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ANALYSIS OF TUMOUR-LOCALIZING HAEMATOPORPHYRIN DERIVATIVE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FAST-ATOM BOMBARDMENT MASS SPECTROMETRY

J. C. M. MEIJERS
Section of Clinical Mass Spectrometry, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (U.K.)
C. K. LIM*
Division of Clinical Cell Biology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (U.K.)
A. M. LAWSON
Section of Clinical Mass Spectrometry, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (U.K.)
and
T. J. PETERS
Division of Clinical Cell Biology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (U.K.)

SUMMARY

Reversed-phase chromatography using a MOS-Hypersil (C_8) column with methanol–1 *M* ammonium acetate buffer (pH 4.6) (60:40) as mobile phase has been developed for the isolation of tumour-localizing haematoporphyrin derivative (HPD). The system effectively resolved the diastereoisomers of haematoporphyrin and its acetyl derivatives. The chromatography peaks were identified by fast-atom bombardment mass spectrometry and were confirmed by chemical synthesis. The main components of HPD before alkaline hydrolysis were diacetylhaematoporphyrin, 8-(1-acetoxyethyl)haematoporphyrin and 3-(1-acetoxyethyl)haematoporphyrin with small amounts of haematoporphyrin, 8-(1-hydroxyethyl)-3-vinyldeuteroporphyrin, 3-(1-hydroxyethyl)-8-vinyldeuteroporphyrin, 8-(1-acetoxyethyl)-3-vinyldeuteroporphyrin, 3-(1-acetoxyethyl)-8-vinyldeuteroporphyrin and protoporphyrin. After hydrolysis with 0.1 *M* sodium hydroxide, the main components were haematoporphyrin, hydroxyethylvinyldeuteroporphyrins, and protoporphyrin.

INTRODUCTION

Haematoporphyrin derivative (HPD) has recently attracted great interest as a potent photosensitizing tumour localizer[1–3] and has been used in clinical trials for the photoradiation therapy of cancers[4–7]. HPD is prepared by treating haematoporphyrin (Fig. 1) with a mixture of acetic and sulphuric acid followed by hydrolysis in 0.1 *M* sodium hydroxide[1,8]. HPD is thus a complex mixture of porphyrins and,

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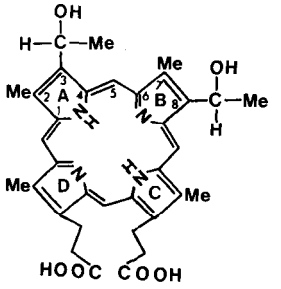

Fig. 1. Structure of haematoporphyrin. Me = methyl.

although a structure has been suggested[9] for the active component, this remains uncertain. High-performance liquid chromatography (HPLC) has been widely used for the analysis of HPD[10–12]. The present paper describes a novel reversed-phase system on a MOS-Hypersil column, with methanol–1 *M* ammonium acetate buffer (pH 4.6) (60:40, v/v) as eluent for the analysis of HPD. The system is superior to other methods, particularly in the resolution of diastereoisomers. The HPLC peaks were identified by fast-atom bombardment mass spectrometry (FAB-MS) and were confirmed by chemical synthesis.

EXPERIMENTAL

Materials and reagents

Haematoporphyrin was from Sigma, Poole, U.K. Ammonium acetate, glacial acetic acid and ethylenediaminetetraacetic acid (EDTA) were AnalaR grade from BDH, Poole, U.K. Methanol was HPLC grade from Rathburn Chemicals, Walkerburn, U.K. HPD was prepared according to a published method[8].

High-performance liquid chromatography

A Varian Assoc. (Walnut Creek, CA, U.S.A.) Model 5000 liquid chromatograph with a UV100 variable-wavelength detector, set at 400 nm, was used. Sample injection was via a Rheodyne 7125 injector, fitted with a 200-μl loop. The separation was carried out on a 25 cm × 5 mm MOS-Hypersil column (Shandon Southern, Runcorn, U.K.) with methanol–1 *M* ammonium acetate (pH 4.6) (60:40) as eluent. Gradient elution was used for fast separation and for eluting highly hydrophobic components. The flow-rate was 1 ml/min. When preparative separation was performed, the peaks were collected in flasks containing 1 mg of EDTA to prevent the formation of metalloporphyrins.

