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Analysis of urinary and faecal porphyrin excretion patterns in human porphyrias by fast atom bombardment mass spectrometry[1]

Jinli Luo, John H. Lamb, C.K. Lim *

MRC Toxicology Unit, Hodgkin Building, University of Leicester, Box 138, Lancaster Road, Leicester, LE1 9HN, UK

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Abstract

We report a new method for obtaining urinary and faecal porphyrin excretion patterns in human porphyrias based on fast atom bombardment mass spectrometry (FAB-MS). Porphyrins were esterified and extracted from urine or faeces as their methyl esters for analysis by FAB-MS. The protonated pseudo-molecular ion $[M+H]^+$ observed for each porphyrin is characteristic of that porphyrin, thus allowing a mixture of porphyrins to be analysed without the need for chromatographic separation. By using tandem MS, identification and characterisation of unknown porphyrins can be achieved. The urinary and faecal porphyrin excretion patterns from various porphyric patients obtained by FAB-MS are in good agreement with those analysed by TLC or HPLC methods. © 1997 Elsevier Science B.V.

Keywords: Porphyrins; Porphyria; Profiling; Fast atom bombardment mass spectrometry

1. Introduction

The porphyrias are disorders of haem biosynthesis characterized by the excessive production, accumulation and excretion of porphyrins and/or porphyrin precursors. Porphyrias are usually inherited diseases but they may also be acquired or induced by exposure to environmental toxic chemicals. Since each type of porphyria is caused by a specific enzyme deficiency in the haem biosynthetic pathway, the porphyrin excretion pattern is characteristic of that porphyria. Porphyrin profiles can thus be used for the differential diagnosis of the porphyrias.

The most important techniques for the analysis of porphyrins in biological materials are spectrophotometry [1,2], fluorimetry [3,4], thin-layer chromatography (TLC) [5,6], high-performance liquid chromatography (HPLC) [7–10] and mass spectrometry (MS) [11,12]. Among these techniques, MS, particularly fast atom bombardment mass spectrometry (FAB-MS) or liquid secondary ion mass spectrometry (LSIMS), has played an

* Corresponding author. Tel: +44 116 2525561; fax: +44 116 2525616.

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important part in identification and characterization of various porphyrins [13,14]. It provides a method for the accurate determination of the molecular weight, and, with tandem mass spectrometry (MS-MS), allows for the structure of a compound to be elucidated.

This paper describes a FAB-MS method for the analysis of porphyrins in biological materials and in particular for the profiling of porphyrin excretion patterns in porphyrias.

2. Experimental

2.1. Materials and reagents

The porphyrin methyl ester chromatographic marker kit and porphyrin free acid chromatographic marker kit in vials containing 10 nmol each of type I uroporphyrin, hepta-, hexa- and penta-carboxylic porphyrins, coproporphyrin and mesoporphyrin were from Porphyrin Products (Logan, UT). Chloroform, methanol, conc. H_2SO_4 and glycerol were AnalaR grade from BDH (Poole, Dorset, UK).

2.2. Esterification of porphyrins in urine [9–14]

Urine (1 ml) was mixed with 10 ml of methanol–conc. H_2SO_4 (90:10, v/v) and left to stand overnight in the dark at room temperature. The resulting porphyrin methyl esters were extracted in chloroform. The chloroform layer was then successively washed with saturated sodium hydrogen carbonate and water. The organic layer was filtered through a filter paper pre-wetted with chloroform and the filtrate was then evaporated to dryness under N_2. The residue was redissolved in chloroform and passed through a Pasteur pipette filled with acid-washed alumina (Merck, UK) which has been pre-conditioned with chloroform. Porphyrin methyl esters were eluted from the column with chloroform. The eluate was evaporated to dryness and the residue redissolved in 100 μl of chloroform–methanol (2:1, v/v) for mass spectrometry analysis. Porphyrin methyl esters are stable when stored dry in the dark [12].

2.3. Esterification of porphyrins in faeces

For the esterification of porphyrins in faeces, methanol–conc. H_2SO_4 (90:10, v/v) was added to the sample (500 mg ml^{-1}) and stirred vigorously with a glass rod to break up the faeces. The mixture was then sonicated in a sonicating water bath for 30 min and kept in the dark at 4°C overnight to allow for complete esterification. The mixture was filtered through filter paper and the solid residual was repeatedly washed with small amount of methanol–conc. H_2SO_4 (90:10, v/v). The combined filtrate was poured into a separation funnel containing chloroform. Water was then added and the mixture was shaken vigorously with regular released of pressure. The aqueous layer was repeatedly extracted with chloroform until no more fluorescence could be detected in the chloroform layer with a UV lamp. The combined chloroform extract was then successively washed with saturated sodium hydrogen carbonate and twice with water. The porphyrin solution was filtered through a filter paper pre-wetted with chloroform. The organic solvent was removed by evaporation under N_2 or for larger volumes at 40–50°C under reduced pressure. The residue was redissolved in chloroform and passed through an alumina column as described in Section 2.2.

