Studies of Porphyrin Glycoconjugates and Amino Acid-, Peptide- and Protein- Adducts by Liquid Chromatography – Mass Spectrometry

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In the name of Allah, Most Gracious, Most Merciful

"Proclaim! (or Read)

In the name of your Lord and Cherisher,
Who created man, out of a mere clot of congealed blood:
Proclaim! And your Lord is Most Bountiful
He who taught (the use of) the Pen
Taught man that which he knew not
But man does transgress all bounds
In that he looks upon himself as self-sufficient
Verily, to you Lord is the return (of all)...."

Translation of the meaning of the Qur'an, Hz Al-'A'lab:1-6
ABSTRACT

The extraction, isolation and characterisation of a group of hitherto unreported protoporphyrin glycoconjugates in the rat Harderian gland using high performance liquid chromatography (HPLC), capillary electrophoresis (CE), on-line HPLC/Electrospray ionisation MS (HPLC/ESI-MS) and tandem MS is described. 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. The function of these glycoconjugates is not clear.

The existence of protoporphyrin glycoconjugates in the urine, plasma and red blood cells (RBC) of a patient with erythropoietic protoporphyria (EPP) was also investigated. Protoporphyrin-1-O-acyl β-xyloside was detected in EPP urine but not in plasma and RBC.

The mechanism of formation of the protoporphyrin glycoconjugates was investigated. Incubations of protoporphyrin and its reduced form, protoporphyrinogen, were carried out with xylose, glucose and their uridine 5’-diphosphate derivatives in the presence and absence of uridine 5’-diphosphate glucuronyl transferase (UDPGT). Negative results were obtained. Incubation of uridine 5’-diphosphoglucuronic acid (UDPGA) with protoporphyrin and UDPGT in Tris-HCl buffer, also yielded no products. Incubation of UDPGA with the Harderian gland homogenate in Tris-HCl buffer (pH7.4), however, resulted in an increase of 61.8% and 78.5% of protoporphyrin-1-O-acyl β-xyloside and protoporphyrin-1-O-acyl β-glucoside conjugates, respectively. This finding, along with an absence of glycoconjugates in incubations with liver homogenate would suggest the involvement of other enzymes which may be present in the Harderian gland.

The reactivities of protoporphyrin and protoporphyrinogen towards amino acids, peptides and proteins were investigated. Protoporphyrin failed to react while protoporphyrinogen formed adducts with cysteine, glutathione, peptides and protein containing a free thiol group when incubated overnight at 37°C in the dark. The adducts formed were separated by HPLC and characterized by HPLC/ESI-MS and matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOFMS). They were formed by the addition of the thio amino acids, peptides and proteins to the vinyl groups of protoporphyrin and consisted of diastereoisomers.
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I would like to thank my parents. my mother for her love and affection that I know is always there and my dad on whose advice I took up the challenge that is PhD.

Finally, and most importantly, no matter how hard I try I cannot even begin to thank the one who taught me all I know….Allah (SWT), The Almighty.
Previously unknown protoporphyrin glycoconjugates identified in the present study.
ABBREVIATIONS

ADP  ALA Dehydratase Deficiency Porphyria
AIP  Acute Intermittent Porphyria
ALA  delta-5-Aminolevulinic Acid Dehydratase
ALASL  delta-5-Aminolevulinic Acid Synthase
APCI  Atmospheric Pressure Chemical Ionisation
API  Atmospheric Pressure Ionisation
CE  Capillary Electrophoresis
CGE  Capillary Gel Electrophoresis
CI  Chemical Ionisation
Copro  Coproporphyrin
Coprogen  Coproporphyrinogen
COPRO-O  Coproporphyrinogen Oxidase
CP  Congenital Porphyria
CZE  Capillary Zone Electrophoresis
Da  Dalton
DMSO  Dimethylsulphoxide
EDTA  Ethylenediaminetetraacetic acid
EIE  Electron Impact
EOF  Electro-osmotic Flow
EPP  Erythropoietic Protoporphyria
ESI  Electrospray Ionisation
ESI-MS  Electrospray Ionisation Mass Spectrometry
FECH  Ferrochelatase
GC-MS  Gas Chromatography Mass Spectrometry
HC  Hereditary Coproporphyria
HEP  Hepatoerythropoietic Porphyria
HMB  Hydroxymethylbilane
HMB-S  Hydroxymethylbilane Synthase
HPLC  High Performance Liquid Chromatography
HPLC-ESI/MS  On-line HPLC-Electrospray Ionisation Mass Spectrometry
IEF  Isoelectric Focusing
MECC  Micellar Electrokinetic Capillary Chromatography
MGBG  Methylglyoxal-bis-(Guanyl) Hydrazone
MPP  3-Methyl-1-Phenyl-2-pyrazolin-5-one
MS  Mass Spectrometry
MS/MS  Tandem Mass Spectrometry
MW  Molecular Weight
NMR  Nuclear Magnetic Resonance
PBG  Porphobilinogen
PCT  Porphyria Cutanea Tarda
PLC  Primary Liver Cancer
Proto  Protoporphyrin IX
PROTO-O  Protoporphyrinogen Oxidase
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<td>Red Blood Cells</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>S/N</td>
<td>Signal to Noise Ratio</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
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<tr>
<td>TIC</td>
<td>Total Ion Current</td>
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<tr>
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Chapter 1

Introduction

1.1 Porphyrrins

Haem is made by the chelation of iron with protoporphyrin IX. Protoporphyrin is a square planar molecule made up of four pyrrolic rings joined together by $\alpha$ carbon atoms to form methine bridges. Protoporphyrin is a highly conjugated compound and an important feature of this complex ring structure is its metal binding capability. The most common of the metals bound are iron and magnesium. The metals are inserted into the centre of this structure and bound to the nitrogen atoms of all four of the pyrrolic rings in such a way that it maintains the planar nature of the overall structure. When ferrous ion is bound to the central nitrogens the resultant complex is called haem (Figure 1.1). It may come as no surprise that just as haem plays a crucial role in the mammalian cell, so chlorophyll is central in the solar energy utilisation in plants, algae and photosynthetic bacteria. In fact the very colour of the plants is due to chlorophyll and the Nobel laureate, Hans Fischer, aptly described chlorophyll and haem as the compounds that made grass green and blood red.

A further characteristic of these, highly conjugated porphyrins, is that they absorb light intensely in the UV region of the spectrum. The main absorbance (the soret band) occurs at approximately 400nm with a further four absorbance bands being observed, although to a much lower intensity, between 500 and 600nm. This
Fig. 1.1 Tetrapyrrolic ring structure of porphyrin(ogen)s, heme and chlorophyll
A, acetic acid; M, methyl; P, propionic acid; V, vinyl; E, ethyl;
R₁, methyl (chlorophyll a), formyl (chlorophyll b);
R₂, CH₃CHC(CH₃)CH₂CH(CH₃)CH₂CH₂CH₂CH₂CH(CH₃)₂

Protoporphyrinogen
(Reduced protoporphyrin)

Protoporphyrin

Heme

Chlorophyll
**Fig. 1.2 Structures of biologically important porphyrins**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of Side Chains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R₁</td>
</tr>
<tr>
<td>Uroporphyrin</td>
<td>A</td>
</tr>
<tr>
<td>Heptacarboxyporphyrin</td>
<td>A</td>
</tr>
<tr>
<td>Hexacarboxyporphyrin</td>
<td>M</td>
</tr>
<tr>
<td>Pentacarboxyporphyrin</td>
<td>M</td>
</tr>
<tr>
<td>Coproporphyrin</td>
<td>M</td>
</tr>
<tr>
<td>Protoporphyrin</td>
<td>M</td>
</tr>
<tr>
<td>Deuteroporphyrin</td>
<td>M</td>
</tr>
<tr>
<td>Mesoporphyrin</td>
<td>M</td>
</tr>
<tr>
<td>Hematoporphyrin</td>
<td>M</td>
</tr>
</tbody>
</table>

A = CH₂COOH, P = CH₂CH₂COOH, M = CH₃, V = CH₃CH, E = C₂H₅, EA = CH₂CHOH
appears to be a characteristic absorbance spectra for all porphyrins. Any change to the porphyrins, however, such as the chelation of a metal to form haem, leads to a change in the absorption spectra of the porphyrin. The distinct change observed is the modification of the four secondary bands to only two which are usually labelled α and β. This characteristic absorption spectra of the porphyrins has been extensively utilised for their study and analysis in biological systems.

Porphyrins are also intensely fluorescent compounds when exposed to UV light with the wavelength similar to their soret band. The fluorescence is observed as two bands in the region of 600 and 650 nm. This fluorescence disappears on chelation of a metal to the porphrin, thus offering a further characteristic that may be utilised for the study of porphyrins and porphyrin-metal complexes.

A variety of biologically important porphyrins exist in nature (Figure 1.2). This variety arises due to the number and nature of side chains bound to the porphyrin macrostructure. The structure of the porphyrin allows eight side chains to be attached as indicated in Figure 1.2. The increased polarity of the side chains and the increased number of these side chains would lead to an increase in the solubility of the porphyrins in water. Due to their solubility certain porphyrins, which are excreted in humans, are excreted in the faeces and others in the urine with some being excreted in both, such as protoporphyrin, uroporphyrin and coproporphyrin, respectively.

1.2 The Haem Biosynthetic Pathway

The haem biosynthetic pathway comprises of a set of eight enzymatic reaction steps for the formation of haem, an essential component of oxygen transport systems, mixed function oxidative reaction, and oxidative metabolic processes. Each step is
controlled by one specific enzyme (Figure 1.3). This pathway is active in all nucleated cells and provides haem and other related structures, in various species. In mammals the principle product of this pathway is haem which is usually bound to various proteins and is central to many biological oxidations. Haem is essential for a vast number of processes such as transport and storage of oxygen in the form of hemoglobin and myoglobin, detoxification reactions in the form of microsomal cytochrome P450, generation of cellular energy, peroxidase mediated reductions to convert hydrogen peroxide into water, biosynthesis of certain steroids, regulation of protein synthesis and cell development (Beard et al. 1996; Harrison and Arosio, 1996; Bloomer, 1998).

Although porphyrins and haem are synthesised in all mammalian cells, by far the majority of them are synthesised in the bone marrow and liver. The haem biosynthetic pathway utilises glycine and succinyl coenzyme A as substrates (Figure 1.3). The intermediate compounds formed during the process, which takes place in the mitochondria and the cytosol of the cell, are the hexa hydro porphyrins, named porphyrinogens. As with any enzyme controlled process within the biological system, disorders develop when the enzyme itself or its activity is affected. Before the disorders relating to the haem biosynthetic pathway can be considered an understanding of the pathway is required.

David Shemin and his colleagues in 1945 revealed evidence of the involvement of an amino acid in the biosynthesis of porphyrin rings of haem. They showed that the nitrogen atoms of haem were labelled when human subjects were fed with $^{15}$N-glycine.
Fig. 1.3 Heme biosynthetic pathway and porphyria

**MITOCHONDRIA**

- ALA Synthase
- ALA Dehydratase deficiency porphyria
- Ferrochelatase
- Proto'gen Oxidase

**CYTOPLASM**

- ALA Dehydratase
- HMB Synthase
- Uro'gen III Synthase
- Uro'gen Decarboxylase
- Copro'gen Oxidase

**Processes**

- Erythropoietic Protoporphyria (EPP)
- Variegate Porphyria (VP)
- Hereditary Coproporphyria (HC)
- Acute Intermittent Porphyria (AIP)
- Congenital Porphyria (CP)
- Porphyria Cutanea Tarda (PCT)
Chapter 1

Using $^{14}$C they also showed that eight of the carbon atoms of haem were derived from the $\alpha$ carbon of glycine, whereas 24 carbon atoms were from the methyl group of acetate and 2 from carboxyl group of acetate. This distinctive pattern of labelling led them to propose that a condensation reaction is taking place between glycine and an activated succinyl compound. It is now well established that the first step in the synthesis of haem is the condensation of glycine and succinyl CoA to form $\delta$-aminolevulinic acid. This committed step is catalysed by ALA synthase (ALA-S, EC2.3.1.37) in the matrix of the mitochondria. The reaction takes place by the use of a cofactor, pyridoxal-5'-phosphate bound to the enzyme. The glycine reacts with the aldehyde group of the pyridoxyl-5'-phosphate followed by condensation of succinyl CoA and the release of CO$_2$ (Retey and Robison, 1982) to form ALA. This is one of the rate-limiting steps in haem biosynthesis and, as might be expected, is highly regulated by a variety of factors. These controlling factors include the genetic control of the synthesis of the enzyme, the transport of the enzyme from the cytosol, where it is synthesised, to the matrix of the mitochondria and the inhibition of the enzyme by the intracellular free haem pool (Battle, 1993). The synthesis of the ALA occurs within the mitochondria and is actively transported to the cytoplasm where the next step of haem synthesis takes place.

In the cytoplasm, two molecules of the substrate are condensed and cyclised by the enzyme ALA dehydratase (ALA-D, EC4.2.1.24) to form a monopyrrole called porphobilinogen (PBG). This is a dehydration reaction and involves the loss of two water molecules in the process. The PBG is synthesised by the binding of the keto group of the ALA to the $\varepsilon$-amino group of the lysine at the active site of the ALA-D enzyme eliminating one water molecule in the process. This is followed by an aldol
The condensation of the second ALA to the first. The end result is the formation of PBG in which the acetic acid is provided by the first ALA molecule and the propionic acid is formed from the second ALA molecule.

The enzymes involved in all steps of the haem biosynthetic pathway require functional sulfhydryl (SH) groups for optimal catalytic activity. These groups may be part of the active site configuration or maintain the structural integrity of the enzyme. Inactivation of the sulfhydryl groups, such as by oxidation for example, leads to a rapid decrease in the activity of the enzyme. ALA-D enzyme is also known to be inhibited, in-vitro by metals. This inhibition has been observed in liver and erythrocytes with metals such as manganese, iron, copper, silver and zinc ions (Gibson et al. 1955). Cobalt and lead has been shown to inhibit ALA-D activity in-vivo (Nakemura et al. 1975, Sassa, 1978). In erythrocytes, the inhibition of ALA-D due to lead poisoning is well established and is used as a measure of lead exposure in humans.

Hydroxymethylbilane synthase (HMB-S, EC4.3.1.8), of which two isoenzymes are known (erythrocyte-specific and tissue-specific), catalyses the third step in haem biosynthesis which involves the condensation of four PBG molecules in a head-to-tail fashion. The end product of this reaction is a linear tetrapyrrole, hydroxymethylbilane (HMB). An ammonium ion is released for each of the methylene bridges formed. Investigations have been carried out in order to study the mechanism of the formation of HMB (Battersby et al. 1980). It has been firmly established that the four pyrrolic rings are attached in a stepwise fashion starting from ring A to D by the HMB-S enzyme. Later researchers (Hart et al. 1987, Jordan and Warren, 1987) have found the involvement of a dipyrrromethane cofactor in the synthesis of HMB. The cofactor
itself is composed of two molecules of PBG which binds to the HMB-S and acts as a primer for HMB synthesis. When the HMB molecule is synthesised it is released leaving behind the cofactor for further HMB synthesis. Hart and co-workers (1988) have found this primer to be covalently bound to the cysteine-242 of the *Escherichia Coli* enzyme. Similar to the ALA-D, HMB-S is also known to be inhibited by trace metals such as copper, iron, magnesium, mercury and lead with lead being a particularly effective inhibitor (Anderson and Desnick, 1980). Maines and Kappas (1977) have also shown the *in-vivo* inhibition of HMB-S by the presence of platinum in kidney.

Following the synthesis of HMB, that has an open ring structure, the next step in the haem biosynthesis involves the closing of this ring to form uroporphyrinogen. Two isomers of uroporphyrinogen can be formed. In the absence of the uroporphyrinogen III synthase (URO-III-S, EC4.2.1.75) the HMB closes chemically to form uroporphyrinogen I (Uro’gen I). This symmetrical porphyrinogen and its metabolites are biologically inactive and accumulate only under pathological conditions. In the presence of URO-III-S the asymmetrical isomer, uroporphyrinogen III (Uro’gen-III) is formed (Figure 1.2) by the intramolecular rearrangement of ring D. The excess of URO-III-S activity compared to that of HMB-S in human erythrocytes ensures the synthesis of Uro’gen-III in favour of the Uro’gen-I isomer. The URO-III-S enzyme has also been shown to be inhibited *in-vitro* by various metals including zinc, cadmium, copper and mercury (Tsai et al, 1987; Clement et al, 1982).

