Isolation and characterization of protoporphyrin glycoconjugates from rat Harderian gland by HPLC, capillary electrophoresis and HPLC/electrospray ionization MS

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It has been widely reported that the Harderian gland, present in most vertebrates, accumulates high levels of porphyrins, particularly protoporphyrin. The present study describes the extraction, identification and characterization of a group of hitherto unreported protoporphyrin glycoconjugates in the rat Harderian gland using HPLC, capillary electrophoresis, on-line HPLC/electrospray ionization MS and tandem MS. The major glycoconjugate was identified as protoporphyrin-1-*O*-acyl *β*-xyloside with a smaller amount of protoporphyrin-1-*O*-acyl *β*-glucoside also detected. In the Harderian glands studied, 50–70 % of the porphyrins present were in the form of protoporphyrin glycoconjugates. This is the first reported occurrence of glycoconjugates of porphyrins in Nature and suggests that previous studies have wrongly identified the major porphyrin in the Harderian gland as the unconjugated protoporphyrin.

Key words: deconjugation, 3-methyl-1-phenyl-2-pyrazolin-5-one derivatives, protoporphyrin-1-*O*-acyl *β*-glucoside, protoporphyrin-1-*O*-acyl *β*-xyloside, transmethylation.

INTRODUCTION

The Harderian gland is a small bi-lobed alveolar gland situated around the posterior half of the eyeball occupying a considerable part of the orbit. It is found in most vertebrates and especially in the rodents [1,2]. It is usually associated with the third eyelid in the inner canthus of the eye. The function of the Harderian gland is not clear [3] and various propositions have been made, including corneal lubrication [4], photoreception [5], production of hormones [6,7], as a source of thermoregulatory lipids [8,9] and as a site of immune response [10,11].

The Harderian gland in rodents synthesizes and stores large amounts of porphyrins. The type and amount of porphyrins present are generally thought to be species-, strain- and gender-specific [12,13]. The major porphyrin component in rodents was reported to be protoporphyrin, with much smaller amounts of harderoporphyrin and other porphyrins [14,15]. In the rat, the porphyrins present in the adult are in the range of μg/mg of wet weight [16].

HPLC has been used widely for the separation of porphyrins [17]. MS, especially positive-ion liquid secondary ionization MS (LSIMS), on the other hand, has been an important method for the characterization of porphyrins [18,19]. The methyl esters of porphyrins are usually used in MS analysis because they ionize better in the ion source and hence provide higher sensitivity of detection [20]. Sufficient sensitivity, however, could be obtained for porphyrins with fewer numbers of carboxyl groups, such as coproporphyrin (four carboxyl groups) and protoporphyrin (two carboxyl groups) without esterification. The development of electrospray ionization MS (ESI-MS), which could be easily coupled to an HPLC system, thus allows these porphyrins to be analysed on-line without the need for prior derivatization into methyl esters.

This article describes the isolation and characterization of a group of hitherto-unreported glycoconjugates of protoporphyrin from the rat Harderian gland, using HPLC, capillary electrophoresis (CE) and HPLC/ESI-MS.

The isolation and characterization of these conjugates, which can account for from 50 to 70 % of the total porphyrins present in the Harderian gland, suggest that the nature of porphyrins in the gland has previously been incorrectly characterized.

EXPERIMENTAL

Materials

Acetic anhydride (> 99 % pure), anhydrous pyridine, 3-methyl-1-phenyl-2-pyrazolin-5-one (MPP), protoporphyrin IX, and all monosaccharides were purchased from Sigma (Poole, Dorset, U.K.). Acetonitrile (HPLC grade), methanol (HPLC grade), DMSO (analytical-reagent grade), sodium hydroxide and potassium hydroxide (analytical-reagent grade) were obtained from Fisher Scientific, Loughborough, U.K. Anhydrous trifluoroacetic acid (TFA, protein-sequencing grade) was obtained from Pierce and Warriner (Chester, U.K.). A 0.05 % (v/v) solution was made by dissolving 1 ml (one ampule) in 2 litres of distilled water.