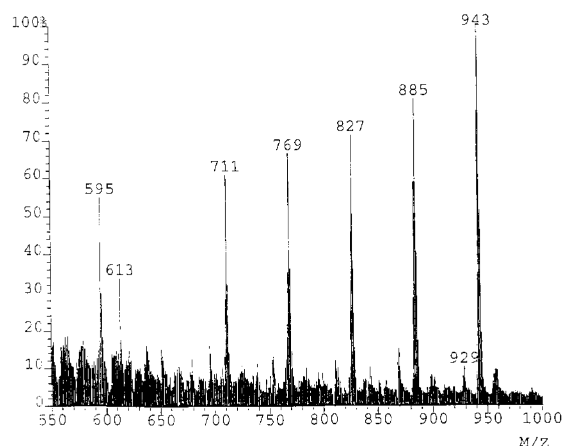

Fig. 1. FAB-MS of a standard mixture of porphyrin methyl esters.

Table 1
Positive-ion FAB-MS of porphyrin methyl esters, $[M+H]^+$ ions and peak assignments

m/z	Compound
943	Uroporphyrin octamethyl ester
885	Heptacarboxylic porphyrin
827	Hexacarboxylic porphyrin hexamethyl ester
769	Pentacarboxylic porphyrin pentamethyl ester
711	Coproporphyrin tetramethyl ester
595	Mesoporphyrin dimethyl ester

2.4. Mass spectrometry (MS)

FAB-MS were obtained on a tandem mass spectrometer (VG70-SEQ) of EBQ_1Q_2 geometry, where E is an electrostatic analyser, B is the magnet and Q_1 is a radio frequency-only quadrupole which acts as a collision cell. Porphyrin methyl esters were dissolved in about 1 ml chloroform–methanol (2:1, v/v) and 1 μl was added to the stainless-steel target probe previously prepared with glycerol (1 μl) as the liquid matrix. All samples were ionized by positive-ion FAB. Xenon atoms from a model B11N (Ion Tech, Teddington, Middlesex, UK) saddle-field fast-atom gun were used as the primary ionizing beam and impacted the sample at 8.5 keV. The secondary ions produced by the xenon atoms were accelerated to 8 keV from the source region and the magnet scanned at 5 s per decade over the mass range *m/z* 1350-50.

In the tandem-MS studies, precursor ions (MH^+) were selected with a resolution of approximately 1000 using EB (equivalent to the first mass spectrometer MS_1) and subjected to collision-activated dissociation using argon as the collision gas. Collision energies (Laboratory frame-of-reference) were varied from 5 to 460 eV, and target gas pressures in the collision cell were typically about 10 mPa (ca. 10^{-6} mbar). Product ion spectra were acquired by scanning Q_2 (equivalent to the second mass spectrometer MS_2) usually over mass range *m/z* 1000-40, and 15 scans were acquired under data system control in the multichannel analysis mode.

3. Results and discussion

In FAB-MS, molecules are desorbed from the matrix surface as either the protonated species $[M+H]^+$ or negative ions ($[M]^-/[M-H]^-$) and with very little or no fragmentation. Since each ion is characteristic for a compound and ion intensity is related to concentration, the technique is applicable to the analysis of mixtures without the need for prior chromatographic separation. This is demonstrated in Fig. 1, the FAB-MS of a standard mixture of porphyrin methyl esters. Porphyrins are usually analysed as the methyl esters because they are much easier to vaporise and ionize in the ion source than the free acids. Each porphyrin can be clearly identified and the assignment of each peak is shown in Table 1. The technique can therefore be used to provide porphyrin profiles in porphyric patients where the porphyrin excretion patterns are typical of the diseases and to detect and identify unusual porphyrin metabolites in these patients. The FAB-MS of the porphyrin methyl esters isolated from the urine of a patient with congenital erythropoietic porphyria (CEP) is a good example (Fig. 2). CEP patients typically excrete a large quantity of uroporphyrin I with a moderate increase in coproporphyrin I due to a uroporphyrinogen III synthase defect. Hepta-, hexa- and penta-carboxylic porphyrins are also elevated [7,9]. These porphyrins can be clearly identified in the spectrum

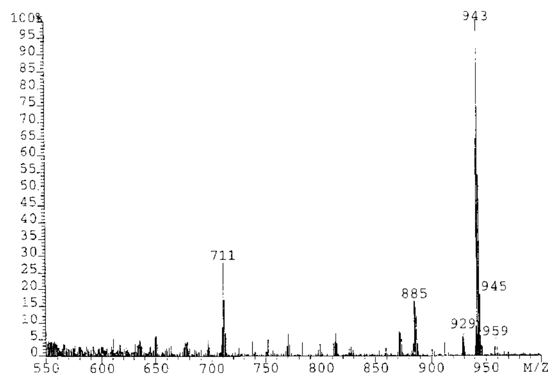

Fig. 2. FAB-MS of the porphyrin methyl esters isolated from the urine of a patient with congenital erythropoietic porphyria.