The last cytosolic enzyme, uroporphyrinogen decarboxylase (URO-D, EC4.1.1.37), carries out decarboxylation of the Uro’gen-III to form coproporphyrinogen III (Copro’gen III). The decarboxylation of the acetic acid side
chains occurs leaving the propionic acid side chains intact. This decarboxylation converts the four acetic acid groups to methyl groups in a stepwise fashion producing the corresponding intermediates with seven, six, five and finally four carboxyl groups. URO-D does not show specificity towards one specific isomer and hence all four isomers of Uro’gen, namely I, II, III and IV are decarboxylated. However the activity of the URO-D is greatest towards the naturally abundant Uro’gen III isomer. URO-D has an absolute requirement for free SH groups for catalytic activity (Elder and Urquhart, 1984; Elder et al, 1983) and is readily inhibited by numerous trace metals which include zinc, cobalt, copper, lead, mercury and platinum. The most potent of these metals is mercury, which effectively eliminates URO-D activity in concentrations as low as 100μM (Woods et al, 1984). It is still not clear whether decarboxylation occurs on one or more site of URO-D enzyme (Verneuil et al, 1983) and further investigations are needed in this regard.

Following the synthesis of Copro’gen III, it is transported back into the mitochondria where the enzyme Copro’gen oxidase (Copro-O, EC1.3.3.3) catalyses the oxidative decarboxylation of two of the four propionic side chains at positions 2 and 4 of the pyrrole rings A and B, respectively. The result of this oxidative decarboxylation is protoporphyrinogen IX (Proto’gen) which consists of two vinyl groups at position 2 and 4. Investigation as to the order of the decarboxylation by Elder and co-workers (1978) have ascertained that the 4-propionic acid group cannot be attacked until the 2-propionic acid has been decarboxylated. These findings are supported by the identification and isolation of a tripropionic acid porphyrin in the Harderian gland of the rat (Kennedy et al. 1970). The finding that only 4-propionic acid 2-vinyldeuteroporphyrin IX (Harderoporphyrin) is present with an absence of its
positional isomer. 2-propionic acid 4-vinyldeuteroporphyrin IX (Isoharderoporphyrin) suggests a sequential decarboxylation of the propionic acids with that at the 2-position being metabolised first (Kennedy et al, 1970). Further support for this sequential decarboxylation is the preference of the COPRO-O to convert harderoporphyrinogen into protoporphyrin more readily than the isoharderoporphyrin (Games et al, 1976). Work carried out by Elder and co-workers have suggested that both decarboxylations occur at a single active site of COPRO-O. The activity of COPRO-O is shown to be inhibited by mercury, however aluminium, lead, manganese and nickel show no effect (Rossi et al, 1992). It is as yet unclear whether mercury inhibits COPRO-O directly or the apparent reduced activity is as a result of mitochondrial membrane damage, to which COPRO-O is associated (Rossi et al, 1992, Woods and Fowler, 1987).

The oxidative decarboxylation of Copro’gen III is followed by oxidation of the tetrapyrrolic ring structure of Proto’gen by the enzyme protoporphyrinogen oxidase (PROTO-O, EC1.3.3.4). PROTO-O, associated with the inner membrane of the mitochondria, removes six hydrogen atoms from Proto’gen to form protoporphyrin IX (Proto), a penultimate precursor to haem. In-vitro Proto’gen can also be oxidised spontaneously in the presence of oxygen to form Proto, however in-vivo where antioxidants are present, the majority of the Proto is due to the action of PROTO-O (Poulson and Polglass, 1975). The PROTO-O is shown to be inhibited by cobalt, copper and the final product, haem.

The final step of the haem biosynthetic pathway is the insertion of ferrous iron into Proto to form haem. The chelation reaction is catalysed by the mitochondrial inner membrane enzyme ferrochelatase (FECH, EC4.99.1.1). FECH, which can also use deuteroporphyrin and mesoporphyrin as its substrates, is shown to be directly
inhibited by trace metals \textit{in-vitro} such as lead, copper, manganese, cobalt and zinc but stimulated by fatty acids (Taketani and Tokunaga, 1981; Taketani et al, 1985). Unlike COPRO-O, there is little evidence to support this effect \textit{in-vivo} for FECH (Woods, 1989) but the similarity of these enzymes, with regards to the association of the enzymes with mitochondrial membrane, would suggest that indirect inhibition may occur. Taketani and colleagues (1985) also proposed that FECH inhibition is likely due to the decreased availability of Fe$^{2+}$, the substrate for ferrochelatase, to utilise. This may be due to auto-oxidation of Fe$^{2+}$ to Fe$^{3+}$ in the presence of oxygen (Punekar and Gokhal, 1991) or by a metal, for example lead, which inhibits the enzymatic reduction of Fe$^{3+}$ to Fe$^{2+}$ within mitochondria (Taketani et al. 1985).

\subsection{1.2.1 Regulation of the Haem biosynthetic pathway}

By far the majority of the total body haem synthesis occurs in the bone marrow accounting for 85\% compared to liver where 13-23\% of total body haem is synthesised (Bloomer, 1998). However, much more is known about the control of haem formation in liver in comparison to erythroblasts. ALA-S, a rate limiting enzyme that converts glycine and succinyl CoA to δ-ALA in the first step of the pathway, exists in two different forms encoded by different genes (Bishop, 1990). The non-erythroid form, or the housekeeping form, is found in the liver (Bloomer, 1998) and is regulated by haem. Haem controls the activity of ALA-S by repressing its synthesis, inhibiting its transport from the cytoplasm into mitochondria and also affecting the activity of ALA-S directly (Gorchein, 1997). Hamilton and co-workers (1991) have shown that reduced synthesis of ALA-S in chick embryo hepatocytes is due to the reduction of half-life of ALA-S mRNA whereas Srivastava and co-workers (1990) indicated that the effect in rats was at the transcriptional level. Furthermore,
haem also regulates its own biodegradation by regulating the synthesis of haem oxygenase mRNA (Moore et al. 1987). Haem oxygenase, present in hepatocytes, metabolises haem to biliverdin, releasing carbon monoxide and iron (Bloomer et al, 1998). The increased degradation of haem depletes the free haem pool which reduces the feedback inhibition of ALA-S thereby increasing the activity of ALA-S. In erythroblasts as well as regulating ALA-S activity, haem also controls haem biosynthesis by regulating erythroblast metabolism and differentiation (Moore et al. 1987). Iron uptake is also regulated by haem in developing erythroid cells by altering the transferrin receptor density (Ponka et al. 1974).

1.3 Porphyria

The name ‘porphyria’ originates from the Greek word ‘porphuros’ or purple, obtained from the intense purple-red colour of porphyrins. The porphyrias are a group of rare and complex metabolic diseases associated with the intermediates of the haem biosynthetic pathway.

The rate at which cells makes haem is controlled by enzymes, the production of which is regulated according to the need of the tissue. Each of the porphyrias is usually associated with the deficiency of one of these enzymes, due either to internal or external factors. Due to this deficiency the cell responds by overproducing some of the precursor intermediates. These overproduced intermediates may then accumulate in the body causing various types of porphyria, dependent on the particular enzyme affected. Eight different types of porphyrias have been described and although most of the porphyrias are inherited as autosomal dominant, some are recessive and others acquired (Grandchamp et al. 1996; Sassa and Kappas, 2000).
The factors which may affect the enzymes of the biosynthetic pathway, and thereby bring about the onset of porphyria may either be external, such as drugs, alcohol, strict dieting, fasting or internal, such as hormones that develop after puberty (Grandchamp et al. 1996; Gorchein, 1997). Furthermore, although porphyria is known to be hereditary, however, due to the involvement of these external factors, the onset is by no means certain and the severity of porphyria varies considerably amongst individuals. This, combined with the fact that the symptoms of porphyria are not always apparent, makes diagnosis of these disorders extremely difficult and may be completely missed until full examination is carried out.

The most important types of porphyrias are the acute porphyrias because attacks of these are often life threatening (Figure 1.4). The less serious, but nevertheless severely debilitating, non-acute porphyrias are mainly dermatological conditions which result in the skin becoming sensitive to light. The photosensitivity of the skin is due to the photosensitizing properties of circulating porphyrins. Although the haem biosynthetic pathway is well understood however, the molecular biology of its function and dysfunction remains to be clarified and is a subject of intense research. The type of porphyria is identified by quantitative analysis of the excretion pattern of the different porphyrins and their precursors present in the urine and faeces since each porphyria has a distinct excretion pattern. A brief description of the porphyrias is presented.
Fig. 1.4 Classification of inherited porphyrias

<table>
<thead>
<tr>
<th>Hepatic</th>
<th>Autosomal recessive</th>
<th>Autosomal dominant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) ALA dehydratase deficiency porphyria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Acute intermittent porphyria (AIP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) Variegate porphyria (VP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4) Hereditary coproporphyria (HC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5) Porphyria cutanea tarda (PCT)</td>
<td>Autosomal dominant</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythropoietic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6) Erythropoietic protoporphyria (EPP)</td>
<td>Autosomal dominant</td>
<td></td>
</tr>
<tr>
<td>7) Congenital erythropoietic porphyria (CEP)</td>
<td>Autosomal recessive</td>
<td></td>
</tr>
<tr>
<td>Hepato-erythropoietic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8) Hepato erythropoietic porphyria (Homozygous form of PCT)</td>
<td>Autosomal recessive</td>
<td></td>
</tr>
</tbody>
</table>

- Acute
- Non-cutaneous
- Cutaneous
1.3.1 Acute Porphyrias

Acute porphyrias are often triggered by exposure to exogenous precipitating factors including drugs, such as amphetamines, cocaine and their derivatives, excess alcohol, smoking, stress, fasting, infection and sex hormones (Kauppinen and Mustajoki. 1992; McColl et al. 1996). Acute attacks, of which about 1% may be fatal, most often occur post puberty and are more common in females (Thadani et al, 2000). It is interesting to note that only 10-15% of gene carriers develop porphyria and therefore it is sometimes found that patients appear to have no family history due to the condition remaining latent for several generations (Thadani et al. 2000).

1.3.1.1 ALA dehydratase deficiency porphyria (ADP)

ALA dehydratase deficiency porphyria, caused by inherited defects in the ALA-D gene, is always inherited in an autosomal recessive fashion. The ALA-D gene has been genetically linked to chromosome 9q34 and spans 16kb (Kaya et al, 1994). The resultant decrease in ALA-D activity leads to an accumulation of ALA with a consequent increase in ALA excretion in urine. As with all acute porphyrias the biochemical features of ADP usually includes neurological dysfunction, however, ADP shows no photosensitivity (Sassa and Kappas, 2000). Although the cause of the neurological dysfunction has not been fully explained however, studies have shown that ALA can either act as a neurotoxin or its accumulation can cause oxidative stress forming reactive species which then produce neuropathological manifestations (Hermes-Lima et al. 1991; Princ et al. 1998). Only a very few cases have been described for ADP making this a very rare inherited disorder.
1.3.1.2 Acute intermittent porphyria (AIP)

In Northern Europe 1/10000 of the population carry the gene for acute intermittent porphyria (AIP) (Moore et al. 1990) making this, the most severe of the acute porphyrias, the most common. The primary defect in AIP lies in the HMB synthase gene leading to a corresponding reduced activity of the HMB-S enzyme. The HMB synthase gene has been mapped to chromosome 11q24.1-q24.2 containing 10kb (Namba et al. 1991; de Verneuil et al, 1992). The result of the defect is an increase in Aminolevulinic acid (ALA) and PBG causing neurological dysfunction such as abdominal pain, vomiting, constipation and psychiatric disturbances. The heterogeneity of the defect is observed by the fact that over 75 different mutations have been reported so far including missense mutations and splice site mutations (Puy et al. 1997). The effect of these mutations may vary widely from a decrease in the enzyme activity to a complete absence of the enzyme. Mortality rates of about 10% exist in acute attacks of AIP, usually due to respiratory paralysis (Battle, 1993).

Urine analysis of porphyrins shows the ALA and PBG raised to high levels during an acute AIP attack. Uroporphyrin (URO) in urine is also raised to lesser degree. Coproporphyrin (Copro), present in urine and faeces and protoporphyrin (Proto), in faeces, is sometimes raised (Lip et al, 1993).

1.3.1.3 Hereditary Coproporphyria

Hereditary coproporphyrria (HC), inherited in an autosomal dominant fashion, occurs due to the reduction in coproporphyrinogen oxidase activity caused by mutations in the COPRO-O gene. The COPRO-O gene was mapped to chromosome 3q12 and consists of 14kb (Cacheux et al. 1994; Delfau-Larue, 1994). Several
different mutation in the COPRO-O gene have been detected leading to the coding of either an unstable enzyme with reduced activity or an enzyme with a decreased affinity for the substrate (Schreiber et al, 1997). HC shows symptoms similar to those described for AIP. However, skin photosensitivity is also present. ALA, PBG and Copro levels are raised in urine, with copro levels also being raised in faeces.

1.3.1.4 Varigate porphyria

Variegate porphyria (VP), also one of the acute porphyrias, occurs due to the reduced activity of protoporphyrinogen oxidase (PROTO-O) enzyme which converts protoporphyrinogen IX to protoporphyrin IX. The mutations, of which a variety have been reported (Warnich et al, 1996; Lam et al, 1997), are present on the PROTO-O gene which is mapped to chromosome 1q22-q23. The size of this gene is unclear with 8kb and 4.5kb being reported by various groups (Taketani et al, 1995; Roberts et al, 1995). These mutations lead to coding of an enzyme with a marked decrease in the activity and stability (Kauppinen et al, 1997; Dailey et al, 1997). The symptoms of VP, which is inherited in an autosomal dominant fashion, are similar to other acute porphyrias. However, the skin sensitivity in VP is much more severe in comparison to HC with increased fragility of the skin. Urine analysis shows high levels of ALA, PBG and Copro. Proto levels are found to be high in bile and faeces. VP in general is a rare disorder, with a prevalence of 1.3 in 100000 in Finland for example (Mustajoki, 1980). One important exception to the rare nature of this disorder is in South Africa, where prevalence rises sharply to 3 in 1000 (Dean, 1971). This is attributed to the lineage of the majority of patient families in South Africa who are thought to be from a single immigrant in the early 17th century (Meissner et al, 1996).
1.3.2 Non-Acute Porphyrias

All non-acute porphyrias, with some acute porphyrias, are cutaneous in which porphyrins are deposited in the upper epidermal layer of the skin. These photosensitising reagents are responsible for the characteristic skin lesions. Skin damage occurs due to the porphyrins’ ability to absorb UV/Visible light, which can penetrate through the epidermis down to the cutaneous blood vessels, resulting in transitions of porphyrin to their excited electronic states. These excited porphyrins may go on to react in two different ways. They may react directly with biological structures such as membrane lipids, nucleic acids and amino acids in proteins called the type I reaction. However, the excited porphyrins may also generate excited singlet oxygen by reacting with molecular oxygen, which then damages biological tissues (type II reaction). Other reactive oxygen species (ROS) such as $O_2^-$, ‘OH and $H_2O_2$ may also be formed causing similar tissue damage (Smith and De Matteis, 1990; Hei et al. 1998; Afonso et al. 1999; Davies et al. 2000).

1.3.2.1 Congenital Porphyria (CP)

One of the rarest form of porphyrias is CP, also called Gunther’s disease which is caused by the genetic defects in the URO-III-S gene. The URO-III-S gene is mapped to chromosome 10q25.3-q26.3 and consists of 60kb (Dubart et al. 1986; Romana et al. 1987). The mutations on the URO-III-S gene are heterogeneous, however, some mutations are unique to individual families (Xu et al. 1995). These genetic mutations lead to the coding of an enzyme with markedly reduced enzymatic activity and also decreased half life of the enzyme (Xu et al. 1995). The symptoms of photosensitivity, due to increased production of uroporphyrin I, start from birth but
late onset can also occur. The major site of expression for uroporphyrin I are the normoblasts of the bone marrow which is then excreted in the urine. Deposition also occurs in bones and teeth with a characteristic fluorescence under UV/Visible light of approximately 400nm (Moore et al. 1990). There appears to be no effective therapy for this disease. However, avoidance of the triggering factors such as sunlight, splenectomy, the administration of chloroquine (Ipren & Fuchs, 1980) and oral activated charcoal (Pimstone et al. 1987) alleviate the symptoms.