The Harderian glands were obtained by dissection from freshly killed Wistar-Han rats (180–500 g) and were stored in a freezer at −20 °C until analysis.

Extraction of porphyrins from Harderian gland

The porphyrins and their conjugates were extracted by homogenizing the Harderian gland (250–300 mg) with 6 × 1 ml of acetonitrile/DMSO (3:1, v/v) in a pestle in a glass homogenizer (Jencons, Leighton Buzzard, Northants, U.K.). The combined extract was then centrifuged (15000 ***g*** for 5 min) and the supernatant used for HPLC separation and isolation of the porphyrins.

Abbreviations used: CE, capillary electrophoresis; ESI-MS, electrospray ionization MS; MPP, 3-methyl-1-phenyl-2-pyrazolin-5-one; TFA, trifluoroacetic acid.

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HPLC and on-line HPLC/EIS-MS

All HPLC separations were carried out on a Varian model 9012 liquid chromatograph (Walton-on-Thames, Surrey, U.K.). A Varian model 9050 UV/Vis detector set at 405 nm was used for detection. A Hypersil BDS column (C_{18}, 250 mm × 4.6 mm, inner diameter; 5 μm partical size; Hypersil, Runcorn, Cheshire, U.K.) with 0.05 % TFA (solvent A) and acetonitrile (solvent B) as the mobile phase at a flow rate of 1 ml/min was used for the separation. The following elution programme was used: from 0 to 30 min, isocratic elution at 35 % solvent A/65 % solvent B; 30–40 min, 35 % A/65 % B to 10 % A/90 % B; 40–50 min, isocratic elution at 10 % A/90 % B. In semi-preparative isolation of the porphyrins the peaks were collected and the purified fractions were evaporated under reduced pressure to dryness.

On-line HPLC/ESI-MS and tandem ESI-MS were carried out on a VG Quattro quadrupole instrument (Micromass, Altrincham, Cheshire, U.K.) fitted with an atmospheric pressure ionization electrospray source. The eluent from the column was split in the ratio 1:9 prior to entering the ESI-MS source.

The source temperature was kept at 120 °C with a cone voltage of 90 V. The capillary and high-voltage electrode potentials were kept at 3.41 and 0.24 kV, respectively. Nitrogen was used as the drying and nebulizing gas at a flow rate of 300 and 40 litres/h, respectively. Full-scan continuum data were acquired over a mass range of 100–1000 Da and processed using the VG Masslynx data system.

Argon was used as the collision gas for tandem ESI-MS. The collision-cell pressure and collision energy were 1.3×10^{-3} mbar and 70 eV, respectively. Product ions were scanned over a mass range of 200–1000 Da and the spectra were collected in the form of continuum data.

Acetylation of purified glycoconjugates

Acetylation of the conjugates was carried out by the addition of 1 ml of anhydrous pyridine and 200 μl of acetic anhydride (> 99 % pure) to the dried porphyrin conjugate fraction. The mixture was vortex-mixed (10 s) and left overnight at room temperature in the dark. This was then evaporated under reduced pressure to dryness and either reconstituted in 150 μl of acetonitrile and analysed immediately by on-line HPLC/ESI-MS or stored at −20 °C until analysis.

Transesterification of purified glycoconjugates

Porphyrin conjugates were transesterified by dissolving the purified and dried HPLC fraction in 1 ml of methanolic potassium hydroxide (2 %, w/v). The mixture was vortex-mixed for 1 min and then neutralized with 300 μl of 2.7 M HCl. The resultant mixture was centrifuged (1200 ***g*** for 5 min) and the supernatant analysed by on-line HPLC/ESI-MS.

Spectrophotometric analysis of porphyrins

A Perkin-Elmer Lambda 2S UV/Vis spectrophotometer (Perkin-Elmer, Bucks., U.K.) with an IBM-compatible 486 PC was used to record the UV-visible spectra of the porphyrins. The spectra of the porphyrins were recorded from λ300 to λ800 nm with acetonitrile/DMSO (3:1, v/v) as a blank.