FAB-MS

FAB-MS was carried out on a Finnigan-MAT-731 double-focussing mass spectrometer. Samples for analysis were dissolved in the matrix on a stainless-steel target stage and bombarded by a xenon atom beam (10 kV, 10 μA) from an atom gun, supplied by M-Scan, Ascot, U.K. Positive-ion spectra were obtained from samples dissolved in either dilute hydrochloric acid or methanol and introduced into a thin film of glycerol–thioglycerol (1:1), coated on the target.

RESULTS AND DISCUSSION

Haematoporphyrin diastereoisomers

Haematoporphyrin has two asymmetric carbon atoms (C-3 and C-8) bonded to the side-chain hydroxyethyl groups (Fig. 1) and therefore exists in the *RR* + *SS* and *RS* + *SR* forms. The haematoporphyrin standard was resolved into two peaks (Fig. 2a) on MOS-Hypersil, eluted with methanol–1 *M* ammonium acetate (pH 4.6) (60:40). In order to investigate these peaks further, an optically active haematoporphyrin isomer was obtained by treating cytochrome *c* with silver sulphate in acetic acid[13]. This compound corresponded to peak 2 (Fig. 2b) of the haematoporphyrin standard. Peak 2 was therefore the *RR* + *SS* form. Small amounts of silver complexes were also detected, but these were well separated from haematoporphyrin diastereoisomers.

The FAB-MS of peaks 1 and 2 were identical, both having a molecular weight of 598 daltons, defined by their protonated molecular ion at *m/z* 599 (M + 1).

Haematoporphyrin derivative before hydrolysis

Fig. 3 shows the separation of HPD prepared by reacting haematoporphyrin with a mixture of acetic and sulphuric acids. Peaks 1 and 2 had the same retention times as the haematoporphyrin diastereoisomers and represented the unreacted haematoporphyrin. The reaction was expected to form mainly the acetylated derivatives with smaller amounts of dehydration products because the two secondary hydroxy groups of haematoporphyrin (Fig. 1) could be acetylated and, to a lesser extent, dehydrated under the reaction conditions used. Peaks 3–6 were indeed the monoacetylated derivatives of haematoporphyrin. They all had the same FAB-MS with the M + 1 signal at 623 (M_r = 622). Partial acetylation of haematoporphyrin[11] should

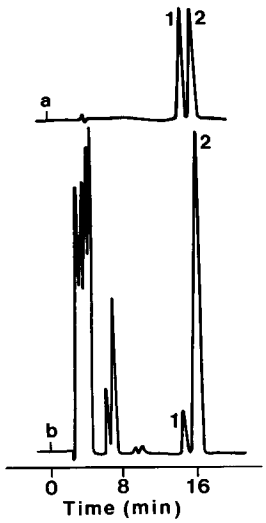

Fig. 2. Separation of haematoporphyrin diastereoisomers. (a) Standard and (b) haematoporphyrin isolated from cytochrome *c*. Column. MOS-Hypersil: Eluent, methanol–1 *M* ammonium acetate (pH 4.6) (60:40). Peaks: 1 = (*RS* + *SR*)-haematoporphyrin: 2 = (*RR* + *SS*)-haematoporphyrin.