1.3.2.2 Porphyria Cutanea Tarda (PCT)

Porphyria cutanea tarda (PCT) is usually observed in men over 40 years of age (Mascaro, 1995) and is the most common of all the porphyrias (Thadani et al, 2000). PCT, also called Cutaneous hepatic porphyria, exists in acquired (PCT type I) and familial forms (PCT type II). PCT type I is usually triggered by the effect of toxic, hormonal, or infectious agents such as alcohol, oestrogenic steroids and hepatitis C virus, respectively on uroporphyrinogen decarboxylase (URO-D) activity (Mascaro, 1995). PCT type II is caused by mutations in URO-D gene inherited as an autosomal dominant trait. The gene has been mapped on to chromosome 1p34 and consists of 3kb (Dubart et al, 1986; Romana et al, 1987). Shortened half life and markedly reduced enzymatic activity (~15-50%) results from a variety of mutations in this gene (Garey et al, 1989; McManus et al, 1996). In familial form URO-D activity is reduced in peripheral blood cells unlike the acquired form where the reduction in enzyme activity is restricted to the liver (Mascaro, 1995). Symptoms of PCT include erythema progressing on to form bullae which may haemorrhage and leave scars. Other symptoms also include hyperfragility of the skin, cutaneous photosensitivity, hyperpigmentation, hypertrichosis and premature aging of the skin. Biochemical
features include an increase in uroporphyrin and 7-carboxylic porphyrin in the urine with a comparatively lesser amounts of 6-, 5- and 4-carboxyl porphyrins (Mascaro, 1995). Treatment usually involves avoidance of precipitating agents, removal of excess iron by venesection and administering of chloroquine (Ashton et al, 1981). Chloroquine forms a complex with uroporphyrin facilitating its release followed by excretion in the urine. It is thought that chloroquine may also inhibit uroporphyrin synthesis (Kordac et al, 1989).

1.3.2.3 Erythropoietic Protoporphyria (EPP)

One of the other non-acute forms of porphyria is Erythropoietic protoporphyria (EPP), endured in Europe by 1:75000 people. It is an inborn error of metabolism owing to the deficiency of ferrochelatase (Todd, 1998). The deficiency of the FECH is due to mutations in the FECH gene located on the 18q21.3 chromosome (Whitcombe et al. 1991). The FECH gene consists of 45kb (Cox, 1997). As with other non-acute porphyrias a variety of different mutations can occur in the FECH gene (Lamoril et al. 1991; Wang et al, 1993) leading to the synthesis of an enzyme with a markedly reduced enzymatic activity (Imoto et al, 1996; Wang, 1996). FECH is the last enzyme of the haem pathway, the function of which is to insert iron into protoporphyrin to form haem. The onset is often from infancy (Gorchein and Foster, 1999). The symptoms of EPP consist of severe pain and burning sensation after exposure to light, usually affecting the face and hands. In the later stages of the disease the characteristic thickening of the skin occurs. In less than 5% of patients, suffering from EPP, protoporphyrin deposition in the liver results in cirrhosis and liver failure (Bloomer, 1979; Doss and Frank, 1989; Kappas et al, 1995).
Many different types of cures have been investigated and reported for this particular disorder including liver transplantation (Meerman et al, 1999) and long term treatment with oral charcoal (Gorchein, 1999). Although beneficial, liver transplant seems to have only a limited use as a cure for EPP since even after the transplant, protoporphyrin levels in blood and faeces remain elevated (Meerman et al, 1999). Photosensitivity, therefore, also remains a problem. The most effective treatment for EPP is thought to be bone marrow transplant, but this procedure itself has a high rate of mortality and morbidity (Meerman et al, 1999).

The basis of the treatment of EPP by activated charcoal is due to the strong adsorption characteristics of protoporphyrin to charcoal. This is an effective treatment as part of the management of EPP, however, side effects, such as diarrhea and flatulence or constipation have been reported (Gorchein, 1999) and make this an unpalatable choice of treatment for the patient. Another treatment for the symptoms of EPP is the oral administration of beta-carotene which reduces the photosensitivity of the skin (Moore et al, 1990) however, side effects such as yellowing of the skin occur.

Preselective gene therapy has also been carried out in mice as a cure for EPP (Pawliuk et al, 1999). Although still in its infancy, it has shown positive results and remains to be seen whether this becomes the therapy of choice for EPP.

1.4 Porphyria and Cancer

Although the exact mechanism has not been elucidated, however, it is well established that a link between porphyria and cancer exists both in rodents and humans. The correlation of porphyria and hepatic cancer, in rodents, has been confirmed by researchers (Davies et al, 2000) who have reported the occurrence of
hepatic cancer and porphyria by the interaction of polychlorinated biphenyls (PCBs) and iron. Davies and co-workers detected the appearance of hepatocellular carcinomas by 12 months when iron and Aroclor 1254, a PCBs mixture, were administered in C57BL/10ScSn mice (Smith et al, 1990; Davies et al, 2000). A similar experiment by Madra and co-workers (1996) produced hepatic porphyria followed by hepatocellular carcinoma in mice. Similar findings have been reported with other polyhalogenated aromatic chemicals such as hexachlorodibenzo-p-dioxins (Sinclair et al. 1990; Smith and De Matteis, 1990; Madra et al, 1995). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, Park et al. 1996; Tritscher et al. 1996; Shertzer et al. 1998) and also hexachlorobenzene (HCB, Smith et al, 1989; Sinclair et al, 1990). Although the mechanism of their carcinogenicity is not clear it is proposed to be through the formation of reactive metabolites (Stewart and Smith, 1987). It is well known that the polyhalogenated aromatic chemicals are good ligands for the Ah-receptors which leads to the expression of a variety of genes (Smith et al, 1990; Faux et al. 1992; Shertzer et al. 1998) including those for cytochrome P450. In particular the chronic induction of cytochromes P450IA1 and P450IA2 is known to occur in microsomes and nuclear membranes (Madra et al. 1996). The induction of these uncoupled cytochrome P450 isoenzymes and the corresponding production of the reduced oxygen metabolites by them, such as hydroxyl radicals and iron-oxygen radicals, indicate that a free radical mechanism contributes to the initiation of uroporphyria and tumour formation (Inoue and Kawanishi, 1987; Imlay et al, 1988). Studies carried out by Inoue and Kawanishi (1987) and also Imlay and co-workers (1988) have found these reduced oxygen metabolites to be genotoxic and mutagenic. These findings have been supported by Faux and co-workers (1992) who have found
evidence of oxidative DNA damage by iron and PCBs in-vivo in C57BL/10ScSn mice. The induced cytochrome P450 isoenzymes, however, may themselves oxidise uroporphyrinogen to uroporphyrin thereby causing uroporphyria and cancer (Jacobs et al, 1989). It has also been proposed that cytochrome P450 isoenzymes may produce a uroporphyrinogen decarboxylase inhibitor under conditions of oxidative stress leading to an increase in uroporphyrinogens thus causing uroporphyria (De Matteis et al, 1988).

Although the interaction of iron and PCBs greatly increase the incidence of the formation of porphyria and cancer, however it has been shown that iron by itself can also potentiate porphyria and cancer (Stevens et al, 1988; Smith et al, 1989; Smith and De Matteis, 1990; Davies et al, 2000). The reason for this may be the induction of the ALA-S, a rate limiting enzyme in the haem biosynthetic pathway, leading to the increased production of uroporphyrinogen which can then be oxidised to uroporphyrin, thus causing uroporphyria (Urquhart et al, 1988). It is also well recognised that non-haem iron may catalyse a variety of radical reactions leading to oxidative stress (Meneghini, 1997). Many of the pathological processes are therefore, thought to be associated with non-haem iron acting as a catalyst of free radical oxidative damage. A summary of the possible mechanisms leading to the development of porphyria and the formation of cancer are outlined in Figure 1.5.

Lithner and Wetterberg (1984) have reported an important parallel occurrence of primary liver cancer (PLC) and porphyria in human patients. They discovered incidences of patients with PLC also having metabolic abnormalities of haem biosynthetic pathway. Further incidences of hepatocellular carcinoma have also been
Fig. 1.5 The involvement of polyhalogenated aromatic chemicals in the development of porphyria and cancer

Polyhalogenated Aromatic Chemicals

Induction

O₂

Cytochrome P450

O₂⁻

Ferritin

OH⁻ or iron-oxygen complex

Lipid peroxidation

DNA damage

Cell membrane damage

Altered gene expression

Uroporphyrinogen

Inhibitor of Uroporphyrinogen Decarboxylase

Uroporphyrin

Uroporphyria

[Porphyrins]⁺

Liver Cancer
reported in 5-16% of patients with PCT, 3% of patients with AIP and 2% of patients with VP (Lim and Mascaro, 1995).

The correlation of porphyria and PLC may have a genetic basis. The genetic mutation associated with certain types of porphyria is also thought to affect an oncogene responsible for the high number of PLC cases. One such type of porphyria is AIP, caused by the decreased activity of HMB-S, the genetic locus of which is present on the long arm of chromosome 11 (Kaczynski et al, 1995). Although the link between porphyria and PLC is certain, however, doubt exists as to whether the cancer causes porphyria or vice-versa. Contradictory findings have been reported regarding the order of the occurrence of porphyria and cancer. Thompson et al in 1970 described a patient who seemed to have porphyria caused by malignant primary hepatoma. In contrast other studies appeared to indicate that malignancy is secondary to porphyria (Kordak, 1972; Solis et al, 1982; Santos et al, 1992).

A relationship between excess porphyrins and cancer in mice has also been postulated (Figge, 1944). Figge and co-workers in 1942 found a match between the amount of protoporphyrin in the Harderian glands and intrinsic susceptibility to cancer of the mammary gland. They showed that inbred strains of mice with a high incidence of spontaneous mammary tumours had a very high concentration of porphyrins, with mice of strains with intermediate degrees of tumour susceptibility having intermediate concentrations of porphyrins and mice of cancer resistant strains exhibiting little or no porphyrins (Strong and Figge, 1941). Therefore, the possibility that porphyrins that accumulate may well be carcinogenic, either in their reduced or oxidised forms, cannot be ruled out.
The carcinogenicity of porphyrins has been implicated by the studies carried out by researchers who have investigated the formation of porphyrin radicals by a variety of peroxidase enzymes (Roberts et al, 1981; Morehouse et al, 1989). Morehouse and co-workers have investigated horseradish, lacto- and myeloperoxidases and found positive results using a variety of naturally occurring and synthetic metal-free porphyrins as substrates. The one-electron oxidation product obtained has been identified as a porphyrin pi-cation radical. These pi-cations have been shown to degrade through a disproportionation mechanism forming di-cations that react with water to form isoporphyrins and mesodihydroxyporphyrins which undergo further reactions to yield ring-open products (Neta et al, 1986; Richoux et al, 1986). Morehouse and co-workers have also shown the enzymatic one-electron reduction of metal-free porphyrins to yield anion free radicals (Morehouse et al, 1987). Furthermore Princ and co-workers in 1998 studied the formation of reactive oxygen species (ROS) promoted by ALA and their effect on porphyrin biosynthesis. Their investigation in the rat cerebellum not only showed the ALA to be a prooxygen but it strongly suggested the involvement of $O_2^\cdot$, $H_2O_2$ and $OH^\cdot$ formed by its autooxidation. The authors have also found this ALA triggered oxidative stress to damage haem biosynthetic pathway enzymes, in particular ALA-D, which could be inhibited by the antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) as well as radical scavengers such as dimethyl sulphoxide (DMSO).

The production of ROS through the iron catalysed oxidation of ALA was suggested by Hermes-Lima et al in 1991. This was confirmed by Fraga et al in 1994 who reported the formation of 8-hydroxy-2'-deoxyguanosine \textit{in-vitro} on the incubation of calf thymus DNA with ALA in the presence of Fe$^{2+}$. This was also
observed by Onuki et al (1994) who found DNA single strand breaks when plasmid pBR322 DNA was incubated with ALA in the presence of Fe$^{2+}$. The results obtained by the above authors lend further support to the involvement of porphyrin radicals and the ROS formed by them in the link between porphyria and cancer.

### 1.5 Harderian gland

In 1694 Johann Jacob Harder described a new gland in the orbits of the eyes in two species of deer. Originally called ‘Harder’s gland’ they were later named Harderian glands. These glands are large, intraorbital glands that are found in all land vertebrates except humans and some other species. Although discovered over 300 years ago, the literature on Harderian gland is scarce. This may be due to their absence in humans and therefore regarded as unimportant or it may be that these glands are often confused as an accessory lacrimal gland (Rodriguez et al, 1992). The structure of the Harderian gland also differs among species and because of this confusion a specific criterion for the characterisation of these Harderian glands was proposed by Sakai in 1981. She described them as tubuloalveolar, ocular glands that secrete lipids by a merocrine mechanism.

Porphyins were detected in the Harderian gland as early as 1924 by Darrien and Turchini but it was thought to be an organ for the storage of porphyins that were formed elsewhere (Darrien and Turchini, 1924). However, later findings by Tomio and Grinstein (1968), among others, obtained evidence for the biosynthesis of porphyins within the Harderian gland. Further, Margolis in 1971 and Thompson et al in 1984 demonstrated the existence of all of the enzymatic complement required to synthesise porphyins from glycine and succinyl coenzyme A. The Harderian glands
of all rodents have been shown to contain porphyrins but the amount of porphyrins in the gland varies in different species and even among individuals of the same species (Payne et al, 1992). In general the gland of the female rodents have been shown to contain higher level of porphyrins than that of the male gland and investigations have shown the porphyrin synthesis to be under hormonal control (Payne et al, 1992). The exception to this observation is the Harderian gland in the rat and in some species of mice where there appears to be no gender variations (Strong, 1942; Margolis, 1971; Shirama et al, 1981). The huge amounts of porphyrins present in the Harderian gland, from which the main component was thought, until now, to be protoporphyrin IX is due to the low levels of ferrochelatase. The increased activity of protoporphyrinogen oxidase and the low ferrochelatase activity leads to the deposition of solid intraluminal porphyrins (Spike et al, 1992).

From these investigations, it is obvious that much information has been gleaned about the structure of the Harderian gland, the porphyrin synthesis and its control within it. However, the function of the Harderian gland remains elusive and is a subject of intense debate. Many different functions have been suggested by various researchers. Sakai in 1981 suggested that these porphyrins functioned as a lubricant for the cornea of the eye as well as maintaining the moisture in the nasal cavity. Other studies, however, linked these porphyrins to a photoreceptive function due to the photoactive nature of these molecules which may play a role in modifying light energy in nocturnal animals (Wetterberg et al, 1970). Wetterberg observed that since the mammalian retina face the Harderian gland, it might serve as a reflector and transducer of light energy. Similarly Hugo and co-workers in 1987 observed an increase in excretion of porphyrin from the Harderian gland of rodents adapted to
living in the dark which are suddenly exposed to light. Thus the porphyrins may also play a role as a ‘light filter’ (Hugo et al, 1987). However, it has also been suggested that the porphyrins may be increasing the amount of visible light reaching the retina by shifting the wavelength of ultraviolet light to visible red thereby aiding nocturnal animals (Wetterberg et al, 1970).

The Harderian gland also secretes lipids (Kennedy, 1970; Seyama et al, 1992). The type of lipids secreted are species specific and the major components include fatty acid alkyl ester (wax) in the rat, 2-O-acylhydroxy fatty acid alkyl ester in the rabbit and 1-alkyl-2,3-diacylglycerol in the guinea pig, mouse and golden hamster. The functions of the lipids are proposed as possible lubricants but they also may act as solvents for pheromones or other biologically active substances (Seyama et al, 1992). The pheromonal role of the Harderian gland secretions has been proposed by work carried out by Theissen and co-workers in 1976 and Payne in 1977 and 1979. The observation that while grooming the Harderian gland secretions are picked up and spread onto the pelage thereby insulating the animal against cold and wetness also suggests the role of these lipids to be of a thermoregulatory nature (Theissen, 1988). Moreover the porphyrins present in the secretions darken the pelage and increase radiant absorption thus increasing the body temperature. The involvement of the Harderian gland in the immune response has also been indicated. Investigation by Burns in 1979 showed that on excision of the Harderian gland in domestic fowl, there was an increase in the excretion of the, closely associated, lacrimal gland. The increased secretory activity of the lacrimal gland was seen to be due to the increased number of type A goblet cells. Furthermore, immunisation with BSA resulted in anti-BSA activity in the Harderian gland in the intact birds with an absence of any activity
in the lacrimal gland. However, when fowls without the Harderian gland were
immunised with BSA, lacrimal gland became immuno-competent. These findings
seem to be in agreement with the previous suggestion that the Harderian gland acts as
a special accessory lymphoid organ under whose control IgM synthesis takes place
(Bryant et al. 1973).