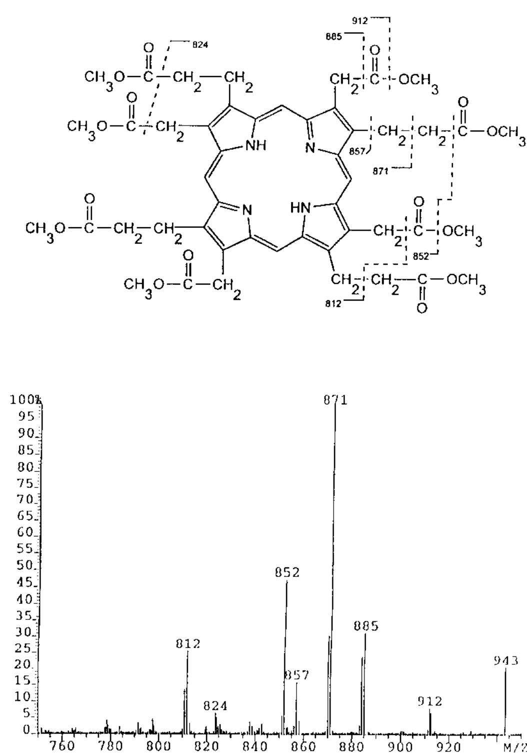

Fig. 3. Product ions of uroporphyrin octamethyl ester (*m/z* 943).

by comparison of the protonated molecular ions $[M + H]^+$ to those of the standards listed in Table 1. Hydroxy- and peroxyacid-uroporphyrin I, which have been reported as metabolites in the urine of CEP [13], were also clearly detected at *m/z* 959 and *m/z* 945, respectively.

Confirmation of porphyrin structures was obtained by tandem MS (MS-MS). The fragmentation pattern of uroporphyrin octamethyl ester is shown in Fig. 3. The major fragmentation pathways of this compound (*m/z* 943) were the successive cleavage of the side-chain methyl ester substituents:

(i) loss of OCH_3 (m/z 912) from an acetate or a propionate group.

(ii) loss of $COOCH_3$ (m/z 885) from an acetate or a propionate group.

(iii) benzylic fission of CH_2COOCH_3 (*m/z* 871) from an acetate group, and, $CH_2CH_2COOCH_3$ (*m/z* 857) from a propionate group.

(iv) loss of $2 \times COOCH_3$ (*m/z* 824)

(v) loss of $COOCH_3 + CH_2COOCH_3$ (*m/z* 812).

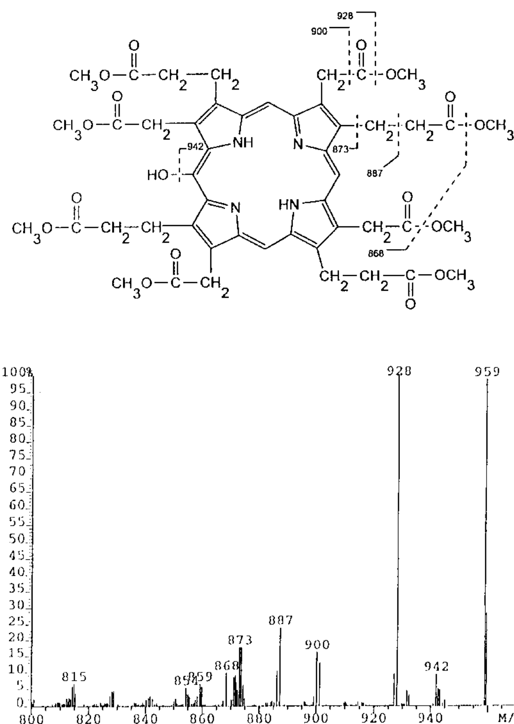

Fig. 4. Product ions of *meso*-hydroxyuroporphyrin octamethyl ester (*m/z* 959).