Preparation of monosaccharide-MPP derivatives

The glycoconjugates were vortex-mixed with methanol (50 μl) and 0.3 M potassium hydroxide (50 μl) for 1 min in order to release the sugar from protoporphyrin. The liberated monosaccharides were then derivatized as described previously by Honda et al. [21,22]. Briefly, the monosaccharides were incubated in 0.5 M methanolic MPP (50 μl) and 0.3 M potassium hydroxide (50 μl) at 70 °C for 30 min. The reaction mixture was allowed to cool to room temperature and neutralized with 0.3 M HCl (50 μl). The resultant mixture was evaporated to dryness under reduced pressure. The residue was then dissolved in chloroform (200 μl) and water (200 μl) and vortex-mixed. The aqueous layer was removed and evaporated to dryness under reduced pressure. The monosaccharide-MPP derivatives were reconstituted in water (200 μl) and analysed by CE and on-line HPLC/ESI-MS.

CE

A 270A Capillary Electrophoresis System (Applied Biosystems, Cheshire, U.K.) with a fused silica capillary (50 μm inner diameter, 72 cm total length, 53 cm effective length; Composite Metal Services, Hallow, Worcs., U.K.) was used. Detection of the derivatives was achieved with an on-column variable-wavelength absorbance detector set at 245 nm. The separation was carried out using di-sodium tetraborate buffer (100 mM, pH 9.5) as the running buffer with the voltage and temperature of the capillary kept at 20 kV and 30 °C, respectively. The samples were loaded on to the capillary by vacuum injection for 3 s. The capillary was washed in between each run with sodium hydroxide (0.1 M) for 3 min and then conditioned with the running buffer for 1 min.

On-line HPLC/ESI-MS of monosaccharide-MPP derivatives

The monosaccharide-MPP derivatives were separated on a Hypersil BDS C_{18} column by gradient elution from 20 to 50 % acetonitrile in 0.1 M ammonium acetate (pH 5.15) at a flow rate of 1 ml/min. All MS conditions were as described above except that the cone voltage was set at 25 V.

RESULTS

Porphyrins from Harderian gland have been extracted following methyl esterification [12,15,23] or as the free acids with HCl [24–26]. These procedures led to deconjugation and degradation of the glycoconjugates. The acetonitrile/DMSO (3:1, v/v) mixture used in the present study effectively and quantitatively extracted porphyrins from the Harderian gland without degradation or deconjugation.

The HPLC mobile phase for the separation of porphyrins in the Harderian gland, like the extractant for their extraction, should not contain methanol, which is the most commonly used organic modifier in reversed-phase HPLC. Partial transmethylation of protoporphyrin glycoconjugates could occur during the chromatographic run and in the subsequent isolation of the compounds by peak collection in the presence of methanol.

The gradient mixture of acetonitrile/0.05 % TFA was developed to provide efficient separation as well as analyte stability. No deconjugation of protoporphyrin glycoconjugates was evident in this mobile-phase mixture. A typical separation is shown in Figure 1.

The analysis of the porphyrins in Harderian gland extract by HPLC/ESI-MS has shown the presence of three peaks with an *m/z* of 563. The peak eluting at 33 min (Figure 2a, peak 3) was confirmed to be the MH^+ ion of free acid protoporphyrin by comparison with authentic standard. Peaks 1 and 2 (Figure 2a) were product ions of the two new major porphyrin metabolites detected at the retention times of 12.5 min (Figure 2c, peak 8) and 17.5 min (Figure 2b, peak 6), respectively. These two more polar metabolites produced MH^+ ions at *m/z* 725 and *m/z* 695,