Although the Harderian gland appears to have numerous and varied functions
nevertheless, due to the similarity of the haem biosynthetic pathway within the
Harderian gland and reticulocytes or liver cells in humans, this gland has been
extensively used as a model to study this pathway, its controlling mechanisms and the
possible reasons that lead to the dysfunction of this pathway. Nature therefore, seems
to have provided us with a readymade organ, which mimics the disorders that some
humans are unfortunate enough to be afflicted with, so it can be studied and a possible
cure found.

1.6 Analysis of porphyrins

A variety of techniques have previously been employed for the analysis of
porphyrins. The common techniques include thin-layer chromatography (TLC)
(Henderson, 1989), high performance liquid chromatography (HPLC) (Lim et al,
1988) and spectrophotometry (Jackson, 1977).

Mass spectrometry is also a widely used technique for the characterisation of
porphyrins (Guo et al. 1989; Luo et al, 1995). Although most analysis of porphyrins
is carried out on their methyl esters (Luo et al. 1997), porphyrins with few carboxyl
groups (between 2 to 4) can usually be analysed without the need for derivatisation.
The techniques used in the current study were HPLC, on-line HPLC-ESI/MS, on-line
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HPLC-ESI/MS/MS, MALDI-TOFMS and capillary electrophoresis (CE) for the identification and characterisation of protoporphyrin and related compounds. The reason for the choice of techniques is outlined below.

1.6.1 **HPLC**

In general HPLC, with ultraviolet (UV) detection, is possibly the most widely used technique in scientific research but as far as the detection of adducts are concerned the use of HPLC (with UV detection) has been limited. The reason for this is the lower sensitivity and the lack of structural information of the adduct that is obtained from it. Nevertheless, this disadvantage is more than compensated for by the adaptability of the technique to the analysis of almost any compound from a single amino acid (Rizzo *et al.* 1996) to large proteins (DeFrutos *et al.*, 1997), the ease of operation, the potential for automation and the greater resolution of adducts (Pfau and Phillips, 1991). Furthermore the combination of HPLC with better and more sensitive detection systems, such as electrochemical detection (Costa *et al.*, 1997) and mass spectrometry (Cadet *et al.*, 1993; Glover *et al.*, 1995; Phillips *et al.*, 1994) means that the lack of sensitivity and the availability of structural information can be overcome and hence this technique is still widely used (Phillips *et al.*, 1994; Jones *et al.*, 1996).

1.6.2 **On-line HPLC-ESI/MS**

Liquid chromatography - Mass Spectrometry (LC-MS) is generally a combination of HPLC and mass spectrometry. These are combined in such a way that mass spectrometry is used on-line as a detection system for the HPLC. In this case either the UV/ECD detection system is eliminated altogether (Lim *et al.*, 1994; Jones *et al.*, 1996; Jones *et al.*, 1996) or used in parallel with mass spectrometry.
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This on-line combination was only possible by the advent of a relatively new technique in mass spectrometry called the Electrospray Ionisation Mass Spectrometry (ESI-MS). In ESI-MS the direct introduction of the solvent into the mass spectrometer is possible, as opposed to GC-MS where the solvent has to be volatilised first and is introduced into the mass spectrometer in a gaseous state.

The obvious advantages of this technique is that large proteins which are difficult to evaporate, or compounds which may degrade at high temperatures used in GC-MS, may easily be analysed.

Porphyrins have been analysed using a variety of mass spectrometric methods such as fast atom bombardment (FAB) (Kurlansik et al, 1983; Naylor et al, 1990; Naylor et al. 1992), electron impact (EI) (Jackson et al. 1965; Shaw et al, 1978), chemical ionisation (CI) (Evershed et al, 1985; Tolf et al. 1986; Berkel et al. 1989; Berkel et al. 1990), laser desorption (Forest et al. 1989; Dale et al. 1996), field desorption (Schronk et al. 1982), plasma desorption (Hunt et al. 1981; Chait et al. 1984), thermospray (Blakley and Vestal. 1983) and atmospheric pressure chemical ionisation (APCI) (Mele et al. 1996). However, the most widely used mass spectrometric technique in the various analytical and research laboratories is the ESI-MS (Berkel et al. 1991; Berkel et al. 1993). This technique is one of the generally labelled ‘soft’ ionisation techniques. This is due to the fact that whereas in other MS techniques, such as EI for example, the compound of interest is bombarded with electrons to fragment it and hence elucidate its structure. In ESI the ionisation of the compound of interest takes place in a different fashion. The ionisation takes place by passing the compound of interest, in solution, through a stainless steel capillary held at a high potential, typically 3-5 kV. The electric field generated by this potential causes
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The solution to spray out from the end of the capillary. Pneumatic pressure is sometimes applied to aid this process. The aerosol thus formed contains highly charged droplets containing the ions of interest. Due to the coulombic repulsion the charge is spread evenly on the surface of the droplet. Nitrogen, or sometimes air, is blown across the skimmer thus evaporating these droplets and causing the charge to surface ratio to increase. Eventually the charge to surface ratio becomes great enough to either eject the charged ions or the droplet explodes into smaller droplets. These droplets are further evaporated until all the ions are released. The ions are then passed through the cone and various skimmers to the quadrupoles, through an increasing vacuum, where they are analysed according to their mass to charge ratio. A schematic of a typical ESI source and droplet formation is shown in Figure 1.6 and 1.7 respectively.

LC-MS provides details of the molecular weight of the compound in question. It may also give some structural information due to in-source fragmentation. However, in order to determine the structure of any unknown compound, tandem MS (MS/MS) is normally utilised. In tandem MS, precursor ion (normally the molecular ion of the compound of interest) is allowed to fragment by bombarding it with an inert gas, usually argon, in the source ionisation chamber. This produces fragments of the molecular ion which can then be studied to determine the structure of the original compound. Recent advances have enabled MS^n to be carried out and have enable fifth generation, or even later, product ions to be studied and these are now routinely carried out in research laboratories.
Fig. 1.6 Schematic of a typical electrospray ion source
(With kind permission from Mr John Lamb, MRC, Leicester, UK)
Fig. 1.7  Droplet production and release of sample ions in an ESI source

Parent droplet ($r \sim 1\mu m$)

Offspring droplets ($r \sim 100$nm)

Local high electric fields

Distortion of the droplet due to shear forces caused by the flight through dense gas

Solvent evaporation

Offspring droplets ($r \sim 10$nm)

Release of sample ions
1.6.3 Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOFMS)

Although relatively new, MALDI-TOFMS has become the preferred technique for the analysis of large macromolecules (ca. ~400 kDa) since its introduction in 1988 (Tanaka et al. 1988; Danis and Karr, 1995). The popularity of this method is due to its increased sensitivity and versatility in detecting large as well as small peptides and the analysis of molecules of biological as well as synthetic origin (Danis et al. 1992). MALDI ionization is a soft ionization method and hence minimal fragmentation occurs. The great importance of this technique is also due to its ability to yield singly charged large peptides, and therefore ions of structural significance unlike other ionization techniques, where multiply charged ions are formed.

In the MALDI system the sample is dissolved in a highly UV absorbing matrix of a low molecular weight, such as alpha-cyano-4-hydroxycinamic acid, upon which a pulsed laser radiation is delivered. The role of the matrix, which is usually in excess, is to facilitate intact desorption and ionization of the sample by transfer of the absorbed energy from itself to the sample thus generating small to very heavy gas phase ions (Figure 1.8a).

The analysis of the ions is carried out by time-of-flight mass spectrometry in which the ions are accelerated towards the detector by applying a potential that causes the ions to drift. The travelling ions are separated according to their mass-to-charge ratios with the lighter ions drifting more quickly and heavier ions drifting more slowly towards the detector (Figure 1.8b). A calibration reference standard of a known mass is used in order to establish an accurate relationship between time of flight and the
mass-to-charge ratio of the ion. Dispersions in the initial position, time and velocity of the ion populations which severely affects the resolving power of the technique can be overcome by employing a delayed extraction procedure (Guilhaus, 1997). Delayed extraction procedure involves the formation of ions in a field-free region followed by the application of a high voltage pulse after a predetermined time delay to accelerate the ions, thereby minimising the energy spread of the ions formed and consequently increasing resolution. Delayed extraction of the ions also yields reduced fragmentation and background noise due to a reduction in the neutral plume initially created by the laser pulse.

1.6.4 Capillary Electrophoresis

The area of Capillary Electrophoresis (CE) is relatively modern and has been an important addition to the analytical techniques available to the scientist. It allows very short run times with efficient separation of many compounds such as flavonoids, sulphonamides, tamoxifen, porphyrins and propolis components (Ng et al., 1993; Ng et al., 1994; Chi et al., 1994; Wu et al., 1993; Barker et al., 1993). The principle by which CE works, although simple, is very different to the techniques mentioned above.

In CE, the separation of solute components is achieved by their interaction with the wall of a narrow bore silica capillary (25-200µm I.D.) while travelling along it. Two ionic buffer reservoirs are placed at both ends of a fused silica capillary tube. After the injection of a small volume of sample, typically nanolitres, a high voltage is applied across the capillary. The voltage is applied by having a probe inserted into the buffers at either end, with one end being the anode and the other cathode (Figure 1.9a). The sample injection is carried out by applying a vacuum or voltage for a short
Fig. 1.8a Matrix-assisted laser desorption ionisation (MALDI) of sample ions

Fig. 1.8b Schematics of a MALDI-TOFMS system
Fig. 1.9(a)  Schematics of a basic CE system

Fig. 1.9(b)  Formation of the double layer on capillary wall

Fig. 1.9(c)  Electro-osmotic flow (EOF) in the capillary

Key: $X^+$ = Analyte
$H^+$ = Buffer Cations
Si-o = Silanol groups on capillary wall
period of time, with the capillary end placed in the sample vial.

The fused silica capillary contains silanol groups on its interior surface, which ionise in the buffer solution and form a double layer (Figure 1.9b) with buffer cations. These ions will migrate to the negatively charged electrode when a voltage is applied across the capillary and hence will produce a flow of liquid along the capillary termed electro-osmotic flow (Figure 1.9c). The electro-osmotic flow along with their charge causes the analytes to migrate. The analytes, depending on their size and charge, will migrate at different rates and hence will be separated as they travel along the capillary (Figure 1.9c).

The voltages applied across the capillary are in the order of 10-30KV. Generally these voltages would cause high temperatures due to Joule heating, however the large surface area of the capillary dissipates heat efficiently and therefore such problems are eliminated (Huang et al. 1989). The high voltages used allow rapid, high resolution separations with the number of theoretical plates being in the order of 100000 to 200000.

Within CE, a variety of different separation methods can be utilised, namely, capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC) (Tsuda. 1987, Ng et al. 1993), iso-electric focusing (IEF) and capillary gel electrophoresis (CGE). This variation makes CE an extremely adaptable and useful analytical technique however, the most commonly used forms are the CZE and MECC.

CZE, the simplest technique, is the separation of analytes using buffers such as potassium phosphate, sodium phosphate and citric acid etc. However, if an ionic
surfactant is added to the buffer, such as sodium dodecylsulphate (SDS), the separation technique of the analytes differs slightly. In order to achieve this the concentration of the surfactant added has to be above its critical micellar concentration. This separation technique is called Micellar Electrokinetic Capillary Chromatography (MECC).

In MECC the analytes are separated due to the combination of two distinct mechanisms, the separation due to EOF and the separation due to the partitioning of the analyte between the free solution and the micelles. The surfactant ions as well as taking part in micelles also adsorb onto the capillary hence taking part in the double layer formation. The addition of the surfactant, therefore, can also modify the speed, or indeed the direction of EOF (Tsuda, 1987).

1.7 Aims of the study

The aims of the study are as follows:

To develop HPLC, CE and HPLC-MS/MS methods for the isolation and characterization of protoporphyrin glycoconjugates in the rat Harderian gland.

To investigate whether protoporphyrin glycoconjugates are also present in human porphyrias, particularly erythropoeitic protoporphyria.

To study the mechanism of formation of protoporphyrin glycoconjugates.

To investigate the reactivity and ability of porphyrins and porphyrinogens to form amino acid-, peptide-, and protein-conjugates or adducts.
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Isolation and characterisation of protoporphyrin glycoconjugates from rat Harderian glands

2.1 Introduction

The Harderian gland is a large bi-lobed alveolar gland situated around the posterior half of the eyeball occupying a considerable part of the orbit (Figure 2.1). It is found in most vertebrates and especially in the rodents (Burns, 1992; Kuhnel, 1992). It is usually associated with the third eyelid in the inner canthus of the eye. The function of the Harderian gland is not clear (Djeridane, 1994) and various propositions have been made including cornea lubrication (Sakai, 1981), photoreception (Pevet et al., 1984), production of hormones (Johnston, 1986; Payne, 1979), a source of thermoregulatory lipids (Thiessen, 1988; Thiessen and Kittrell, 1980) and a site of immune response (Montgomery and Maslin, 1992; Burns, 1979).

The Harderian gland in the 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 (Kennedy, 1970; Shirama et al., 1981). The major porphyrin component in rodents was reported to be protoporphyrin, with much smaller amounts of harderoporphyrin and other porphyrins (Spike et al., 1990; Johnston et al., 1985). In the rat, the porphyrins present in the adult are in the μg/mg wet weight range (Cardalda et al., 1997).
Fig. 2.1 Location of the Harderian gland in the rodent eye 
(Thiessen, 1992) 
(Dark Area around the eye = Harderian gland)
High-performance liquid chromatography (HPLC) has been widely used for the separation of porphyrins (Lim et al., 1988). Mass spectrometry (MS), especially positive ion liquid secondary ion mass spectrometry (LSIMS), on the other hand, has been an important method for the characterization of porphyrins (Guo et al., 1989; Luo and Lim, 1995). 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 (Luo et al., 1997). Sufficient sensitivity, however, could be obtained for porphyrins with fewer number of carboxyl groups, such as coproporphyrin (4 carboxyl groups) and protoporphyrin (2 carboxyl groups) without esterification. The development of electrospray ionization mass spectrometry (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 chapter describes the isolation and characterization of a group of hitherto unreported glycoconjugates of protoporphyrin from the rat Harderian gland, using high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and high performance liquid chromatography – electrospray ionisation mass spectrometry (HPLC/ESI-MS).

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

2.2 EXPERIMENTAL

2.2.1 Materials and Reagents

Acetic anhydride (99+%), anhydrous pyridine, 3-methyl-1-phenyl-2-
pyrazolin-5-one (MPP), di-sodium tetraborate, protoporphyrin IX and all monosaccharides were purchased from Sigma Chemical Co. (Dorset, UK.).

Acetonitrile (CH$_3$CN, HPLC grade), methanol (MeOH, HPLC grade), dimethyl sulphoxide (DMSO, analytical reagent grade), toluene (ACS reagent), sodium hydroxide, potassium hydroxide (analytical reagent grade), ethylenediaminetetraacetic acid (EDTA, disodium salt), Ascorbic acid (free acid) and concentrated HCl (S.G. 1.18, analytical reagent grade) were all obtained from Fisher Scientific, Loughborough, UK. Anhydrous trifluoroacetic acid (TFA, protein sequencing grade) was obtained from Pierce and Warriner (Chester, UK.). Bond elute C18 solid phase extraction cartridges were form Jones Chromatography, Hengoed, U.K.

A 0.1% TFA solution was made by dissolving 1ml ampule in 1 litre of distilled water which was used directly or further diluted as required. All working solutions were sparged with helium for 1 minute before use. A 2% (w/v) potassium hydroxide solution in methanol was made by dissolving 2g of potassium hydroxide in 100ml of Methanol.

The Harderian glands were obtained by dissection from freshly sacrificed Wistar-Han rats (180 - 500g) and stored at -20°C until analysis.

2.2.2 Extraction of porphyrins from Harderian gland

The porphyrins and their conjugates were extracted by homogenizing the Harderian gland (250-300mg) with 6 x 1ml 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 (15000g for 5 min) and the supernatant used for HPLC
separation and isolation of the porphyrins.