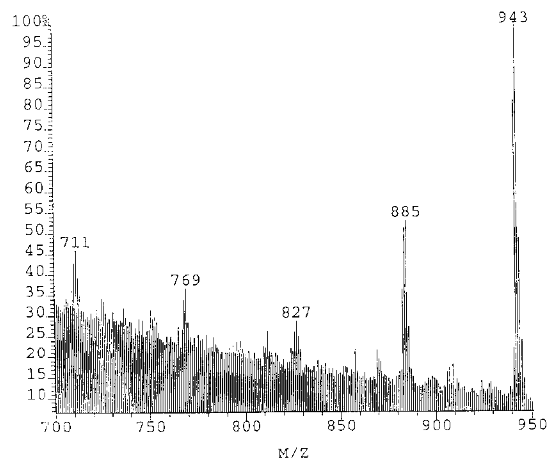

Fig. 5. FAB-MS of the porphyrin methyl ester isolated from the urine of a patient with porphyria cutanea tarda.

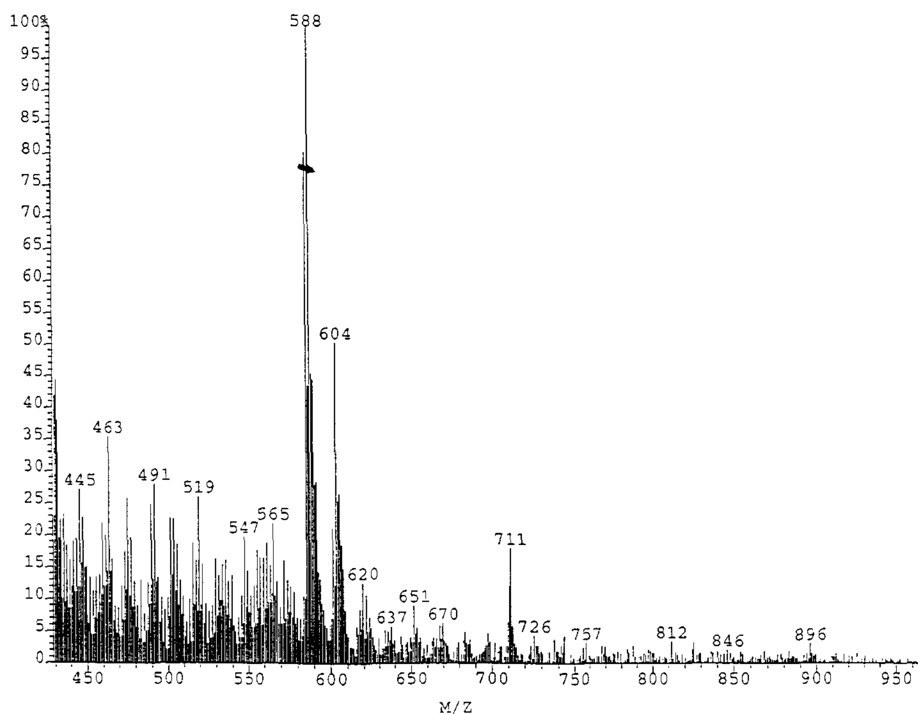

Fig. 6. FAB-MS of the porphyrin methyl esters isolated from a patient with hereditary coproporphyria.

The route of fragmentation involving successive cleavage of the side-chain substituents is common to all the porphyrin methyl esters. The fragmentation of a hydroxylated uroporphyrin metabolite (*m/z* 959) is shown in Fig. 4. The presence of the hydroxy group was clearly demonstrated by an additional product ion at *m/z* 942, corresponding formally to the loss of an OH group (17 Da). The loss of an OH group from the *meso*-position of a porphyrin is unusual. It is possible that this occurred by initial protonation of the OH group, followed by elimination of H_2O.

In patients with porphyria cutanea tarda (PCT), large amounts of uroporphyrin (*m/z* 943) and heptacarboxylic porphyrin (*m/z* 885) are excreted in the urine [7]. The FAB-MS profile of the urine sample from a patient with PCT is shown in Fig. 5. The pattern, with major peaks at *m/z* 943 and *m/z* 885, is characteristic for PCT and can be used to differentiate this disease from other porphyrias.

FAB-MS can also be used to analyse faecal porphyrins. Fig. 6 shows the faecal porphyrin profile of a patient with hereditary coproporphyria where excess coporporphyrin III (*m/z* 711) is excreted in the faeces.

4. Conclusions

FAB-MS is a useful technique for the rapid screening of metabolites in the urine and faeces of patients with porphyria. It provides characteristic porphyrin profiles without the need for lengthy chromatographic separations. Tandem FAB-MS gives additional information on the structure, thus assisting in the identification of new porphyrin metabolites. The porphyrin profiles obtained with FAB-MS are similar to those provided by TLC or HPLC.

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