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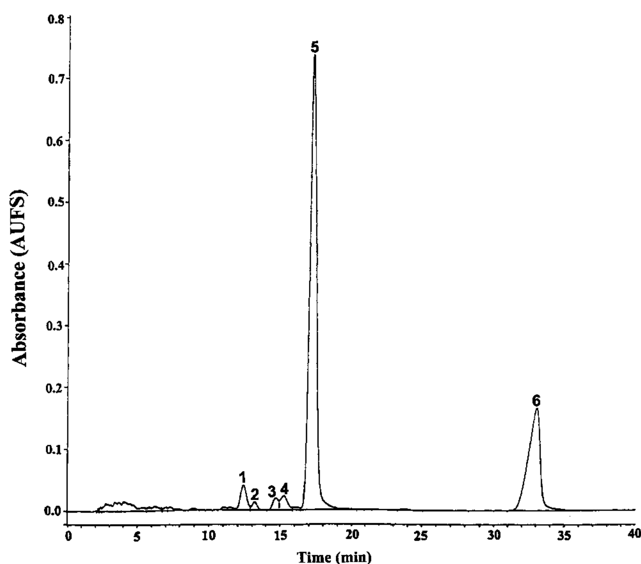

Figure 1 HPLC separation of porphyrins in Harderian gland extract

Column: Hypersil BDS (250 mm × 4.6 mm inner diameter; 5 μm partical size). Eluent: isocratic elution with acetonitrile/0.05% TFA (65:35, v/v) for 30 min followed by linear gradient elution from 65 to 90% acetonitrile in 10 min. Detector: UV/Vis 405 nm. Peaks: 1–5, protoporphyrin glycoconjugates; 6, protoporphyrin. AUFS, absorbance unit full scale.

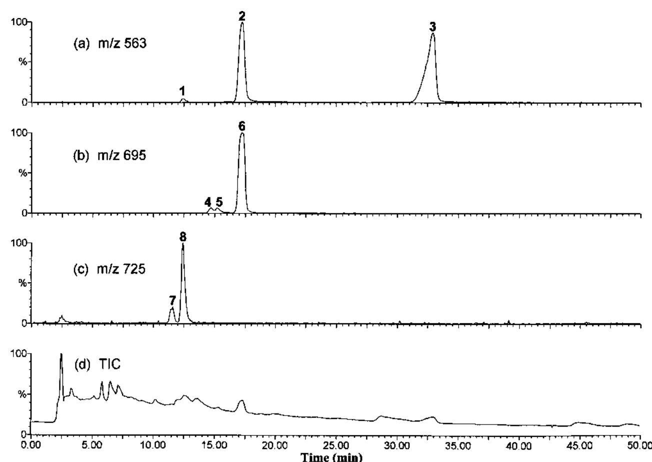

Figure 2 On-line HPLC/ESI-MS chromatograms of porphyrins in Harderian gland extract

(**a**) Mass chromatogram of ion of *m/z* 563; (**b**) mass chromatogram of conjugates of *m/z* 695; (**c**) mass chromatogram of conjugates of *m/z* 725; and (**d**) total ion current (TIC) chromatogram.

respectively, and by in-source collision-induced dissociation gave the corresponding product ions at *m/z* 563 (Figure 2a, peaks 1 and 2).

The mass difference of 132 Da between the MH^+ ion at *m/z* 695 and the product ion at *m/z* 563 indicated the possibility of a pentose conjugated with protoporphyrin. Tandem ESI-MS was carried out and this indeed confirmed that the ion at *m/z* 563 with the same retention time as the possible conjugate (17.5 min) was a product ion of the MH^+ 695 ion.

The mass difference of 162 Da between the MH^+ ion at *m/z* 725 and the product ion at *m/z* 563 indicated the presence of a hexose conjugate. The ion at *m/z* 563 was also confirmed by tandem ESI-MS to be a product ion of the MH^+ 725 ion. Peaks 4 and 5 (Figure 2b) also gave a MH^+ ion at *m/z* 695. These minor components are isomers of peak 6 (Figure 2b) and will be discussed further below.