2.2.3 *HPLC of Harderian gland glycoconjugates*

All HPLC analysis and separations were carried out on a Varian Model 9012 liquid chromatograph (Walton-on-Thames, Surrey, UK). A Varian Model 9050 UV/Vis detector set at 405nm was used. The separation was achieved on a Hypersil BDS C18 column (5μm, 4.6 x 250mm) from Shandon Scientific, Runcorn, Cheshire, UK. The Harderian gland extracts in acetonitrile/DMSO (3:1 v/v) were injected onto the column via a Rheodyne 7125 injector (Cotati, CA, USA) fitted with a 200μl loop.

2.2.3.1 Optimisation of HPLC for glycoconjugate analysis

2.2.3.1.1 Use of Ammonium acetate buffer for glycoconjugate separation

The glycoconjugates were analysed using a 0.01M ammonium acetate buffer as the aqueous mobile phase with acetonitrile as the organic phase. The ammonium acetate buffer of pH 4.0, 5.0 and 6.0 was used in order to try and resolve the glycoconjugates. Analysis with a 0.1M ammonium acetate buffer was also attempted.

2.2.3.1.2 Use of TFA solvent for glycoconjugate separation

An aqueous mobile phase of 0.1, 0.05, 0.01 or 0.001% TFA in water (v/v) was used as the aqueous phase for the analysis of the glycoconjugates with acetonitrile as the organic phase. The stock solution of 0.1% TFA (v/v) was made by the addition of a 1ml ampule in 1 litre of distilled water which was further diluted with water to obtain the required TFA solution.

Various acetonitrile compositions were also tested for optimum sensitivity and
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resolution.

2.2.3.2 Quantification of Harderian gland glycoconjugates by HPLC

Protoporphyrin standards were prepared from stock solution of protoporphyrin dissolved in acetonitrile / DMSO (3:1 v/v). Consecutive dilutions were carried out to obtain standards of between 0.1 and 100μM. 50μl of each standard was injected onto the column. The calibration curve obtained was used to quantify the Harderian gland glycoconjugates.

2.2.3.3 Isolation of the Harderian gland glycoconjugates

The Harderian gland glycoconjugates were isolated using two different HPLC systems. The first was with a Hypersil BDS C18 column (5μm, 4.6 x 250mm) with 0.05% TFA (v/v) (Solvent A) and acetonitrile (Solvent B) as the mobile phase and the following gradient elution program: 65%B from 0 to 30 minutes, 65%B to 90%B from 30 to 35 minutes and isocratic at 90%B for a further 10 minutes. The individual peaks were collected in 50ml Starstedt plastic tubes and combined in a 500ml glass bottle which was then frozen at -20°C until purification.

A Pye-Unicam PU 4010 pump (Cambridge, UK) attached to a semi-preparative Hypersil BDS C18 column (5μm, 10 x 250mm, Hypersil, Runcorn, Cheshire, UK) was also used for the isolation of the glycoconjugates. A Linear UVVis 204 detector (Nevada USA) set at 405nm was used as the detection system. The results were recorded on a Hewlett Packard HP3394A (Hewlett Packard, Wokingham, Surrey, U.K.) chart recorder. 500μl of the Harderian gland extracts in acetonitrile/DMSO (3:1 v/v) were injected onto the column via a Rheodyne 7125 injector (Cotati, CA, USA) fitted with a 1ml loop. The separated components were
collected in a 500ml glass bottle and stored at -20°C until purification by Solid-Phase extraction technique. The tubes and bottles containing the fractions were shielded from light at all times. The Harderian gland extract was kept at 4°C in the dark between each injection.

2.2.3.4 Solid-Phase extraction of the isolated Harderian gland fractions

The frozen Harderian gland extract fractions were allowed to thaw at room temperature and SPE carried out as follows. The organic solvent from the collected fractions was evaporated under nitrogen at room temperature. The remaining aqueous phase was diluted with 1M ammonium acetate buffer (pH 5.16, 1:9 v/v) and loaded onto a C18 SPE cartridge. The 20ml C18 Bond Elute cartridge was preconditioned with 20ml of acetonitrile followed by 40ml of 1M ammonium acetate buffer (pH 5.16). The loaded glycoconjugates were eluted using aliquots of 2ml of 0.1%TFA in acetonitrile at a time until no fluorescence was detectable on the cartridge under UV lamp. The eluents were pooled and evaporated to dryness under reduced pressure.

2.2.4 On-Line HPLC/ESI-MS and ESI-MS/MS of Harderian gland glycoconjugates

On-line LC-MS was carried out on a VG Quattro quadrupole instrument (Micromass Ltd., Altrincham, UK.) fitted with an atmospheric pressure ionisation electrospray source. The source temperature was kept at 120°C with the cone voltage of 90V. The capillary and HV electrode potentials were kept at 3.41 and 0.24kV, respectively. Nitrogen was used as the drying and nebulising gas at a flow rate of 300 and 40 L/h, respectively. Full-scan continuum data was acquired in the positive ion
mode over a mass range of 100-1000Da and processed using a VG Masslynx data system. The scanning rate was 1 scan per 3 seconds with 0.1 second interscan delay.

For LC-MS/MS acquisitions argon was used as the collision gas with the gas cell pressure of $1.3 \times 10^{-3}$ mBar and the collision energy of 70 eV. The product ions were scanned over a mass range of 200-750Da and the spectra were collected in the form of continuum data.

The solvent delivery system was a Varian Model 9012 liquid chromatograph. The separation was achieved with the HPLC system described in Section 2.2.3.3. The flow rate of 1ml/min was split (1:9) with 100µl being injected into the source.

Due to the incompatibility of DMSO with mass spectrometric analysis the Harderian gland extract was diluted with water (1:1 v/v) prior to injection.

### 2.2.4.1 Optimisation of LC-MS for glycoconjugate analysis

#### 2.2.4.1.1 Effect of Cone voltage on glycoconjugate ionisation and analysis

100µl aliquots of Harderian gland extract, diluted with water (1:1 v/v), were analysed by LC-MS with varying cone voltages from between 30 to 120V. The source temperature was kept constant at 120°C with the capillary and HV voltage kept at 3.41 and 0.24kV. All HPLC conditions were kept constant as described above. The acquired data was integrated with the VG Masslynx integration data software. A peak area against cone voltage curve was drawn for protoporphyrin and each conjugate.
2.2.4.1.2 Effect of Source temperature on glycoconjugate ionisation and analysis

100μl aliquots of Harderian gland extract, diluted with water (1:1 v/v), were analysed by LC-MS at various source temperatures from between 60 to 140°C. The cone voltage was set at 90V with all other conditions being kept constant as above. A graph of peak area against source temperature was drawn for protoporphyrin and each conjugate.

2.2.4.2 Quantification of Harderian gland glycoconjugates by on-line HPLC/ESI-MS

Quantification of the Harderian gland glycoconjugates by HPLC/ESI-MS was achieved from a calibration curve, obtained by the analysis of protoporphyrin standards (0.1-100μM), in a similar way as Section 2.2.3.2.

2.2.5 Glycoconjugate acetylation

Acetylation of the conjugates was carried out by a method described previously by Weber and Khorana in 1972. Anhydrous pyridine (1ml) and acetic anhydride (200μl) were added to the purified dried glycoconjugate placed in a 5ml dupont glass tube wrapped with aluminium foil. The mixture was vortex mixed until all the glycoconjugate had dissolved and left overnight at room temperature in the dark.

The acetylated product was isolated by evaporating the reaction mixture to complete dryness under reduced pressure. Any residual drop of pyridine left was removed by the addition of 1ml of toluene followed by further evaporation. The acetylated product was either, reconstituted in 150μl of CH₃CN and analysed
immediately by on-line HPLC/ESI-MS, or stored at -20°C until analysis.

2.2.6 Spectrophotometric analysis of glycoconjugates

2.2.6.1 UV/Vis Spectrophotometry

A Perkin-Elmer Lambda 2S UV/Vis spectrophotometer (Perkin-Elmer Ltd, Buckinghamshire, U.K.) with an IBM compatible 486 PC was used to record the UV-Visible spectra of the porphyrins. The spectra were processed using Perkin-Elmer Lambda2 software. The spectra of the porphyrins in acetonitrile/DMSO (3:1 v/v) in a 1ml glass cuvette (1cm path length) were recorded from \( \lambda \) 300 to 800 nm. Acetonitrile/DMSO (3:1 v/v) was used as blank.

2.2.6.2 Fluorescence spectrophotometry

A Perkin-Elmer LS50B luminescence spectrofluorimeter (Norwalk, CT, USA) with the Perkin-Elmer Fluorescence Data Manager software on an IBM compatible Pentium 75 computer was used for recording the fluorescence spectra of glycoconjugates in acetonitrile/DMSO (3:1 v/v). A 3ml quartz cuvette with a 1cm pathlength was used. The excitation wavelength was 405nm with the excitation and emission slits being 5 and 10mm, respectively. The fluorescence spectra were recorded from \( \lambda \) 350 to 800 nm. An acetonitrile/DMSO (3:1 v/v) mixture was used as blank.

2.2.7 Trans-esterification of purified glycoconjugates

The purified and dried HPLC glycoconjugate fraction, in a 5ml glass dupont tube wrapped in aluminium foil, was dissolved in 1.1ml of methanol/DMSO mixture (4:1 v/v). 100\( \mu \)l was immediately analysed by HPLC to check for the degradation of
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the conjugate. To the remaining 1ml of dissolved fraction 2mg of ascorbic acid and a trace of EDTA (disodium salt) was added and vortex mixed for 10 seconds. A 2% (w/v) methanolic potassium hydroxide (1ml) was added to the reaction mixture. The reaction was carried out by vortex mixing for 1 minute. The alkaline reaction mixture was neutralised with 300μl of HCl (2.7M) followed by centrifugation at 1200g for 5 minutes. The supernatant was separated and analysed by HPLC and HPLC/ESI-MS.

2.2.8 Partial Esterification of Protoporphyrin

Protoporphyrin IX was partially esterified by the addition of 100μl of 1% (v/v) sulphuric acid in methanol to three 5ml glass Dupont tubes each containing 1.3mg of protoporphyrin IX standard. The resultant mixture was whirly mixed and incubated in the dark at 4°C. The protoporphyrin esters in the incubation mixtures were extracted in chloroform after 1, 2 and 4.5 hours as follows.

The mixture was transferred into a separating funnel and 2ml of chloroform added. A further 2ml of methanol and 2ml of water was also added in order to aid separation of the organic and aqueous layer. The esterified protoporphyrin was extracted into chloroform by mixing vigorously in the separating funnel. The chloroform layer was collected and a further 2ml of chloroform added to the separating funnel. The mixture was again vigorously mixed and the chloroform layer separated. The chloroform extract was combined and subsequently washed with 5ml of saturated hydrogen carbonate followed by 5ml of water. The chloroform was dried under nitrogen at room temperature.

The dried protoporphyrin esters were reconstituted in 1ml of 1% (v/v) DMSO in acetonitrile by whirly mixing followed by centrifugation at 1200g for 5 minutes.
The supernatant was separated and analysed.

2.2.9 Nuclear Magnetic Resonance (NMR) spectroscopy of glycoconjugates

Approximately 3.5mg of the purified dried fractions were dissolved in 0.5ml of DMSO and transferred into Wilmad 5mm NMR tubes (Coventry, UK). These were then analysed by NMR spectroscopy with a Bruker 400MHz spectrometer (Coventry, UK.) over 12 hours.

NMR was also carried out on acetylated glycoconjugates which were dissolved in 0.5ml redistilled chloroform.

2.2.10 Capillary electrophoresis of Harderian gland glycoconjugates

CE analyses were carried out on an Applied Biosystems Model 270A Capillary Electrophoresis System (Applied Biosystems, Cheshire, U.K.) with UV/Vis absorption detection employing a standard deuterium tungsten lamp. A fused silica capillary (50μm inner diameter, 72cm total length, 53cm effective length; Composite Metal Services, Hallow, Worcs., U.K.) was used. The samples were loaded on to the capillary by vacuum injection for 3 seconds. The capillary was washed in between each run with 0.1M sodium hydroxide for 3 minutes and then conditioned with the running buffer for 1 minutes. All other conditions, including buffers, capillary voltage and temperature were as described below in the relevant section.

2.2.10.1 Reproducibility of CE

Methylglyoxal-bis(Guanyl)hydrazone (MGBG) was used as a test compound in order to determine the reproducibility of the capillary and the performance of the CE system in general. MGBG (0.5mg) was dissolved in 1ml of 200mM Sodium
dihydrogen orthophosphate (pH 4.4) running buffer. The MGBG solution was
filtered using a 0.2μm Watman filter and vacuum injected onto the capillary for 3
seconds. The capillary voltage and temperature was set to 15kV and 30°C,
respectively. The UV detection wavelength was set to 283nm.

2.2.10.2 Preparation of Monosaccharide-MPP derivatives

The glycoconjugates were vortex-mixed with methanol (50μl) and 0.3M
potassium hydroxide (50μl) for 1 minute in order to release the sugar from
protoporphyrin. The liberated monosaccharides and the monosaccharide standards
were then derivatised as described previously by Honda et al. (1989, 1991). Briefly,
the monosaccharides were incubated in 0.5M methanolic MPP (50μl) and 0.3M
potassium hydroxide (50μl) at 70° for 30 minutes. The reaction mixture was allowed
to cool to room temperature and neutralised with 0.3M HCl (50μl). The resultant
mixture was evaporated to dryness under reduced pressure. The residue was then
dissolved in chloroform (200μl) and water (200μl) and vortex-mixed. The aqueous
layer was removed and evaporated to dryness under reduced pressure. The
monosaccharide-MPP derivatives were reconstituted in water (200μl) and analysed by
capillary electrophoresis and on-line HPLC/ESI-MS.

2.2.10.3 Capillary electrophoresis of Monosaccharide-MPP derivatives

The separation was carried out using di-sodium tetraborate buffer (100mM,
ph9.5) as the running buffer with the voltage and temperature of the capillary kept at
20kV and 30°C, respectively. The samples were loaded on to the capillary by
vacuum injection for 3 seconds. The capillary was washed in between each run with
0.1M sodium hydroxide for 3 minutes and conditioned with the running buffer for 1
minutes. The results were recorded on a Hewlett packard HP3394A chart recorder (Hewlett-Packard Company, Wokingham, Surrey, UK.).

2.2.10.4 HPLC and on-line HPLC/ESI-MS of Monosaccharide-MPP derivatives

HPLC separation of monosaccharide-MPP derivatives was carried out on a Varian Model 9012 liquid chromatograph (Walton-on-Thames, Surrey, UK.) with a Varian Model 9050 UV/Vis detector set to 245nm as the detection system. A Hypersil BDS C_{18} column (5μm, 4.6 x 250mm, Hypersil, Runcorn, Cheshire, UK.) with 0.1M ammonium acetate (pH 5.15) and acetonitrile as the mobile phase at a flow rate of 1ml/min was used. The monosaccharide-MPP derivatives were eluted by increasing the acetonitrile from 20% to 50% (v/v) in 20 minutes followed by 50% to 90% in 5 minutes and kept at 90% for a further 10 minutes. The samples were injected onto the column via a Rheodyne 7125 injector (Cotati, CA, USA) fitted with a 200μl loop.

For HPLC/ESI-MS of the derivatives the eluent from the column was split in the ratio 1:9 and directly fed into the ESI-MS source. All HPLC/ESI-MS conditions were as described in Section 2.2.4 except the cone voltage was set to 25V and the full-scan continuum data was acquired over a mass range of 50-800Da.

2.2.11 Stability of Harderian gland glycoconjugates

The stability of the glycoconjugates was checked in both organic and aqueous solvents and compared to the stability of protoporphyrin. The glycoconjugates were extracted in acetonitrile/DMSO mixture (3:1 v/v) from the Harderian gland as described in Section 2.2.2. The extract was stored at -20°C until analysis by HPLC. Immediately prior to HPLC analysis the entire extract was vortex mixed for approximately 10 seconds. A 200μl aliquot was transferred to a 1.5ml eppendorf and
centrifuged at 15000g for 3 minutes. The supernatant was separated and 100μl injected onto the column.