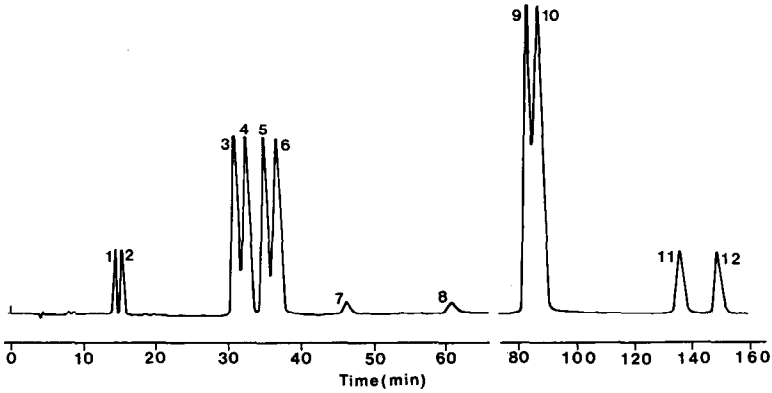

Fig. 3. Isolation of haematoporphyrin derivative prepared by allowing haematoporphyrin to react with a mixture of acetic and sulphuric acids. HPLC conditions as in Fig. 2. Peaks: 1 = (*RS* + *SR*)-haematoporphyrin; 2 = (*RR* + *SS*)-haematoporphyrin; 3 = (*RS* + *SR*)-3.-(1-acetoxyethyl)-8-hydroxyethyldeuteroporphyrin: 4 = (*RR* + *SS*)-3-(1-acetoxyethyl)-8-hydroxyethyldeuteroporphyrin; 5 = (*RS* + *SR*)-8-(1-acetoxyethyl)-3-hydroxyethyldeuteroporphyrin; 6 = (*RR* + *SS*)-8-(1-acetoxyethyl)-3-hydroxyethyldeuteroporphyrin; 7 = 8-(1-hydroxyethyl)-3-vinyldeuteroporphyrin; 8 = 3-(1-hydroxyethyl)-8-vinyldeuteroporphyrin; 9 = (*RS* + *SR*)-diacetyldeuteroporphyrin; 10 = (*RR* + *SS*)-diacetylhaematoporphyrin; 11 = 8-(1-acetoxyethyl)-3-vinyldeuteroporphyrin; 12 = 3-(1-acetoxyethyl)-8-vinyldeuteroporphyrin.

lead to the formation of 3-(1-acetoxy) and 8-(1-acetoxy) isomers (Fig. 4). Four isomers were detected, as each haematoporphyrin diastereoisomer (*RS* + *SR* and *RR* + *SS*) gave two acetyl derivatives. Partial acetylation of (*RS* + *SR*)-haematoporphyrin gave peaks 3 and 5 while the (*RR* + *SS*) isomer gave peaks 4 and 6 (Fig. 5). Thus, peaks 3 and 5 and peaks 4 and 6 were pairs of positional isomers and peaks 3 and 4 and peaks 5 and 6 were diastereoisomers.

In reversed-phase chromatography of dicarboxylic porphyrins the dominant retention mechanism is hydrophobic interaction between the porphyrin side-chain substituents and the hydrocarbonaceous stationary phase surface[14]. Retention of deuteroporphyrin derivatives is governed by the relative hydrophobicity of the groups attached at the C-3 and C-8 positions. Compounds with the more hydrophobic group at the C-8 position are retaining longer than those with the group at the C-3 position[11]. Assuming similar behaviour in the present system and bearing in mind that the acetoxyethyl group is more hydrophobic than the hydroxyethyl group, peak 3

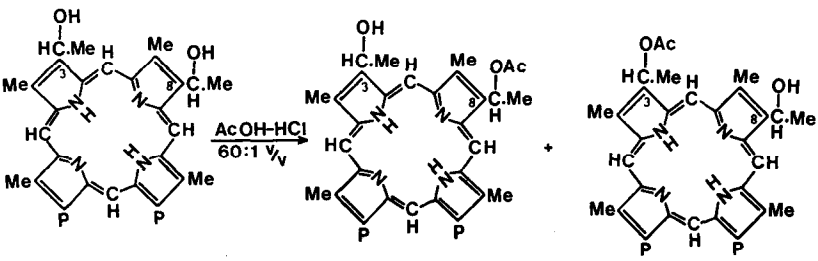

Fig. 4. Partial acetylation of haematoporphyrin. P = CH_2CH_2COOH; Me = methyl; Ac = acetyl.

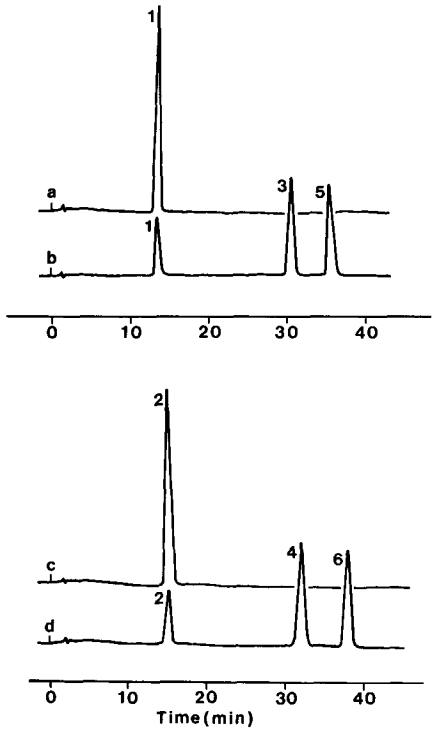

Fig. 5. Separation of products formed by partial acetylation of (*RS* + *SR*)- and (*RR* + *SS*)-haematoporphyrin diastereoisomers. (a) and (c) standards, (b) and (d) products. HPLC conditions as in Fig. 2. Peak identification as in Fig. 3.

