is strongly desirable for toxicological studies. Finally, high-resolution mass spectrometry is adaptable to medium throughput analyses and increasingly ubiquitous in a variety of laboratory settings. Here, we evaluated ESI-qTOF-MS-based methods for quantitatively profiling a series of diagnostically important porphyrins from a toxicologically relevant biological matrix (liver), starting with sample preparation and addressing all steps through quantitation.

We first examined uHPLC as a potential method for porphyrin quantification. The most widely used solvent system for these analyses, aqueous 1M ammonium acetate, can provide very good resolution of porphyrins and even their structural isomers if optimized [33]. Isomer resolution is important for a subset of specialized applications. For example, interruption of coproporphyrinogen oxidase, with coproporphyrinogen III as its natural substrate, leads to accumulation of coproporphyrinogen III [34]. However, the unnatural

coproporphyrinogen I can accumulate if the enzyme preceding coproporphyrinogen oxidase is inhibited [35]. Because coproporphyrinogens I/III have the same mass and strongly similar structures, a high resolution separation method is required in order to distinguish them. Using uHPLC, we observed that isomer pairs are indeed resolvable (Figure 1, S1), and for the case of isomer resolution, the high ionic strength 1M ammonium acetate solvent appears to be necessary.

However, there were two potential complications to using uHPLC as an analytical method. First, we observed that background metabolites that absorb in the UV/vis provided significant interference with detection and quantification of the target compounds (not shown). Second, when a broad set of porphyrin standards was used, splitting of several peaks in the uHPLC trace was observed. We speculated that analyte-analyte interactions, or competitive binding of analytes to the column, could be at work. Hence, the method would have to be further optimized for this solvent/column/instrument combination, and for the presence of background compounds from the liver matrix to render it fully applicable. However, our objective was to identify a flexible method that minimized the need for analyte- or matrix-specific optimizations.

Detecting analytes by their exact mass using a standard ESI-qTOF-MS instrument was therefore deemed preferable. Importantly, compound identification and quantification (though not isomer resolution) can be achieved using their exact masses. As a consequence, a well resolved separation is not needed. Similarly, neither high reproducibility in the uHPLC retention times nor strongly absorbing background metabolites are problematic for the analysis, since the UV/visible trace is effectively not used. The uHPLC separation was nonetheless retained as part of the method, in order to fan out the complex mixture of analytes and background species. This maximized the sensitivity of detection and minimized the likelihood of saturating either the electrospray source or mass detector. The standard 1M ammonium acetate used with uHPLC was incompatible with downstream MS since the concentrated salt repeatedly clogged the electrospray source. We therefore chose a simple, inexpensive, aqueous methanol/acetic acid solvent system that was highly compatible with LC-MS. Though the quality of the separations was clearly not as good, this did not affect our ability to identify and quantify standards via the integrated extracted ion chromatograms (EICs) selected for each compound's exact mass (Figures 2–3 and Table 2).

A second major area of concern was sample preparation. Porphyrins are a challenging class of compounds since the analytes of interest – ranging from highly water soluble uroporphyrin on the one hand, to the multiply decarboxylated and therefore more hydrophobic heme and biliverdin products on the other – are challenging to net in a single extraction step. At the same time, ionization suppression of the analytes, due to salts or analyte interactions with one other or the matrix, had to be minimized. A versatile method that would be applicable to challenging tissue and blood samples in addition to urine was likewise desirable. Finally, to the extent possible, the method should allow for quantitative isolation of a range of porphyrins and breakdown products.

For successful extraction of liver tissues, we observed that at least 3 factors were critical. First, the tissue needed to be well homogenized via mechanical means (for example, a

rotating mortar and pestle). Chemical or osmotic lysis of the cells or other treatments were not needed. Second, acidification of the homogenates in strong HCl was necessary (1M HCl, 50% final sample volume, pH ~0.9), likely because the more hydrophilic species could be driven into the organic phase (DMSO) under these conditions. Finally, isolation and elution of the porphyrin species on a syringe-style C18 column eliminated significant amounts of background metabolites as well as Cl^- , both of which contributed to significant ionization suppression in the analytes. The sensitivity of the mass spectrometer (to picomoles of sample) and the efficiency of elution from the C18 media moreover allowed for the analysis of very small (<100 μL) volumes of liver extract produced from 0.2 g tissue samples. However, if it were necessary to detect vanishingly small quantities of metabolites, sample concentration/drying following the elution step is expected to improve the observed recovery yield.