Stability check of the glycoconjugates in aqueous media was carried out by dissolving the purified dried fractions in a 0.9M HCl solution and storing them at −20°C until analysis. When required, the solution was allowed to thaw at room temperature and vortex mixed for 10 seconds. A 50μl aliquot was transferred into a 1.5ml eppendorf to which 50μl of 0.9M sodium hydroxide and 100μl acetonitrile/DMSO (3:1 v/v) were added. The resultant solution was vortex mixed for 10 seconds and 180μl injected onto the column.

The HPLC conditions used for the stability study were as described in Section 2.2.3. with the UV/Vis detection at 405nm.

2.2.12 Solubility of Harderian gland glycoconjugates

The solubility of glycoconjugates in organic and aqueous solvents was tested in two ways. Firstly, by extraction from the Harderian gland with either a pure organic solvent or an organic/aqueous mixture. The Harderian gland was homogenised in 2ml of acetonitrile/DMSO mixture (3:1 v/v) and divided into two 1ml aliquots in 5ml Dupont glass tubes. 1ml of water was further added to one aliquot and 1ml acetonitrile/DMSO mixture (3:1 v/v) was added to the other. Both homogenates were vortex mixed for 2 minutes and then centrifuged at 1200g for 10 minutes. The supernatants were separated and 50μl injected onto the column for analysis.

A spectrophotometric method for the determination of the solubility of the glycoconjugates was also used. A quantity of the purified dried conjugate or protoporphyrin was initially dissolved in 400μl water followed by sonication for 5
minutes. A set volume of acetonitrile was added to the solution followed by vortex mixing (10 sec) and sonication for 2 minutes. The mixture was then vortex mixed for 10 seconds, transferred into a 1ml glass cuvette (1cm pathlength) and the corresponding absorbance measured. The procedure was repeated and a solubility curve of absorbance against percentage organic obtained.

A solubility curve of absorbance against HCl concentration was also obtained in a similar fashion. A 2M HCl solution was added stepwise to the water containing either the conjugate or the protoporphyrin.

The absorbance for the above experiment was measured using a Perkin-Elmer 2S UV/Vis spectrophotometer (Perkin-Elmer Ltd, Buckinghamshire, UK.) set at 405nm.

2.3 RESULTS AND DISCUSSION

2.3.1 Extraction of porphyrins from Harderian gland

The most commonly used method for the extraction of porphyrins from Harderian gland was to convert the porphyrins into their corresponding methyl esters with methanolic sulphuric acid followed by extraction into chloroform or dichloromethane (Kennedy, 1970; Spike et al, 1990). This procedure led to the loss of the conjugated group by transmethylation.

Extraction with a mixture of acetic acid / ethyl acetate followed by methyl esterification (Day et al, 1978) and extraction with 1.5M (Rodriguez et al, 1992; Buzzell et al, 1991) or 3M HCl (Brun and Sandberg, 1985) could also lead to deconjugation. It is therefore not surprising that the protoporphyrin glycoconjugates had been missed in all previous analyses.
The acetonitrile / DMSO (3:1 v/v) mixture, used in the present study, effectively and quantitatively extracted porphyrins from the Harderian gland without degradation and deconjugation. The methanol / DMSO (4:1 v/v) mixture for the extraction of porphyrins in tissues should also be avoided. Partial transmethylation was observed when this mixture was used for the extraction of the porphyrins in the Harderian gland.

2.3.2 **HPLC mobile phase**

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 2.2.

2.3.2.1 Optimisation of glycoconjugate analysis

The effect of pH of the aqueous phase on the resolution of the glycoconjugates and their isomers was investigated by using different concentrations of TFA in water. The concentrations of TFA tested were 0.1% (v/v, pH 1.9), 0.05% (v/v, pH 2.2), 0.01% (v/v, pH 2.8) and 0.001% (v/v, pH 3.8). The optimum TFA concentration was found to be 0.05% with the corresponding pH of 2.2.
Fig. 2.2 HPLC separation of porphyrins in Harderian gland extract

Peaks 1 - 5 = Protoporphyrin glycoconjugates
Peak 6 = Protoporphyrin IX
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The glycoconjugate analysis and separation was also optimised by isocratic elution using various amounts of organic phase. The concentration of acetonitrile (organic phase) tested ranged from 55% to 70% (v/v). The optimum acetonitrile concentration was found to be 65% (v/v).

It was found that in comparison to the ammonium acetate system, the use of TFA system gave a much better resolution and therefore this system was used.

2.3.2.2 Quantification of Harderian gland glycoconjugates by HPLC

Total porphyrins in the rat Harderian gland have been reported to be in the μg/mg wet weight range (Cardalda et al., 1997; Tomio and Grinstein, 1968) from which 87% is reported to be protoporphyrin (Tomio and Grinstein, 1968). Although we failed to detect any coproporphyrin or harderoporphyrin however, quantification by HPLC has determined the average protoporphyrin amount in Harderian glands with an average weight of 210mg (n=5) to be 80.2μg, corresponding to 0.38μg/mg wet weight. These findings are therefore, in agreement with the previous reported results. However, of the 80.2μg of protoporphyrin present, 53% was in the form of glycoconjugates.

2.3.3 LC-MS and LC-MS/MS of glycoconjugates

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 minutes (Figure 2.3a, peak 3) was confirmed to be the MH⁺ ion of free acid protoporphyrin by comparison with authentic standard. Peaks 1 and 2 (Figure 2.3a) were product ions of the two new major porphyrin metabolites detected at the retention times of 12.5 minutes (Figure 2.3c, peak 8) and 17.5 minutes (Figure 2.3b.
peak 6), respectively. These two more polar metabolites produced MH$^+$ ions at m/z 725 and m/z 695, respectively, and by in-source collision-induced dissociation gave the corresponding product ions at m/z 563 (Figure 2.3a, 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 of the possible conjugate (17.5 min) was a product ion of the MH$^+$ 695 ion (Figure 2.4a).

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 (Figure 2.4b) to be a product ion of the MH$^+$ 725 ion. Peaks 4 and 5 (Figure 2.3b) also gave a MH$^+$ ion at m/z 695. These minor components are isomers of peak 6 (Figure 2.3b) and will be discussed further in the later section.

### 2.3.3.1 Cone voltage and source temperature optimisation of glycoconjugate analysis

Figure 2.5 shows the results obtained for the optimum cone voltage for the analysis of glycoconjugates. The optimum cone voltage for protoporphyrin is shown to be 80V. Due to a very stable structural configuration of protoporphyrin, a high cone voltage needed to ionise protoporphyrin is not unusual. Similarly high cone voltages are required for all haem related compounds. This stability is also observed in the difficulty involved in the fragmentation of protoporphyrin by tandem mass spectrometry in which only the loss of side groups is observed.

The optimum voltage for the glycoconjugates varied from 60V for the major
Fig. 2.3 On-line HPLC/ESI-MS chromatograms of porphyrins in the Harderian gland extract (TIC = Total Ion Current)

(a) m/z 563

(b) m/z 695

(c) m/z 725

(d) TIC

Time (min)
Fig. 2.4 ESI-MS/MS product ion spectra of protoporphyrin glycoconjugates

(a) Protoporphyrin-pentose conjugate (MH$^+$ 695)

(b) Protoporphyrin-hexose conjugate (MH$^+$ 725)
Fig. 2.5 Optimum cone voltage for on-line HPLC/ESI-MS analysis of protoporphyrin glycoconjugates
protoporphyrin-pentose glycoconjugate. The conjugation of sugars to protoporphyrin leads to relatively lower cone voltages required to ionise them. This is possibly due to the presence of numerous oxygen atoms on the sugar moiety that help to acquire and stabilise the positive charge.

Differences also exist in the optimum ionisation of the isomers of each conjugate. These probably indicate the relative stability of each isomer and its ability to stabilise the positive charge.

The optimum temperature for the ionisation of the protoporphyrin and glycoconjugates was found to be 120°C with only slight variations from the optimum ionisation (Figure 2.6). This temperature was therefore used for all analyses of the protoporphyrin and its glycoconjugates.

2.3.3.2 Quantification of Harderian gland glycoconjugates by LC-MS

Quantification of protoporphyrin present within the Harderian glands (190mg, n=5) by on-line LC-MS yielded 59.4μg, corresponding to a concentration of 0.31μg/mg wet weight, thus also in agreement with previously reported findings. For this particular set of glands studied 50.1% of the protoporphyrin present was in the form of glycoconjugates.

2.3.4 Site of Conjugation

2.3.4.1 Spectrophotometric analysis of glycoconjugates

There are two possible sites where protoporphyrin can form conjugates with monosaccharides. The first is by N-substitution and the second by forming β-
Fig. 2.6 Optimum source temperature for on-line HPLC/ESI-MS analysis of protoporphyrin glycoconjugates
Fig. 2.7 UV-Visible absorption spectra of (a), protoporphyrin IX standard and (b), protoporphyrin-pentose conjugate in CH$_3$CN/DMSO (3:1 v/v)
Fig. 2.8 Transmethylation of protoporphyrin glycoconjugate
glycosidic 1-O-acyl conjugates with the propionic acid groups.

It has been previously shown (Smith and Farmer, 1982) that N-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 2.7). N-substitution was therefore ruled out.

2.3.4.2 Partial esterification of protoporphyrin

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. The possibility that only one isomer was being made is extremely unlikely. It is much more probable that the two isomers obtained were not resolved. Structural considerations of the protoporphyrin show no likely preference for one isomer as opposed to the other and hence make the latter option much more likely. Furthermore, attempts to resolve the isomers by varying the chromatographic conditions were unsuccessful.

2.3.4.3 Transesterification of purified glycoconjugates

Treatment of the conjugates with methanolic KOH resulted in transmethylation in all cases. The resulting protoporphyrin monomethyl ester was analysed by HPLC and confirmed by HPLC/ESI-MS analysis. The HPLC results clearly showed a shift in retention time compared to the glycoconjugates, consistent with a more non-polar methyl ester (Figure 2.8). The result was confirmed by HPLC/ESI-MS which showed a MH⁺ ion at m/z 577, corresponding to the
replacement of the monosaccharide group with a methyl group (Figure 2.9).

2.3.4.4 Acetylation of purified glycoconjugates

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 acetylation method utilised is an extremely simple and efficient method of acetylating only the hydroxyl groups present and goes to 100% completion. The products were analysed by HPLC/ESI-MS.

The conjugate with the MH$^+$ ion at m/z 695 (Figure 2.3b, peak 6) gave a MH$^+$ ion at m/z 821, an increase in mass of 126Da (Figure 2.10a). This increase corresponds to the addition of three acetyl groups and strongly suggests conjugation of protoporphyrin with a pentose. The nature of the pentose, a ketopentose or an aldopentose, could not be distinguished by acetylation.

Similarly, the conjugate with the MH$^+$ ion at m/z 725 (Figure 2.3c, peak 8) gave a MH$^+$ ion at m/z 893, an increase in mass of 168Da (Figure 2.10b). This increase corresponds to the addition of four acetyl groups suggesting conjugation of protoporphyrin with a hexose.

2.3.5 Stability and solubility of glycoconjugates

The comparison of the stability of the protoporphyrin-xylose conjugate in acidic and organic solvents, showed it to degrade when stored at -20°C in acidic conditions, over six weeks (Figure 2.11). The glycoconjugate showed no observable degradation, however, when stored in organic media, such as the extraction solvent of acetonitrile/DMSO (3:1 v/v). The degradation of protoporphyrin-xylose in acidic
Fig. 2.9 On-line HPLC/ESI-MS chromatograms of transmethylated protoporphyrin-pentose conjugate

(a) Total ion chromatogram (TIC)

(b) Mass chromatogram of Protoporphyrin IX (m/z 563)

(c) Mass chromatogram of Protoporphyrin monomethyl ester (m/z 577)
Fig. 2.10 On-line HPLC/ESI-MS chromatograms of acetylated protoporphyrin glycoconjugates

(a) Acetylated protoporphyrin-pentose conjugate (m/z 821)

(b) Acetylated protoporphyrin-hexose conjugate (m/z 893)
Fig. 2.11 Stability of protoporphyrin-xylose glycoconjugate in acidic and organic media
Fig. 2.12a Solubility comparison of Protoporphyrin and Protoporphyrin-xylose glycoconjugate in HCl

![Graph showing solubility comparison of Protoporphyrin and Protoporphyrin-xylose glycoconjugate in HCl](image)

Fig. 2.12b Solubility comparison of Protoporphyrin and Protoporphyrin-xylose glycoconjugate in CH$_3$CN/DMSO (3:1 v/v)

![Graph showing solubility comparison of Protoporphyrin and Protoporphyrin-xylose glycoconjugate in CH$_3$CN/DMSO (3:1 v/v)](image)
conditions is explained later in this chapter.

The solubility of the glycoconjugate, compared to unconjugated protoporphyrin, is markedly increased in both the aqueous (acidic) and organic solvents as shown in Figure 2.12a and b, respectively. The increase in solubility is relatively higher in the aqueous solvent as compared to the organic solvent. This observation would be expected due to the addition of a very polar molecule i.e. a sugar, to the protoporphyrin. The increase in solubility may indeed be one of the reasons for their synthesis in the Harderian gland which is, like any other biological environment, aqueous in nature. This increase in solubility may well be employed as an aid in order to eliminate protoporphyrin from the gland.

2.3.6 NMR analysis of glycoconjugates

The most common technique used to confirm the overall structure of any unknown compound is Nuclear Magnetic Resonance spectroscopy. However, the analysis of the two major isolated and purified glycoconjugates i.e. protoporphyrin-xylose, and protoporphyrin-glucose yielded very ‘noisy’ spectra from which little information could be gleaned (Figure 2.13b and c). The unclear spectra obtained could be due to a number of factors for example, the low amount of the isolated glycoconjugates, the presence of a mixture of isomers of the glycoconjugates or the presence of contamination. The results were compared to the protoporphyrin standard (Figure 2.13a).

The NMR spectra nevertheless, did show some indications as to the nature of the compound. Two doublet resonances observed at 5.3ppm are indicative of a -CH group bonded (or close) to two oxygen atoms. This resonance may be obtained from
the sugar molecule of the glycoconjugate. Further resonances are observed at 3.8ppm and are consistent with -CHOH groups, also from the sugar moiety. These resonances are absent in the protoporphyrin spectrum (Figure 2.13a). Similar results were also obtained from the protoporphyrin-glucose conjugate spectrum (Figure 2.13c). However due to the lower amount of this conjugate present in the Harderian gland the spectrum was even more unclear and furthermore, some expected resonances, such as those from the vinyl group, were absent.

The unclear results obtained by NMR analysis lead us to use alternative means for the characterisation of these glycoconjugates.

2.3.7 Identification of the protoporphyrin bound monosaccharides

2.3.7.1 HPLC and HPLC/ESI-MS

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. The process of derivatisation of reducing sugars under mild conditions, is almost quantitative and yields strong UV absorbing, electrochemically sensitive derivatives. The structure of the monosaccharide-MPP derivative has already been determined and is shown to be monosaccharide bis-MPP (Honda et al. 1989, Figure 2.14).

The derivatised monosaccharides were analysed by HPLC with UV detection at 245nm based on a method by Honda et al (1989). The results showed that with the exception of mannose, which co-eluted with the unreacted MPP reagent at 9 minutes and xylose which co-eluted with arabinose at 18 minutes, all the derivatives resolved satisfactorily (Figure 2.15). Honda and his colleagues used phosphate buffers for
Fig. 2.13a $^1$H-NMR of Protoporphyrin Standard

![NMR Spectrum of Protoporphyrin Standard](image)
Fig. 2.13b $^1$H-NMR of purified Protoporphyrin-xylose conjugate
Fig. 2.13c $^1$H-NMR of purified Protoporphyrin-glucose conjugate
Fig. 2.14 Proposed structures for the MPP-carbohydrate derivatives
(Honda et al, 1989)

(a) MPP-pentose derivative

(b) MPP-hexose derivative
Fig. 2.15 HPLC analysis of MPP derivatives of monosaccharide standards

1 = Unreacted MPP and Mannose derivative (co-elution)
2 = Ribose derivative
3 = Glucuronic acid derivative
4 = Glucose derivative
5 = Xylose and Arabinose derivatives (co-elution)
Fig. 2.16 On-line HPLC/ESI-MS analysis of MPP derivatives of monosaccharide standards

(a) Glucuronic acid derivative (m/z 525)

(b) Glucose derivative (m/z 511)

(c) Ribose (3), Xylose (4) and Arabinose (4) derivatives (m/z 481)

(d) Unreacted MPP (m/z 175)

(e) Total ion current
separation of the monosaccharide-MPP derivatives. However, due to the general incompatibility of phosphate buffers with mass spectrometry, we employed ammonium acetate as the aqueous phase. The corresponding on-line HPLC/ESI-MS confirmed the identity of the peaks seen in HPLC as shown in Figure 2.16. It was observed that although the resolution of unreacted MPP reagent was very good on the HPLC system however, it suffered markedly on the LC-MS system. This anomaly has also been observed with protoporphyrin, and is thought to be due to the interaction of these compounds with the capillary wall being employed in the on-line HPLC/ESI-MS system.