was assigned the (*RS* + *SR*)-3-(1-acetoxyethyl)-8-hydroxyethyldeuteroporphyrin; peak 4 the (*RR* + *SR*)-3-(1-acetoxyethyl)-8-hydroxyethyldeuteroporphyrin; peaks 5 and 6 were the (*RS* + *SR*) and (*RR* + *SS*) diastereoisomers of 8-(1-acetoxyethyl)-3-hydroxyethyldeuteroporphyrin, respectively.

Partial dehydration of haematoporphyrin by heating (60°C) in dimethylformamide[11] or dimethyl sulphoxide gave two compounds (Fig. 6), corresponding to peaks 7 and 8 (Fig. 7). They were the 8-(1-hydroxyethyl)-3-vinyldeuteroporphyrin and 3-(1-hydroxyethyl)-8-vinyldeuteroporphyrin, respectively, as previously reported[11]. The FAB-MS spectra were identical with their M + 1 signals at 581 (M_r = 580).

The two major peaks 9 and 10 (Fig. 3), were expected to be the O,O'-diacetylhaematoporphyrin diastereoisomers. Peak 9 was the *RS* + *SR* and peak 10 the *RR* + *SS* form, as they could be prepared by acetylation[11] of (*RS* + *SR*)- and (*RR* + *SS*)-haematoporphyrin, respectively. The FAB-MS showed the M + 1 signal at 683 indicating a molecular weight of 682, *i.e.* that of diacetylhaematoporphyrin.

Acetylation of peaks 7 and 8 gave peaks 11 and 12, respectively. Peak 11 is therefore 8-(1-acetoxyethyl)-3-vinyldeuteroporphyrin and peak 12 the 3-(1-acetoxyethyl)-8-vinyl isomer (Fig. 3).

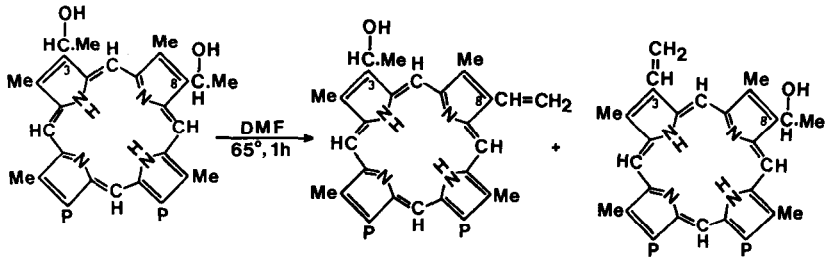

Fig. 6. Partial dehydration of haematoporphyrin. P = CH_2CH_2COOH; Me = methyl.

Haematoporphyrin derivative after hydrolysis

Before use, HPD is treated with 0.1 *M* sodium hydroxide which hydrolyses the acetylated mixture and produces a material which is more effective as a tumour localizer[15]. The HPLC separation of hydrolysed HPD is shown in Fig. 8. A 30-min linear gradient elution, from 60% (v/v) methanol in 1 *M* ammonium acetate (pH 4.6) to 100% methanol, was used in order to elute the highly hydrophobic components. The main components were haematoporphyrin diastereoisomers, hydroxyethylvinyldeuteroporphyrin isomers, and protoporphyrin. These compounds are not tumour localizers[10,15]. A small amount of strongly retained compounds was eluted at above 95% methanol. It has been shown that the tumour-localizing component of HPD is highly hydrophobic[16] and associated with the late-eluted peaks. Dougherty *et al.*[9] have provided NMR and, particularly, FAB-MS evidence that these localizing components are ethers, formed from two haematoporphyrin molecules. FAB-MS analysis of the hydrolysed HPD (Fig. 9) showed signals at *m/z* 1143, 1161 and 1179. We interpret these as cluster ions, formed from the various porphyrins present and