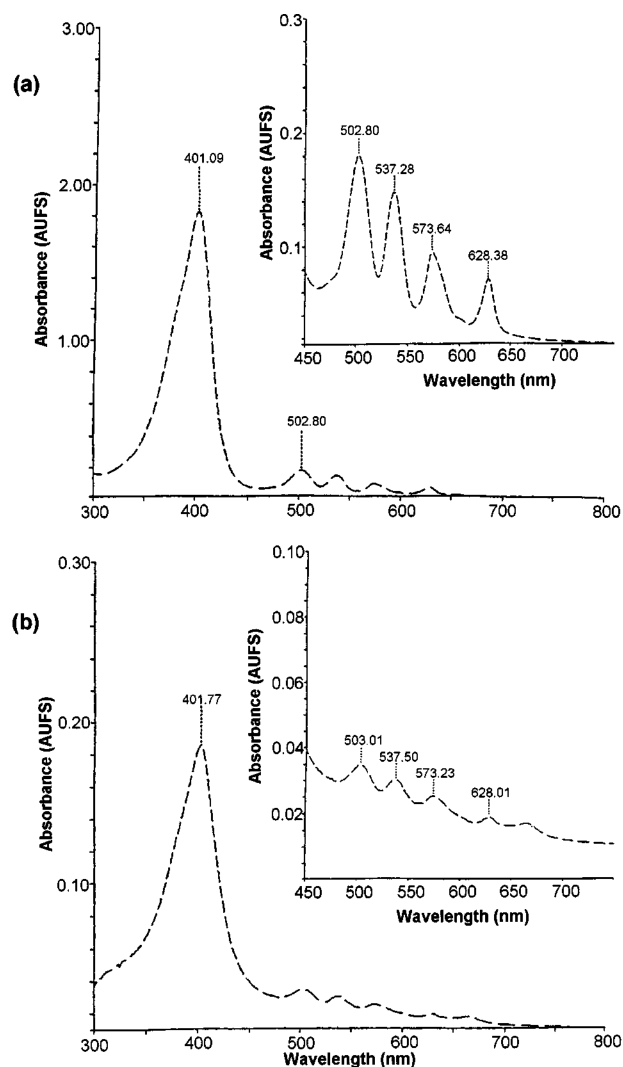

Figure 3 UV-visible absorption spectra

(**a**) Protoporphyrin IX standard and (**b**) protoporphyrin-pentose conjugate in acetonitrile/DMSO (9:1, v/v). AUFS, absorbance unit full scale.

An indication of the nature of the sugar conjugated with protoporphyrin was obtained by acetylation of the hydroxyl groups with anhydrous pyridine/acetic anhydride. The conjugate with the MH^+ ion at *m/z* 695 (Figure 2b, peak 6) gave a MH^+ ion at *m/z* 821, an increase in mass of 126 Da. This increase corresponds to the addition of three acetyl groups and strongly suggests conjugation of protoporphyrin with a pentose.

The conjugate with the MH^+ ion at *m/z* 725 (Figure 2c, peak 8) gave a MH^+ ion at *m/z* 893, an increase in mass of 168 Da. This increase corresponds to the addition of four acetyl groups, suggesting conjugation of protoporphyrin with a hexose. There are two possible sites where protoporphyrin can form conjugates with monosaccharides. The first is by N-substitution and the second by forming β-glycosidic 1-*O*-acyl conjugates with the propionic acid groups. It has been shown previously [27] that N-