The analysis of the MPP-sugar derivative from the major glycoconjugate showed a peak eluting at 18 minutes, however, due to the co-elution of xylose and arabinose, the identity of this monosaccharide could not be confirmed. Therefore, a different analytical method was needed.

2.3.7.2 Capillary Electrophoresis

The reproducibility of the CE capillary, and the system in general, proved to be good. Figure 2.17a shows a graph of retention time for each consecutive injection. Although the retention time increased slightly but with the coefficient of variation of 2.05%, the reproducibility was satisfactory. Figure 2.17b shows the area of MGBG peak with repeated injections. The coefficient of variation obtained was 7.72%. Since the coefficient of variation within 10% is generally acceptable, this value, therefore, was accepted and the glycoconjugate analyses carried out.

In 1991 Honda and his colleagues developed a capillary electrophoretic method for the analysis of the MPP derivatives of sugars. This method used the
Fig. 2.17 Reproducibility of the CE system

a) Variation of retention time with repeated injections

b) Variation of peak area with repeated injection
Fig. 2.18a  Capillary electropherograms of MPP derivatives of pentose monosaccharides
(Peaks: 1=MPP, 2=xylose, 3=arabinose, 4=ribose, 5=lyxose)

(a) Pentose monosaccharide standards

(b) Monosaccharide obtained from conjugate with MH⁺ at m/z 695
(Retention time = 17.5 min)

(c) Monosaccharide obtained from conjugate with MH⁺ at m/z 695
(Retention time = 15 min)
Fig. 2.18b Capillary electropherograms of MPP derivatives of hexose monosaccharides
(Peaks: 1=MPP, 2=glucose, 3=mannose, 4=galactose)
complexation of the MPP derivatives with borate, present in the buffer, to provide a very high resolution analytical method, capable of resolving the different stereoisomers of sugars. However, this method was incapable of resolving aldopentoses from aldohexoses.

From the HPLC/ESI-MS analysis it had already been observed that the major glycoconjugate consisted of pentose and the minor glycoconjugate consisted of hexose. The lack of resolution of pentoses and hexoses therefore, could be overcome by separate analysis of these standards. The monosaccharides, conjugated with protoporphyrin, were identified by releasing the monosaccharides with methanolic KOH followed by conversion and separation of the sugars as their MPP derivatives by CE.

Figure 2.18a shows the electropherograms of the monosaccharide-MPP derivatives of (a) a mixture of pentose monosaccharide standards, (b) the monosaccharide derived from the conjugate with the MH$^+$ at m/z 695 at 17.5 minutes (Figure 2.3b, peak 6) and (c) the monosaccharide derived from the conjugate with the MH$^+$ at m/z 695 at 15 minutes (Figure 2.3b, peaks 4 and 5). Figure 2.18b shows the electropherograms of the monosaccharide-MPP derivatives of (a) a mixture of hexose monosaccharide standards and (b) the monosaccharide derived from the conjugate with MH$^+$ at m/z 725 (Figure 2.3c, peak 8). The results clearly show that the monosaccharides are xylose and glucose, respectively.

According to the monosaccharide-MPP derivative structure (Figure 2.14), the molecular weight of the xylose and glucose derivatives would be 480 and 510, 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
Fig. 2.19 On-line HPLC/ESI-MS chromatograms of MPP-monosaccharide derivatives

(a) Mass chromatogram of m/z 481
MPP derivative of monosaccharide obtained from conjugate with MH⁺ ion at m/z 695 (Retention time = 17.5 min)

(b) Mass chromatogram of m/z 511
MPP derivative of monosaccharide obtained from conjugate with MH⁺ ion at m/z 725
Fig. 2.20 Structures of protoporphyrin glycoconjugates identified in the present study

Protoporphyrin IX

Protoporphyrin-1-O-acyl β-xyloside

Protoporphyrin-1-O-acyl β-glucoside
Fig. 2.21 Isomerisation of the protoporphyrin-xylose glycoconjugate by incubation in 0.9M HCl
glucose, respectively (Figure 2.19).

2.4 CONCLUSION

From the above results, the two major glycoconjugates of protoporphyrin were identified as protoporphyrin monoxyloside and protoporphyrin monoglucoside with the sugar residue attached via 1-O-acyl β-glycosidic bond (Figure 2.20), similar to that found in the bilirubin glycoconjugates (Jansen, 1981; Kennedy et al, 1970) and other naturally occurring glycoconjugates.

The two small peaks (Figure 2.3b, peaks 4 and 5) with the m/z of 695 are the isomerisation 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 (Jansen, 1981; Kennedy et al, 1970) and was confirmed by incubation of the purified protoporphyrin monoxyloside in 0.9M HCl for 3 hours at room temperature when the same two peaks were formed (Figure 2.21). Similarly, the peak with the MH+ ion at m/z 725 (Figure 2.3c, peak 7) was derived from isomerisation 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 (Kennedy et al, 1970). In the present study harderoporphyrin was not detected even when selected ion recording (SIR) MS was used to improve the sensitivity of detection.

The results show that 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 to 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 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 needed to be elucidated which is detailed in a later chapter.
Chapter 3

Protoporphyrin Glycoconjugates in the Urine, Plasma and RBC of a Patient with Erythropoietic Protoporphyrinia

3.1 Introduction

We have reported the presence of previously undetected protoporphyrin glycoconjugates in the rat Harderian gland. These novel glycoconjugates, which can account for 50 to 70% of the total porphyrin present in the Harderian gland, have been isolated and fully characterised using HPLC, CE and HPLC/ESI-MS. The major glycoconjugate has been identified as protoporphyrin-1-O-acyl β-xyloside with smaller amount of protoporphyrin-1-O-acyl β-glucoside also detected.

The present study was carried out in order to investigate the existence of such glycoconjugates in erythropoietic protoporphyria (EPP), a condition caused by the reduced activity of the enzyme ferrochelatase, with the resultant accumulation of protoporphyrin (Murphy 1999).

3.2 Experimental Section

3.2.1 Materials

Protoporphyrin IX was purchased from Sigma Chemical Co. (Dorset, U.K.). Acetonitrile (CH₂CN, HPLC grade), methanol (MeOH, HPLC grade) dimethyl sulphoxide (DMSO, analytical reagent grade), sodium hydroxide, potassium hydroxide (analytical reagent grade) and ammonium acetate (analytical reagent grade)
Chapter 3

were obtained from Fisher Scientific, Loughborough, UK.

A 0.01M Ammonium acetate solution was made by dissolving 0.77g of ammonium acetate in a litre of distilled water and the pH adjusted to 5.0 with glacial acetic acid.

Anhydrous trifluoroacetic acid (TFA, protein sequencing grade) was obtained from Pierce and Warriner (Chester, UK). A 0.1% v/v solution was made by dissolving 1ml (one ampule) in a litre of distilled water.

3.2.2 Extraction of porphyrins from EPP Urine

The plasma, red blood cells (RBC) and 24 hour urine were obtained from a patient with erythropoeitic protoporphyria (EPP) and stored at -80°C until analysis.

Frozen urine from a patient with EPP was allowed to thaw to room temperature and centrifuged at 1200g for 10 minutes. The precipitate was dissolved in 500μl of CH₃CN / 0.01M ammonium acetate (pH 5.0, 65:35 v/v) and analysed by HPLC or HPLC/ESI-MS. The supernatant (50ml) was loaded onto a Bond Elute C₁₈ solid phase extraction cartridge which had been preconditioned successively with acetonitrile (40ml) and water (80ml). The porphyrins were eluted using 1ml portions of acetonitrile containing 10% TFA/H₂O (0.1% v/v) until no fluorescence was detectable on the cartridge under a UV lamp. The eluates were pooled and evaporated to dryness under reduced pressure. The residue was reconstituted in 400μl of CH₃CN / 0.01M ammonium acetate (pH 5.0, 65:35 v/v) and analysed by HPLC or HPLC/ESI-MS.
3.2.3 Extraction of porphyrins from EPP RBC and Plasma

Patient plasma and RBC were thawed and divided into 200μl aliquots. Porphyrins were extracted in 400μl CH₃CN/DMSO (3:1 v/v). The mixture was vortex mixed for 3 minutes followed by centrifugation (15000g) for 10 minutes at 10°C. The supernatant was analysed by HPLC and on-line HPLC/ESI-MS. In order to eliminate any degradation, the plasma and RBC were shielded from light throughout the extraction procedure.

3.2.4 HPLC and on-line HPLC - Electrospray Ionisation Mass Spectrometry

All HPLC separations were carried out on a Varian Model 9012 liquid chromatograph (Walton-on-Thames, Surrey, UK) as outlined in Chapter 2 with the detector set at 405nm for detection. A Hypersil BDS column (C₁₈, 250mm x 4.6mm I.D.; 5μm particle size; Hypersil, Runcorn, Cheshire, UK) with a flow rate of 1ml/min was used for the separation. In order to optimise the separation two mobile phase systems were used. The first was an ammonium acetate (0.01M, pH 5.0,) / acetonitrile system (35:65 v/v) with isocratic elution for 30 minutes. The second system consisted of 0.1% TFA/H₂O (v/v) and acetonitrile as the mobile phase (A) and (B) respectively. The following elution programme was used: from 0 to 50 minutes, 75%A, 25%B to 30%A, 70%B; from 50 to 55 minutes, 30%A, 70%B to 10%A, 90%B; from 55 to 65 minutes, isocratic elution at 10%A, 90%B. The eluent from the column was split in the ratio 1:9 prior to entering the ESI-MS source. Full-scan continuum data was acquired over a mass range of 100-1000Da and processed using VG Masslynx data system. All other LC-MS conditions used were as described in Chapter 2.
3.3 Results and Discussion

3.3.1 HPLC/ESI-MS of porphyrins in Harderian gland extracts and EPP urine

In order to optimise the glycoconjugate analysis in urine and blood ammonium acetate / acetonitrile HPLC mobile phase system was tested. This optimisation led to differing retention times of the glycoconjugates found in the Harderian glands. The optimum system was 0.01M ammonium acetate (pH 5.0) with acetonitrile at a ratio of 35:65 v/v run isocratically for 30 minutes. The analysis of the porphyrins in Harderian gland extract by on-line HPLC/ESI-MS using this mobile system has shown the presence of three peaks with a m/z of 563. The peak eluting at 18 minutes (Figure 3.1a, peak 3) was confirmed to be the MH⁺ ion of free acid protoporphyrin by comparison with authentic standard. Peaks 1 and 2 (Figure 3.1a) were product ions of the two new major porphyrin metabolites detected at the retention times of 10 minutes (Figure 3.1c, peak 7) and 15 minutes (Figure 3.1b, peak 5) corresponding to protoporphyrin-1-O-acyl β-glucoside and protoporphyrin-1-O-acyl β-xyloside, respectively. These two more polar metabolites produced MH⁺ ions at m/z 725 and m/z 695, respectively, and by in-source collision-induced dissociation gave the corresponding product ions at m/z 563 (Figure 3.1a, peaks 1 and 2).

Although RBC and plasma failed to show the presence of these glycoconjugates, the analysis of the centrifuged 24-hour urine sample, without extraction, under the same conditions, produced two peaks with an m/z of 563 (Figure 3.2a, peak 1 and 2). The retention times of 15 and 18 minutes corresponded to the product ion of protoporphyrin-1-O-acyl β-xyloside and free acid protoporphyrin, respectively, as compared to the analysis of the Harderian gland (Figure 3.1). The
Fig. 3.1 On-line HPLC/ESI-MS analysis of Harderian gland extract (Ammonium acetate/acetonitrile system)

(a) m/z 563

(b) m/z 695

(c) m/z 725

(d) TIC
Fig. 3.2 On-line HPLC/ESI-MS analysis of 24-hour urine
(Ammonium acetate/acetonitrile system)

(a) m/z 563

(b) m/z 695

(c) m/z 725

(d) TIC
Fig. 3.3 On-line HPLC/ESI-MS analysis of urine pellet obtained from 24-hour urine (Ammonium acetate/acetonitrile system)

(a) m/z 563
(b) m/z 695
(c) m/z 725
(d) TIC
protoporphyrin-1-O-acyl β-xyloside, with an MH⁺ ion at m/z 695, was not seen due to the small amount of the conjugate present (Figure 3.2b). Further, protoporphyrin-1-O-acyl β-glucoside, was also absent as indicated by the absence of the MH⁺ ion at m/z 725 at 10 minutes (Figure 3.2c). Since the amount of the major conjugate is small in the urine sample the absence of the smaller conjugate was anticipated.

The analysis of the urine sediment, obtained from the centrifugation of the preceding urine sample, quite clearly showed two peaks at m/z 563 at 15 minutes and 18 minutes, corresponding to the product ion of protoporphyrin-1-O-acyl β-xyloside and free acid protoporphyrin, respectively (Figure 3.3a, peaks 1 and 2). Moreover, the MH⁺ ion of protoporphyrin-1-O-acyl β-xyloside at m/z 695 eluting at 15 minutes can also be detected (Figure 3.3b, peak 3).

The results therefore, show that the naturally occurring protoporphyrin glycoconjugates, found in the Harderian gland, are also present in EPP. Although the amount of these conjugates are low, it nevertheless shows that glycoconjugates of protoporphyrin can be formed in conditions in which protoporphyrin is highly elevated, as in EPP.

3.4 Conclusion

In conclusion, the protoporphyrin-1-O-acyl β-xyloside, present in the Harderian gland, have also been detected in EPP urine but not in RBC or plasma. It is well documented that EPP patients suffer from liver damage ranging from mild liver function abnormality to total liver failure (Murphy 1999). These complications occur due to the accumulation and deposition of excessive amount of protoporphyrin in the liver. The conjugation of the protoporphyrin may be a mechanism employed to
increase the hydrophilicity of protoporphyrin in order to increase excretion and hence reduce deposition.
Investigation of the Mechanism of formation of Protoporphyrin glycoconjugates

4.1 Introduction

The majority of protoporphyrin present in the Harderian gland is in the form of glycoconjugates. This may indicate a possible function for these glycoconjugates. The elucidation of the mechanism of formation of these glycoconjugates may give us an indication of their function. Synthesis through an enzymatic route would almost certainly suggest a possible function for these glycoconjugates. The glycoconjugate formation may, however, be through a non enzymatic route, such as chemical reaction. In this case the glycoconjugates are more likely to be an accidental by-product due to the amount of protoporphyrin present within the Harderian gland. The knowledge of the mechanism of formation of these glycoconjugates may enable us to ascertain the reason for such huge protoporphyrin synthesis in the Harderian gland. Moreover, this information may also give us an indication as to the function of the Harderian gland itself.

4.2 Experimental

4.2.1 Materials and Reagents

Acetic anhydride (99+%), anhydrous pyridine, 3-methyl-1-phenyl-2-pyrazolin-5-one (MPP), protoporphyrin IX, magnesium chloride (MgCl₂), Tris-HCl, uridine 5'-diphosphate (UDP), uridine 5'-diphosphogluconic acid (UDPGA),
uridine 5'-diphosphoglucuronyl transferase (UDPGT) and all monosaccharides were purchased from Sigma Chemical Co. (Dorset, UK.).

Acetonitrile (CH$_3$CN, HPLC grade), methanol (MeOH, HPLC grade), dimethyl sulphoxide (DMSO, analytical reagent grade), sodium hydroxide, potassium hydroxide (analytical reagent grade) and concentrated (conc.) HCl (S.G. 1.18, analytical reagent grade) were all obtained from Fisher Scientific, Loughborough, UK. Anhydrous trifluoroacetic acid (TFA, protein sequencing grade) was obtained from Pierce and Warriner (Chester, UK.).

A 0.1% TFA solution was made as described in Section 3.2.1. Working solutions of 1mM protoporphyrin in CH$_3$CN/DMSO (3:1) and 25mM UDP and UDPGA in distilled water were freshly made as required. MgCl$_2$ (1.23mM) was made up in distilled water and used as required.