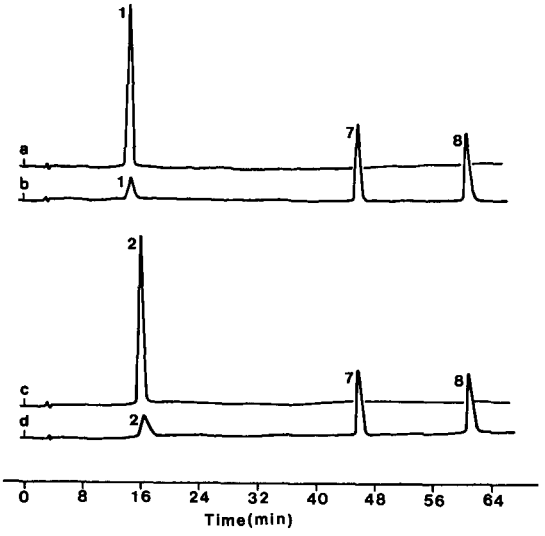

Fig. 7. Separation of partial dehydration derivatives of haematoporphyrin. (a) and (c) standards, (b) and (d) products. HPLC conditions as in Fig. 2. Peak identification as in Fig. 3.

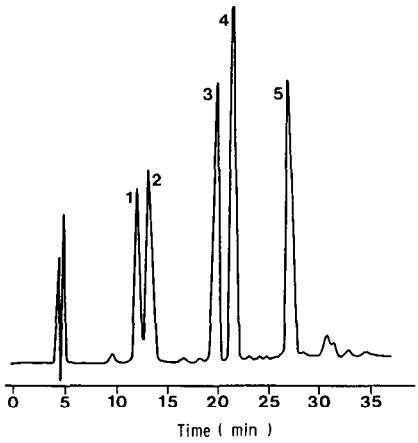

Fig. 8. Separation of haematoporphyrin derivative after hydrolysis with 0.1 *M* NaOH. Column, MOS-Hypersil; eluent and elution, linear gradient from 60% methanol in 1 *M* ammonium acetate (pH 4.6) to 100% methanol in 30 min. Peaks: 1 = (*RS* + *SR*)-haematoporphyrin; 2 = (*RR* + *SS*)-haematoporphyrin; 3 = 8-(1-hydroxyethyl)-3-vinyldeuteroporphyrin; 4 = 3-(1-hydroxyethyl)-8-vinyldeuteroporphyrin; 5 = protoporphyrin.

not due to covalently bonded di-HPD ethers. This and other studies clearly demonstrated that porphyrin cluster formation is a common phenomenon in FAB-MS.

Protoporphyrin, for example, gave an ion signal at *m/z* 1125, indicating the association of two protoporphyrin molecules (Fig. 10). As this molecule lacks a hy-

