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Examination of the Fragmentation Behavior of Hemin and Bilin Tetrapyrroles by Electrospray Ionization and Collision-induced Dissociation

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Abstract : Bilin tetrapyrroles are metabolic products of the breakdown of porphyrins within a species. In the case of mammals, these bilins are formed by the catabolism of heme and can be utilized as either biomarkers in disease or as an indicator of human waste contamination. Although a small subset of bilin tandem mass spectrometry reports exist, limited data is available in online databases for their fragmentation. The use of fragmentation data is important for metabolomics analyses to determine the identity of compounds detected within a sample. Therefore, in this study, the fragmentation of bilins generated by positive ion mode electrospray ionization is examined by collision-induced dissociation (CID) as a function of collision energy on an FT-ICR MS. The use of the FT-ICR MS allows for high mass accuracy measurements, and thus the formulas of resultant product ions can be ascertained. Based on our observations, fragmentation behavior for hemin, biliverdin and its dimethyl ester, phycocyanobilin, bilirubin, bilirubin conjugate, mesobilirubin, urobilin, and stercobilin are discussed in the context of the molecular structure and collision energy. This report provides insight into the identification of structures within this class of molecules for untargeted analyses.

Keywords : Bilins, Tetrapyrroles, Collision-induced dissociation, Electrospray ionization, Fourier transform ion cyclotron resonance, Metabolomics

Introduction

Heme (iron protoporphyrin IX) is an essential tetrapyrrole vital to many organisms ranging from bacteria to mammals.[1] In mammals, heme is catabolized to bilirubin; the action of microflora on bilirubin glucoronides[2] leads to the production of a series of linear tetrapyrroles known as bilins. An outline of the metabolic degradation of heme to the bilins is shown in Figure 1.

Interest in bilins has increased since they were discovered to have diagnostic value as urinary biomarkers for sepsis,[3] as fecal biomarkers for cirrhosis,[4] as markers of human waste contamination,[5,6] and as evidence of the exposure of bow flies (*P. regina*) to vertebrate fecal waste.[7] Recent work in our laboratory suggests that two of the bilins, stercobilin and stercobilinogen, are promising biomarkers for autism spectrum disorders (ASD) in a murine model.[8]

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Relatively few reports on the fragmentation behavior of bilins in the positive ion mode exist. Jones-Lepp first reported a collision-induced dissociation (CID) tandem mass spectrum for urobilin from its hydrochloride.[5] Cao *et al.* reported positive ion CID mass spectra for two compounds derived from fecal samples, urobilin (L form, also known more commonly as stercobilin) and urobilinogen (L form, also known as stercobilinogen) as well as bilirubin standard.[4] We reported positive ion CID mass spectra of bilirubin, *i*-urobilin and its dimethylester, and stercobilin and its dimethylester, at multiple collision energies, collision gases, and mass analyzers.[9] More recently, the CID analysis of stercobilin was extended to deuterated forms, gaining additional insight into its fragmentation behavior.[10] Owings *et al.* reported MS/MS of three different bilins with *m/z* 591 (D-urobilinogen, *i*-urobilin, and third unidentified bilin).[7] Inspired by these reports, here the positive ion CID mass spectra of heme and several other bilins are reported; for several of these compounds, this is the first report of MS/MS using any activation method. Where previous reports of CID of these compounds exist, the present work will be compared to the earlier reports.