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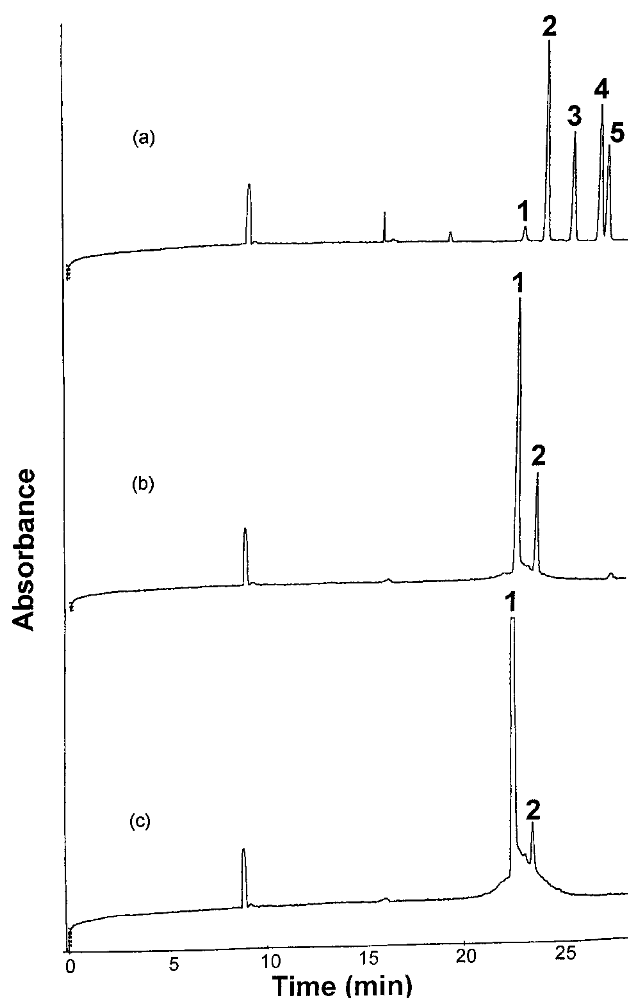

Figure 4 Capillary electropherograms

(**a**) MPP derivatives of pentose monosaccharide standards; (**b**) MPP derivative of monosaccharide obtained from the conjugate with MH^+ at m/z 695 eluting at 17.5 min and (**c**) MPP derivative of monosaccharide obtained from the conjugate with MH^+ at m/z 695 eluting at 15 min. Peaks: 1, MPP; 2, xylose; 3, arabinose; 4, ribose; 5, lyxose.

substitution of porphyrins shifts the Soret band significantly towards higher wavelengths. The UV-visible spectra of the conjugates showed the absorption maxima of the Soret peak to be almost identical to that of the unmodified protoporphyrin standard (Figure 3). N-Substitution was therefore ruled out.

Treatment of the conjugates with methanolic KOH resulted in transmethylation in all cases. The resulting protoporphyrin monomethyl ester was confirmed by HPLC/ESI-MS analysis. It gave a MH^+ ion at m/z 577, corresponding to the replacement of the monosaccharide group with a methyl group. The monosaccharides conjugated with protoporphyrin were identified by releasing the monosaccharide with methanolic KOH followed by conversion and separation of the sugars as their MPP derivatives by CE.

Figure 4 shows the electropherograms of the monosaccharide-MPP derivatives of (Figure 4a) a mixture of pentose monosaccharide standards, (Figure 4b) the monosaccharide derived from the conjugate with the MH^+ at m/z 695 at 17.5 min (Figure 2b, peak 6) and (Figure 4c) the monosaccharide derived from the conjugate with the MH^+ at m/z 695 at 15 min (Figure 2b, peaks 4 and 5). The results clearly show that the monosaccharide is xylose. The monosaccharide derived from the conjugate with MH^+ at m/z 725 (Figure 2c, peak 8) was similarly identified by CE to be glucose by comparison with a mixture of hexose monosaccharide standards consisting of glucose, mannose and galactose.

The structure of the monosaccharide-MPP derivative has already been determined and shown to be monosaccharide bis-MPP [21]. According to this structure, the molecular masses of the xylose and glucose derivatives would be 480 and 510 Da, respectively. The derivatives gave the MH^+ ions at m/z 481 and m/z 511 when analysed by HPLC/ESI-MS, thus confirming that they are derived from xylose and glucose, respectively.

From the above results, the two major glycoconjugates of protoporphyrin were identified as protoporphyrin monoxyloside and protoporphyrin monoglucoside with the sugar residue attached via a 1-*O*-acyl β-glycosidic bond (Figure 5), similar to that found in the bilirubin glycoconjugates [28,29] and other naturally occurring glycoconjugates.