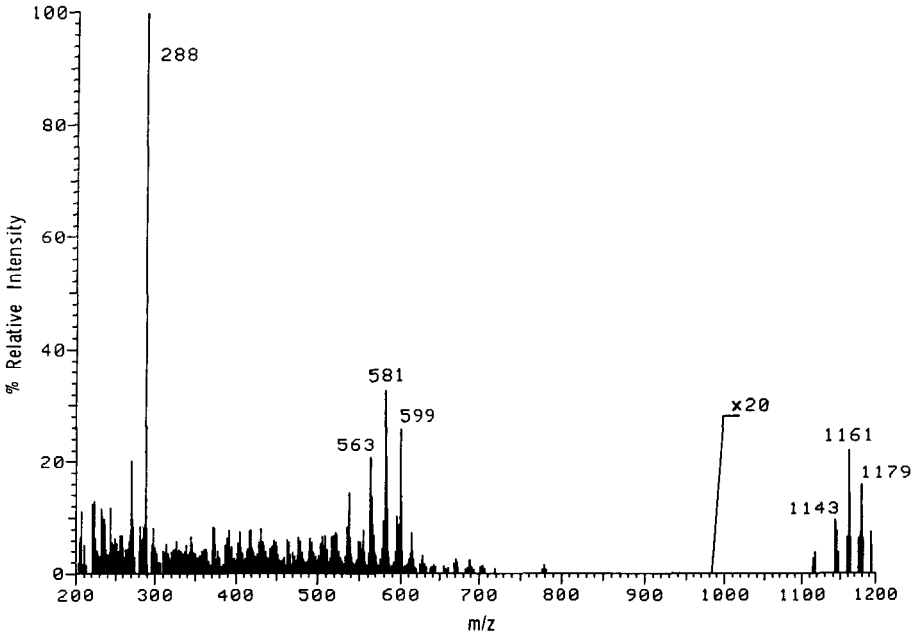

Fig. 9. Positive-ion fast-atom bombardment mass spectrum of haematoporphyrin derivative after alkaline hydrolysis.

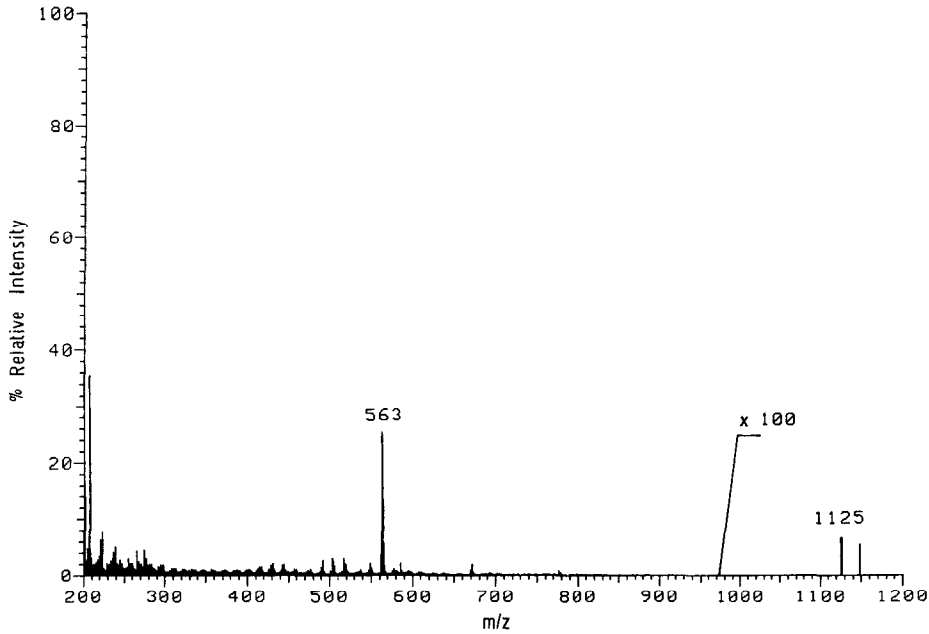

Fig. 10. Positive-ion fast-atom bombardment mass spectrum of protoporphyrin.

droxyethyl group, it would be unable to form an ether linkage. Ether formation of appropropriate porphyrins can take place under alkaline hydrolysis conditions and might be expected to lead to the production of polymers. However, these are minor components. Cluster formation has been shown for all the porphyrins studied, and it has been suggested that porphyrin cluster plays a role in the tumour-localization process[2,10,17]. There is a possibility that tumour-localizing HPD is simply due to specific porphyrin clusters.

CONCLUSIONS

The following conclusions can be drawn from the present study:

(1) Reversed-phase chromatography on MOS-Hypersil with methanol–1 *M* ammonium acetate (pH 4.6) (60:40) is effective for the isolation of HPD.

(2) Tumour-localizing HPD before alkaline hydrolysis is a mixture of acetylated and dehydrated derivatives of haematoporphyrin.

(3) Hydrolysis of HPD with 0.1 *M* sodium hydroxide gave haematoporphyrin, hydroxyethylvinyldeuteroporphyrins, and protoporphyrin as the main components and small amounts of highly hydrophobic compounds.

(4) FAB-MS is an important technique for the analysis of HPD. Electron-impact MS is unsuitable because dehydration of the hydroxyethyl groups led to similar spectra for many of the compounds.

(5) Cluster formation is a common phenomenon of porphyrins in FAB-MS.

(6) The possibility that tumour-localizing HPD is due to specific porphyrin clusters cannot be ruled out.

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