Bacterial cells, a less complex biological matrix analyzed for comparison, did not require column purification for quantitative detection. The inner-membrane-bound portion of the Gram positive pathogen, *Staphylococcus aureus*, was isolated by enzymatically digesting its outer cell wall. The resulting protoplasts, similar to whole Gram negative bacteria, were disrupted by sonication and analyzed. Unlike the liver matrix, standards injected into this fraction did not show significant ionization suppression relative to standards analyzed in ACN (data not shown). Although the C18 columns could be used for purification, it was shown that sufficient sonication of the bacterial cells gave MS peak areas that were equal to areas obtained after the purification step. Thus, for the bacterial specimens, this step could be omitted. We speculate that less dense biological materials, such as urine or potentially blood, might behave similarly to the bacterial cells, though this was not explicitly tested.

Using the above-described method, interruption in step 4 of porphyrin biosynthesis (catalyzed by uroporphyrinogen decarboxylase) was correctly identified in hexachlorobenzene-treated Sprague-Dawley rats. These rats had levels of uroporphyrin and heptacarboxylporphyrin that were highly elevated above those detectable in untreated rats. Hexacarboxylporphyrin and coproporphyrin were also seen to be slightly elevated, although to a lesser extent (Scheme 1). This pattern of metabolites is consistent with expectation, based on prior work [5, 19–21].

CONCLUSIONS

High resolution mass spectrometry afforded by a quadrupole time of flight instrument of the type available in many laboratories provided a simple, high throughput, quantitative means of diagnosing and monitoring porphyrin profiles in two complex biological media. The use of uHPLC with an MS-compatible solvent system, a highly acidic extraction step, and the use of C18 columns as a pre-cleaning step for the liver samples were all essential. Strong acid and subsequent sample clean up were not important for maintaining the sensitivity of the analysis from bacterial cells. The procedures developed here, from sample preparation to data analysis, have been subjected to the necessary controls and are ready for implementation on any appropriate mass spectrometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Separation of 12 porphyrin standards via uHPLC in a commonly used high-ammonium acetate solvent system: 9% acetonitrile 1M ammonium acetate/methanol. 20 μ L of a mixture of standards (0.5 mM each) dissolved in 4:1 ACN:DMSO was injected onto the uHPLC column and resolved over 20 min. Analytes: (A) uroporphyrin I; (B) uroporphyrin III; (C) heptacarboxylporphyrin; (D) hexacarboxylporphyrin; (E) pentacarboxylporphyrin; (F) coproporphyrin I; (G) coproporphyrin III; (H) biliverdin; (I) N-methyl protoporphyrin IX; (J) hemin; (K) mesoporphyrin IX; (L) protoporphyrin IX. Note that while resolution of peaks is good, several peaks are split, possibly due to interactions of standard compounds with one another. See Scheme 1 for structures, Table 2 for analyte molecular weights, and Table 1 for a sampling of retention times.

Figure 2.

Extracted ion chromatograms used to identify and quantify 10 porphyrin standards after application of the entire method. The 10 porphyrins were spiked into a 100 mg liver sample. The liver was homogenized and porphyrins extracted using the method described, and the sample was purified and concentrated on the Waters Sep-Paks before injection onto the uHPLC-MS. The 0.1% formic acid water/methanol chromatography solvent system is used, and 20 μ L of sample was injected onto the column. Analytes: (A) uroporphyrin I/III (mixture); (B) heptacarboxylporphyrin I; (C) hexacarboxylporphyrin I; (D) pentacarboxylporphyrin I; (E) coproporphyrin I/III (mixture); (F) biliverdin; (G) hemin; (H) N-methyl protoporphyrin IX; (I) mesoporphyrin IX; (J) protoporphyrin IX. See Scheme 1 for structures and Tables 1 and S1 for molecular weights and retention times.