Experimental

Materials

The following materials were utilized throughout the experiments, all solvents are HPLC grade unless otherwise

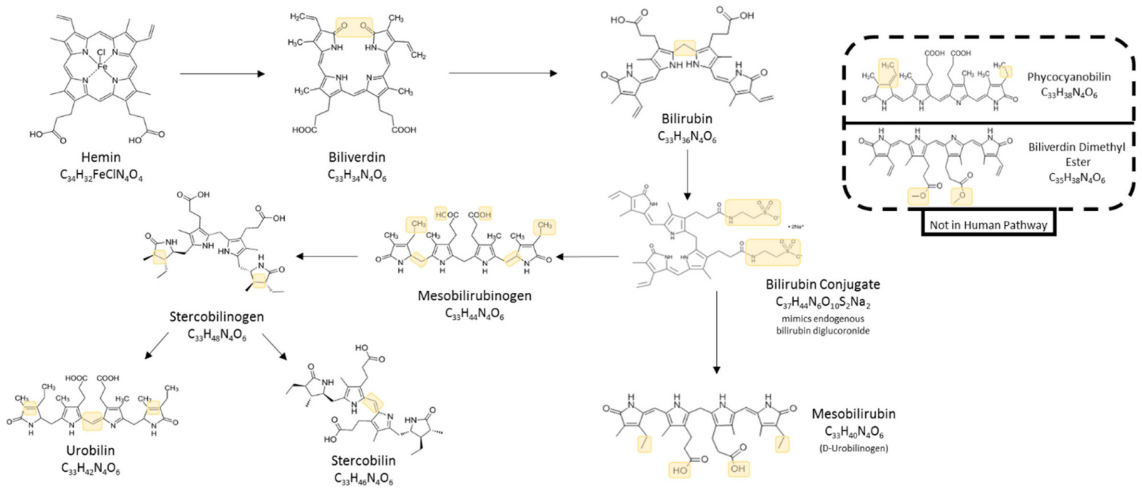

Figure 1. Outline of the metabolic degradation of heme; metabolites indicated subsequent to the bilirubin conjugate are produced by microflora in the colon. Highlighted are the portions of the chemical structures which exhibited a change from the previous compound. Phycocyanobilin and biliverdin dimethyl ester were both investigated, but are not present in the human metabolic pathway, with their respective precursor being biliverdin.

Table 1. Comparison of the fragmentation pattern of all of the bilins tested at 10V. Reported are the observed exact masses of fragment ions in *m/z* with reported ppm error in parenthesis (nd = not detected).

	Hemin	Biliverdin	Phycocyanobilin	Bilirubin	Bilirubin Conjugate	Mesobilirubin	Urobilin	Stercobilin
1 outer pyrrole lost	nd	nd		nd	nd	nd	466.23242 (-2.64)	470.26360 (-2.87)
2 outer pyrroles lost	nd	nd	nd	nd	nd	nd	343.16451 (-2.10)	345.18012 (-2.21)
Half product	nd	297.12265 (-2.42)	301.15405 (-2.06)	299.13830 (-2.40)	406.14235 (-1.90)	301.15402 (-2.15)	303.16967 (-2.12)	305.18528 (-2.26)
1 inner pyrrole left	nd	nd	nd	nd	nd	nd	nd	180.10124 (-3.69)

stated: hemin, equine (Aldrich Chem. Co., St. Louis, MO, USA), biliverdin hydrochloride (Cayman Chemical Company, Ann Arbor, MI, USA), bilirubin, mixed isomers (Sigma, St. Louis, MO, USA), urobilin hydrochloride, stercobilin hydrochloride, bilirubin conjugate, biliverdin dimethyl ester, phycocyanobilin, and mesobilirubin (Frontier Scientific, Logan, UT, USA), anhydrous methanol 99.8% acetyl chloride and acetonitrile (Sigma Aldrich, St. Louis, MO, USA), ammonium acetate, water, and formic acid, 88% (FA) (Fisher, Pittsburgh, PA, USA), and ammonium hydroxide 28.0-30.0% (J.T. Baker, Phillipsburg, NJ, USA)

Methods

To prepare stock solutions for positive ion mode, 5.0×10^{-6} M solutions of hemin, biliverdin, phycocyanobilin, bilirubin conjugate, biliverdin dimethyl ester, urobilin, and stercobilin were made in 1 mL of 20% acetonitrile (ACN) and 5 µL of FA was added. Bilirubin and mesobilirubin were made as a 5.0×10^{-6} M solutions in 1 mL of 50:50 ACN: 5 mM ammonium acetate. For negative ion mode, all bilin tetrapyrroles were made as 5.0×10^{-6} M solutions in 1 mL of 50:50 ACN: 5 mM ammonium acetate. For the hemin sample, 5 µL of ammonium hydroxide was added.