Protoporphyrin has two propionic acid groups. There are, therefore, two possible positions where the monoglycoside could be formed. Since only one major monoconjugate of each sugar was detected, it is likely that the two isomeric forms were not separated under the conditions used. Attempts to resolve the isomers by varying the chromatographic conditions were unsuccessful.

The two small peaks (Figure 2b, peaks 4 and 5) with the m/z of 695 are the isomerization products of the 1-*O*-acyl β-xyloside due to the lability of the 1-*O*-acyl bond, which is prone to migrate from the C-1 position at the xylosyl residue to other positions. This behaviour has been observed before for bilirubin glycoconjugates [28,29] and was confirmed by incubation of the purified protoporphyrin monoxyloside in 0.9 M HCl for 3 h at room temperature when the same two peaks were formed. Similarly, the peak with the MH^+ ion at m/z 725 (Figure 2c, peak 7) was derived from isomerization of the sugar moiety.

Harderoporphyrin was shown to be a significant component of the porphyrins in the Harderian gland of rats and levels as high as 29 % of total porphyrins have been reported [30]. In the present study harderoporphyrin was not detected even when selected ion recording (SIR) MS was used to improve the sensitivity of detection.

DISCUSSION

The combination of HPLC, HPLC/ESI-MS, and CE has proved extremely effective in fully characterizing a new group of protoporphyrin conjugates found in the rat Harderian gland. These conjugates constitute 50–70 % of the total porphyrins present in the Harderian gland. They have not been detected before, possibly due to the extraction methods being employed and the lack of suitable analytical techniques for their isolation and characterization. The function of these conjugates is not known. It may be that conjugation is to improve the aqueous solubility of protoporphyrin for excretion and hence is a means of regulating the amount of protoporphyrin within the gland, or it may have other yet unknown functions. It is interesting that only monoconjugates and no diconjugated protoporphyrin have been detected. This is the first identification of a naturally occurring glycoconjugate of a porphyrin. Whether these conjugates are also present in other biological sources, particularly in human porphyrias, is certainly worth investigating using the techniques described here. The mechanism of formation of these glycoconjugates remains to be elucidated.

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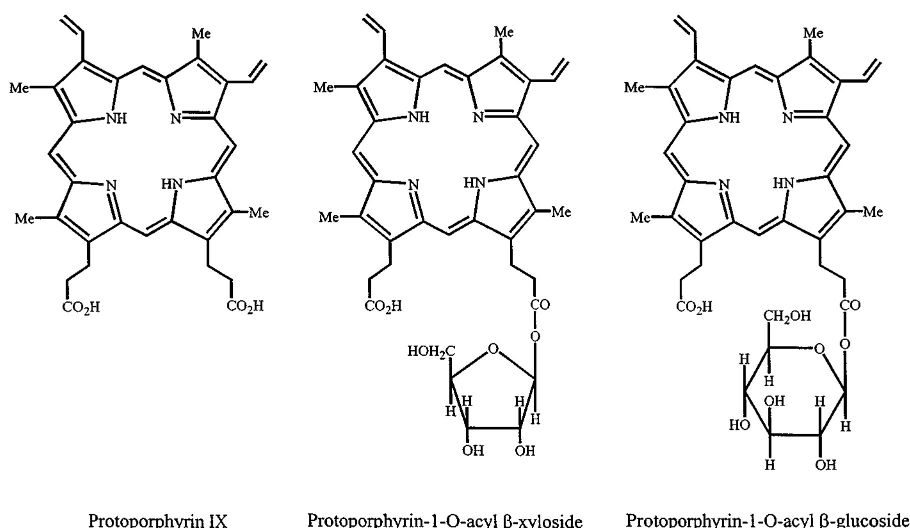

Figure 5 Structures of protoporphyrin glycoconjugates identified in the present study

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