Figure 3.

Analysis of rat livers from animals treated with hexachlorobenzene via high resolution MS. HCB is a well-characterized porphyrogenic agent that induces a deficiency in uroporphyrinogen decarboxylase (UROD) and has been shown to cause a characteristic accumulation of uroporphyrin and heptacarboxylporphyrin in hepatic tissue (see text). The formic acid water/methanol solvent system is used. 15 μ L of column purified sample was injected onto the column. The expected retention times of the analytes are indicated on the MS trace, and the identities of compounds corresponding to the EIC peaks (determined by mass) are labeled. Analytes: (A) uroporphyrin I/III (mixture); (B) heptacarboxylporphyrin I; (C) 2-vinyl, 4-hydroxymethyl deuteroporphyrin IX; (D) protoporphyrin IX; (E) hexacarboxylporphyrin I; (F) pentacarboxylporphyrin I; (G) biliverdin; and (H) coproporphyrin I/III (mixture).

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).

Table 1

Typical uHPLC elution times and integrated MS peak areas for 6 of the standards analyzed in 9% acetonitrile/1M ammonium acetate/methanol and 0.1% formic acid water/methanol solvent systems.^a

Standard	9% ACN in 1M Ammonium Acetate/MeOH		0.1% Formic Acid in Water/MeOH	
	Elution Time (min)	EIC peak area ^a	Elution Time (min)	EIC peak area ^a
Uroporphyrin I	6.0	6.5600×10^2	12.8, 13.3	1.0277×10^5
Heptacarboxyl porphyrin	8.0	1.6780×10^3	13.7, 14.3	1.3682×10^5
Hexacarboxyl porphyrin	10.0	3.5680×10^3	14.6, 15.1	1.7464×10^5
Pentacarboxyl porphyrin	11.6	1.3084×10^5	15.5, 16.0	2.3252×10^5
Coproporphyrin I	13.2	1.9694×10^5	16.5, 17.0	4.9799×10^5
Mesoporphyrin	19.2	1.3705×10^5	18.3, 18.8	1.7540×10^6

^aPeak areas were determined by integrating over the entire EIC of the compound, which sometimes appeared as a two-peak structure, particularly in the formic acid solvent system. Areas are given per 5 μ M of compound injected onto the column.

Table 2

Representative results from standard curves for compounds resolved in 0.1% formic acid in water/MeOH

standard	Molecular weight (g/mol)	Elution Time (min)	m _{porph} (EIC counts × 10 ³ /mM) ^{a,b}	Linear measurement range (mM) ^c
Uroporphyrin	830.23	12.8, 13.3	76.8	0.1 – 5+
Heptacarboxyl porphyrin	786.24	13.7, 14.3	118	0.1 – 5+
Hexacarboxyl porphyrin	742.25	14.6, 15.1	133	0.1 – 5+
Pentacarboxyl porphyrin	698.26	15.5, 16.0	150	0.1 – 5+
Coproporphyrin	654.27	16.5, 17.0	192	0.1 – 10+
Mesoporphyrin	566.29	18.3, 18.8	325	0.1 – 10+
Protoporphyrin IX	562.26	21.4	130	0.1 – 100+
N-methyl protoporphyrin IX	576.27	18.8, 19.8	150	0.1 – 5+
Hemin	615.19	17.5	116	0.1 – 100+
Biliverdin	582.25	16.1	192	0.1 – 2.5+
2-vinyl, 4hydroxymethyl (IS)	566.29	18.5	175	0.1–5+

^a All least squares R² values for linear fits to the data were above 0.97.^b EIC = extracted ion chromatogram.^c True lower limits of detection, representing the absolute sensitivity of the mass spectrometer detector and the degree of sample noise, are likely significantly lower than these numbers. At the other end of the concentration range, saturation was reached for certain metabolites. A plus sign indicates that no suppression was reached at the concentrations investigated.