Methyl-esterification of the inner carboxyl groups on hemin, biliverdin, bilirubin, urobilin, and stercobilin were made by combining 10 µg of bilin to 500 µL of freshly made methanolic HCl. Methanolic HCl was produced by combining 160 µL acetyl chloride dropwise to 1mL of anhydrous methanol.

Instrumentation

Mass spectra were recorded on a Bruker Solarix 12T FT-ICR MS (Billerica, MA, USA) utilizing electrospray ionization (ESI) in positive ion mode. Tandem MS experiments were completed by CID using argon as a collision gas with collisional energies varying from 5 V-40 V depending on the sample.

Results and Discussion

Full scan ESI mass spectra of unpurified hemin ($C_{34}H_{32}FeClN_4O_4$) mixture indicates the detection of $[M-Cl]^+$ species at *m/z* 616.17641 (theoretical *m/z* 616.17674, -0.54 ppm error). The positive ion ESI shows primarily the loss of carboxylic acid side chains at lower energies with increasing losses of side chains as the collisional energy was ramped to 40V. Through this, the iron coordinated ring stayed intact. It may be of interest in further studies to utilize a higher energy to determine if the breaking of the coordinated ring could be obtained.

The fragmentation of biliverdin, bilirubin, and mesobilirubin all exhibited similar behaviours. Unpurified standards of the aforementioned bilins were observed at their respective $[M+H]^+$ peaks. Bilirubin ($C_{33}H_{36}N_4O_6$) was detected at *m/z* 585.26838 (theoretical *m/z* 585.27076, -4.07 ppm error), biliverdin ($C_{33}H_{34}N_4O_6$) at *m/z* 583.25283 (theoretical *m/z* 583.25511, -3.91 ppm error), and mesobilirubin ($C_{33}H_{40}N_4O_6$) at 589.29997 (theoretical *m/z* 589.30206, -3.55 ppm error). In these bilins, the loss of outer pyrroles was not observed with any of the collisional energies utilized. Instead, the half product, from dissociation of the C-C bond between the central bridge methylene carbon to an inner pyrrole, is the primary fragment ion generated. Mesobilirubin proved to be the least stable of the group, and fragmentation above 10 V was unable to be completed.

The full scan ESI mass spectra of unpurified bilirubin conjugate ($C_{37}H_{44}N_6O_{10}S_2Na_2$) was unusual in that no ions consistent with the intact molecular structure were observed. Two peaks were observed at *m/z* 819.24335 and *m/z* 797.26150, respectively. Accurate mass measurements suggest the former peak is most likely due to loss of C_2 from the protonated bilirubin conjugate (theoretical *m/z* 819.24285, 0.61 ppm error), but loss of neutral sodium from the radical cation of bilirubin conjugate cannot be discounted completely (theoretical *m/z* 819.24525, -2.33 ppm error). For the latter peak, exact mass measurements favor $[M - 2Na + H]^+$ as the identity (theoretical *m/z* 797.26331, error -2.27 ppm). As observed in Table I, at 10V the loss of an outer pyrrole is not observed, but as voltage was increased, the loss of outer pyrroles was detected as well as more effects on the taurate groups. Although many dissociation channels are accessible because of the taurate groups, the half product observed still exhibits one intact taurate group that changes as higher voltages are applied.

Although not a part of the human metabolic pathway, full scan ESI of unpurified phycocyanobilin ($C_{33}H_{38}N_4O_6$) was examined for comparison of possible fragmentation patterns shared with other bilins. Phycocyanobilin was observed at its corresponding $[M+H]^+$ peak at *m/z* 587.28641 (theoretical *m/z* 587.28452, 3.22 ppm error). Although phycocyanobilin's nearest precursor in its metabolic pathway is biliverdin, phycocyanobilin exhibits the loss of one of its outer pyrroles as well as the half product, whereas biliverdin only exhibits its respective half product. As collision energy increases, more losses are seen from the half product of phycocyanobilin.

Although we have previously reported on the fragmentation patterns of unpurified urobilin and stercobilin, each bilin was repeated in this study to ensure the validity of the results observed previously. Both bilins were observed at their $[M+H]^+$ peaks at *m/z* 591.31771 and *m/z* 595.34901 respectively (theoreticals: *m/z* 591.31526 and *m/z* 595.34658, 4.14 and 4.08 ppm error, respectively). As in our previous report, both urobilin and stercobilin exhibited the loss of one and two outer pyrroles, as well as their half products.[9] Stercobilin was the only substance within the tetrapyrroles for which 10 V could generate a protonated fragment for one inner pyrrole with its intact COOH side chain.

Methyl esterification was completed for hemin, biliverdin, bilirubin, urobilin, and stercobilin to validate that the half product was observed in the fragementation spectra. Also completed was a standard of bilivderdin dimethyl ester to ensure the methyl esterification procedure was carried out properly. In the case of hemin, although a species with the addition of 28 Da was observed, the species could not be isolated for fragmentation. The addition of acid in the methyl esterification process for bilirubin gives rise to a product that indicates that bilirubin has been converted to biliverdin. The addition of acid to bilirubin has previously been reported to cause tautomerization yielding violins.[11] Urobilin, stercobilin, and biliverdin each exhibited the addition of 28 Da to their $[M+H]^+$ peak, as well as an addition of 28 Da to what was hypothesized to be the loss of an outer pyrrole and an addition of 14 Da to what was hypothesized to be the half product of each of the bilins. Comparison of the standard biliverdin dimethyl ester to that synthesized by methyl esterification in the laboratory exhibited many of the same peaks within the CID mass spectra. Additional fragmentation pathways were observed in the purchased standard not observed in the laboratory-synthesized compound possibly due to higher sensitivity with the standard compound.

The CID of bilirubin, biliverdin, and mesobilirubin exhibited similarities in their fragmentation behaviours. In these compounds, the loss of an outer pyrrole was not observed at any of the collisional energies utilized, and the half product of each compound was the predominant fragment ion observed. In contrast, the other listed bilins (except for hemin, which is a cyclic tetrapyrrole) exhibited the loss of outer pyrroles as well as the half product being present. Although the molecular structure of a compound plays an important role in the fragmentation patterns exhibited, from the data presented herein, it not the only factor that plays a role. The solvent conditions utilized

within studies may play a larger role than previously considered on the structures' fragmentation. This may be due to conformational changes in certain solvents, and warrants further investigations. Another possibility for the differential fragmentation patterns may arise from the collisional cross-sections of the molecules, which may also be influenced by solvent effects. Should the noted compounds exhibit more compact or open structures, this may make an impact on the fragmentation that occurs. To further investigate this claim, ion mobility mass spectrometry (IM-MS) or cross-sectional areas by FT-ICR (CRAFTI) could be utilized to further investigate the size and shape of the gas-phase ions and their potential impact.[12]

Conclusions

CID fragmentation of various bilin tetrayrroles within the combined human-microbiome metabolomic pathway (as well as some outside the pathway) were investigated to determine patterns in fragmentation behavior. While many of the bilins generate both the loss of outer pyrroles as well as half products, bilirubin, biliverdin, and mesobilirubin only exhibited their respective half products. It is shown that the molecular structure alone can not be utilized to determine what fragmentation should be observed. Solvent conditions affecting the conformation of the molecule as well as collisional cross-sections may play larger roles then previously thought in fragmentation pathways.

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