Liver tissue and Harderian glands were obtained as described in Chapter 2 and stored at -80°C until required.

4.2.2 Reaction of Protoporphyrin with Monosaccharide

Protoporphyrin (300µl, 1mM) dissolved in acetonitrile/DMSO (3:1 v/v) was added to xylose (300µl, 0.9mM) and glucose (300µl, 0.9mM). The mixture was vortex mixed for 30 seconds and divided into two equal aliquots. Both aliquots were incubated for 24 hours with one being placed in the dark at 4°C while the other in ambient light at room temperature.

Incubations were also carried out with protoporphyrin dissolved in 0.1M HCl and 0.1M NaOH. 300µl of 0.5mM protoporphyrin, xylose and glucose were mixed,
aliquoted and incubated as above.

Protoporphyrin dissolved in acetonitrile/DMSO (3:1 v/v), 0.1M HCl and 0.1M NaOH, without the addition of sugars, was also incubated as described above and used as controls. All the samples and controls were kept at 4°C until immediately prior to HPLC analysis.

4.2.3 Preparation of Liver and Harderian gland homogenates

The frozen Harderian gland and liver tissues were thawed out when required. Harderian gland (250-350mg) and Liver (300mg) was homogenised in 4 ml of 50mM Tris-HCl buffer (pH 7.4) in a pestle-in-glass homogeniser. The homogenate was then divided equally into four aliquots in 5ml dupont glass tubes wrapped in aluminium foil and stored at -80°C until required.

4.2.4 Incubation of Protoporphyrin with Harderian gland Homogenates

A 1ml aliquot of Harderian gland homogenate in Tris-HCl buffer was allowed to thaw to room temperature and 25μl of MgCl2 (1.23mM), 100μl of UDPGA (25mM) and 5μl of protoporphyrin (1mM) was added. The reaction mixture was vortex mixed for 5 minutes and incubated at 37° either overnight or for 1 hour.

Control reactions were carried out in a similar fashion with the absence of either the homogenate or the magnesium chloride. A further aliquot was used without the addition of any of the reagents. In order to keep the volume of the controls and reaction mixtures equal Tris-HCl was used as a make up solvent. The controls were treated and incubated in the same way as the reaction mixture.

Similar incubations were carried out with UDP (100μl, 25mM) and glucose
(100μl, 25mM) or UDP (100μl, 25mM) and xylose (100μl, 25mM) being substituted for UDPGA.

The mixtures were centrifuged at 15000g for 5min and the supernatant transferred into a clean, foil wrapped tube and analysed by HPLC after purification by solid phase extraction. In order to recover any possibly undissolved protoporphyrin reaction product, the pellet was reconstituted with 200μl of CH$_3$CN/DMSO (3:1v/v) and vortex mixed for 1 minute. The mixture was centrifuged at 15000g for 5 min and the supernatant analysed by HPLC.

4.2.5 Incubation of Protoporphyrin with Liver Homogenate

Three aliquots of liver homogenate were allowed to thaw to room temperature. To the first aliquot 25μl MgCl$_2$ (1.23mM), 100μl glucose (25mM) and 100μl UDP (25mM) was added. The reaction was initiated by the addition of 5μl protoporphyrin (1mM). The reaction mixture was thoroughly mixed and incubated in the dark at 37°C for 2 hours.

To the second and third aliquot 100μl xylose (25mM), instead of glucose, and 100μl UDPGA (25mM), instead of UDP and sugar were used. A liver homogenate aliquot containing 25μl MgCl$_2$ and 5μl protoporphyrin was the control mixture. Tris-HCl buffer was used as the make up solvent.

The reaction was stopped by the addition of 2.4ml of CH$_3$CN/DMSO (3:1 v/v). The mixture was vortex mixed for 2 minutes and centrifuged at 2000g for 10 minutes at 10°C. The supernatant was separated and analysed by HPLC.

Blanks corresponding to the above mixtures were also incubated. These
blanks contained all the substrates as described above except the liver homogenate. 1ml of Tris-HCl buffer was added to each instead of the liver homogenate.

4.2.6 Incubation of Protoporphyrin in Liver Homogenate in the presence of UDPGT.

A set of five 1ml aliquots of liver homogenate were allowed to thaw to room temperature. To each of the five homogenates 100μl Triton X-100 (10%) and 25μl MgCl₂ (1.23mM) was added. The first homogenate aliquot contained 200μl UDPGT (1mg/ml) and 100μl UDPG (25mM), with the corresponding controls containing 200μl UDPGT (1mg/ml) and 100μl Tris-HCl buffer, and 200μl Tris-HCl buffer and 100μl UDPG (25mM). The fourth homogenate aliquot contained 200μl UDPGT (1mg/ml) and 100μl UDPX (25mM) with the corresponding control containing 200μl Tris-HCl and 100μl UDPX (25mM). The reaction was initiated by the addition of 5μl protoporphyrin (1mM) to each of the five reaction mixtures and controls. The samples were vortex mixed and incubated at 37°C in the dark for 1 hour.

The reaction was terminated by vortex mixing with 2.8 ml of CH₃CN/DMSO (3:1 v/v). The mixtures were centrifuged at 1200g for 10 minutes at 10°C. The supernatant was separated and analysed immediately by HPLC.

4.2.7 Incubation of Protoporphyrin in Tris-HCl buffer containing UDPGT

The incubation procedure was the same as that described for liver homogenate (Section 4.2.6) with the exception that 1ml of liver homogenate (in Tris-HCl) was replaced with 1ml of Tris-HCl. Two sets of reactions were done. In the first, the substrate used was UDPG with the corresponding controls and the second used UDPX as the substrate, also with corresponding controls.
4.2.8 Incubation of protoporphyrinogen with UDPGA, UDP/Xylose, and UDPG in the presence of UDPGT.

UDPGT (1mg/ml, 200µl) was added to two 1ml aliquots of Tris-HCl buffer containing, protoporphyrinogen (1mM, 20µl) and magnesium chloride (1.23mM, 25µl). The reaction was initiated by the addition of UDPX (25mM, 100µl) to one aliquot and the addition of UDPG (25mM, 100µl) to the other. The reaction mixtures were thoroughly mixed, incubated at 37°C for 1 hour in the dark and analysed by HPLC. A control containing Tris-HCl buffer (50mM, 100µl) in place of UDPX or UDPG was also incubated and analysed.

4.2.9 HPLC and LC-MS analysis of the incubates

All the reaction mixtures and controls were analysed by HPLC and/or HPLC-ESI/MS. In order to be able to detect all possible conjugates or adducts in the reaction mixtures, the separation conditions were modified slightly from those described in Chapter 2. TFA in water (0.1% v/v) and acetonitrile was used as mobile phase A and B, respectively with the following gradient: 0 to 50 minutes, 25 to 70%B; 50 to 55 minutes, 70 to 90%B followed by isocratic elution at 90%B for a further 35 minutes.

4.3 Results and Discussion

4.3.1 Preparation of liver and Harderian gland homogenates

The homogenisation of the Harderian gland and liver carried out by an automatic homogeniser with a metal blade lead to the formation of protoporphyrin-iron complex. This complex was seen as a doublet eluting at the same retention time
as protoporphyrin at approximately 55 minutes (Figure 4.1a). It was found therefore, that the optimum homogenisation technique for these protoporphyrin containing tissues was by pestle-in-glass homogeniser (Figure 4.1b).

4.3.2 Reaction of protoporphyrin and protoporphyrinogen with monosaccharides

No reaction products were detected after incubation of protoporphyrin with xylose or glucose either in the dark at 4°C or in ambient light at room temperature. Similar results were obtained for incubations in acidic or basic media. This rules out the possibility that the conjugates were formed chemically by the reaction of protoporphyrin and monosaccharides.

The possibility that the glycoconjugates were formed from protoporphyrinogen instead of protoporphyrin was investigated by the incubation of protoporphyrinogen with glucose and xylose. The analysis of the incubation mixture showed a number of unknown peaks eluting before protoporphyrin (Figures 4.2a and 4.2b).

The peaks eluting at 29.8 and 31.9 minutes have been shown to be due to complete and partial hydrolysis of the vinyl groups, respectively, of protoporphyrin. All other peaks were present in the controls and hence were disregarded. No reaction of protoporphyrinogen with any of the sugars was apparent.

4.3.3 Incubation of protoporphyrin with liver and Harderian gland homogenates

4.3.3.1 Harderian gland Incubations

The glycoconjugates are found only in the Harderian gland. This raised the possibility that the particular enzyme involved in their synthesis may only be present
in the Harderian gland. These incubations were carried out in order to investigate this possibility.

UDPGA, a high energy phosphate compound derived from the oxidation of UDP/glucose, is used extensively as a glucuronide donor in phase II reactions of drug metabolism in humans. This process involves the transfer of glucuronic acid from UDPGA to an electron rich atom such as N, O or S on the substrate forming an amide, ester or thiol bond. The reaction is catalysed by the UDP glucuronyl transferase enzyme. UDP glucuronyl transferase enzyme is known to have a broad substrate specificity and therefore these reactions can occur with a wide variety of drugs and molecules. Furthermore, the UDP glucuronyl transferase enzyme may also utilise other activated sugars as substrates, such as UDP/xylose or UDP/glucose. Although the majority of these phase II reactions occur in the liver, however other sites of these reactions such as lung and kidney are also known. This raised a distinct possibility that the glycoconjugates formed in the Harderian gland may well be formed from the UDP activated monosaccharides. Since the glucuronide formation is the most common reaction, involving UDPGA, and although not detected, it was possible that glucuronide was also formed as a precursor to the glycoconjugates found in the Harderian gland.

**1 hour incubation**

The 1 hour Harderian gland incubations with UDPGA, UDP/xylose and UDP/glucose were carried out in order to detect any changes to the amount of glycoconjugates already present in the gland. The results showed no significant differences in the xylose or glucose glycoconjugates as compared to the control (Figure 4.3). This could indicate either that the substrates used are not the correct
Fig. 4.1(a) Harderian gland homogenisation by steel blade homogeniser

![Graph showing chemical compounds](image-url)

- Protoporphyrin IX
- Glucose-Xylose Glycoconjugates
- Protoporphyrin IX-iron complex (Heme)
- Protoporphyrin-Glucose Glycoconjugates

Absorbance (mAUFS)

Time (min)
Fig. 4.1(b) Harderian gland homogenisation by pestle in-glass homogeniser
Fig. 4.2(a) Incubation of Protoporphyrinogen with Xylose

Protoporphyrin IX
Fig. 4.2(b) Incubation of Protoporphyrinogen with Glucose

Protoporphyrin IX
substrates for the enzyme involved or that the incubation time was not long enough for the glycoconjugate synthesis.

**Overnight Incubations**

The Harderian gland was incubated with UDPGA overnight along with a control, in which no Harderian gland was added. Harderian gland was incubated by itself as a further control. The tissue from the overnight incubation samples was removed by centrifugation after the reaction had been completed. However, due to the aqueous nature of the reaction solvent it was entirely possible that protoporphyrin along with any possible conjugates formed would have been precipitated out. The precipitate therefore, was reconstituted in CH$_3$CN/DMSO (3:1 v/v) and analysed along with the supernatant.

Two unknown peaks were detected in the reaction mixture eluting at 16.6 and 19.5 minutes (Figure 4.4a). However, due to the small amount present LC-MS could not be carried out and hence further characterisation could not be achieved. The supernatant of Harderian gland incubations showed the presence of haem and UDPGT however, no reaction products were detected (Figure 4.4d).

The results also showed higher concentration of protoporphyrin in the precipitate than the supernatant as expected of a non-polar compound such as protoporphyrin (Figures 4.4a and d). The precipitate of the reaction mixture showed the presence of protoporphyrin-hexose and protoporphyrin-pentose conjugates as well as unreacted protoporphyrin together with protoporphyrin-iron complex. These compounds were also detected in the control Harderian gland incubation (Figure 4.4b). However, the reaction mixture showed an increase in all the glycoconjugates
compared to the control (Figure 4.4e). The protoporphyrin-xylose conjugate showed a 61.8% increase (Figure 4.4a, peak 2) whereas the protoporphyrin-glucose conjugate showed an increase of 79.0% (Figure 4.4a, peak 1). The isomers of the glycoconjugates also showed a general increase ranging from 21.0% for the protoporphyrin-glucose conjugate isomer (Figure 4.4a, peak 1a) to 19.3% and 25.9% for protoporphyrin-xylose conjugates, peak 2a and peak 2b, respectively.

The protoporphyrin also showed an increase of 68% (Figure 4.4a, peak 3). The higher concentration of protoporphyrin in the reaction mixture, relative to the control, was expected since no additional protoporphyrin was added to the control. This, combined with the fact that the volume of the control was kept the same as the reaction mixtures by the addition of Tris-HCl buffer, lead to a decrease of the protoporphyrin concentration in the control. The increase in the glycoconjugates however, is most likely due to the additional synthesis taking place in the Harderian gland.

4.3.2.2 Liver Incubations

UDPG and UDPX were incubated in the liver homogenate in a 50mM Tris-HCl buffer at pH 7.4. The buffer used was based on previously published literature (Duffy et al. 1997; Chowdhury et al. 1986; Bogan et al. 1997, Killard et al. 1996; Bogan et al. 1995). Additional UDPGT was added to the incubation mixture. The analysis of the reaction mixture showed no apparent reaction taking place. The HPLC chromatogram showed the unreacted protoporphyrin eluting at 53 minutes. A small peak eluting at 36 minutes was detected in both, the UDPX and UDPG reaction mixtures (Figures 4.5a and b) as well as the controls. This peak was probably UDPGT or an impurity in that presentation as confirmed by HPLC analysis of
Fig. 4.3 The Effect of Different Reagents on the Amounts of Glycoconjugates When Incubated With the Harderian Gland (1 hour incubation)
Fig. 4.4(a) Harderian Gland Homogenate Incubation with UDPGA (Precipitate Extract)

1) Protoporphyrin-glucose (major)
1a) Protoporphyrin-glucose (minor)
2) Protoporphyrin-xylose (major)
2a) Protoporphyrin-xylose (minor 1)
2b) Protoporphyrin-xylose (minor 2)
3) Protoporphyrin IX
4) Protoporphyrin-iron complex (haem)

Unknown Peaks
Fig. 4.4(b) Harderian Gland Homogenate Control Incubation (Precipitate Extract)

1) Protoporphyrin-glucose (major)
1a) Protoporphyrin-glucose (minor)
2) Protoporphyrin-xylose (major)
2a) Protoporphyrin-xylose (minor 1)
2b) Protoporphyrin-xylose (minor 2)
3) Protoporphyrin IX
4) Protoporphyrin-iron complex (haem)
Fig. 4.4(c) UDPGA Incubation in the Absence of the Harderian Gland (Precipitate Extract)
Fig. 4.4(d) Harderian Gland Homogenate Incubation with UDPGA (Supernatant)

Absorbance (mAUFS)

UDPGT

Protoporphyrin IX

Protoporphyrin IX-iron complex (Haem)

Time (min)
Fig. 4.4(e) The Increase of Protoporphyrin Glycoconjugates on incubation of UDPGA with Harderian Gland (24 hours)
UDPGT dissolved in Tris buffer (50mM, pH 7.4) (Figure 4.5c). The analysis of these samples 24 hours later showed this peak to have disappeared which would be consistent with the degradative properties of UDPGT.

4.3.4 Incubation of protoporphyrin with UDPGA and UDP/Xylose in the presence of UDPGT

UDP-Glucuronic Acid is the main substrate for UDPGT enzyme. The apparent absence of a UDPGA or a glucuronic acid conjugate in the Harderian gland and the liver incubations needed to be investigated further.

UDPGA was incubated in the presence of MgCl₂, protoporphyrin and UDPGT. A control, in which the enzyme was omitted, was run in parallel. The HPLC analysis showed two peaks eluting at 17 and 20 minutes (Figure 4.6a). These peaks were absent in the control (Figure 4.6b). The analysis also showed other peaks eluting before protoporphyrin. However, these peaks were present in both the reaction mixture and the controls and hence were disregarded.

Glucuronic acid is an acid derivative of glucose. The increased polarity of glucuronic acid would, therefore, suggest that the elution time for a possible protoporphyrin-glucuronic acid glycoconjugate would be earlier than the protoporphyrin-glucose glycoconjugate, found in the Harderian gland, under similar analytical conditions. However, the elution time difference between the protoporphyrin-glucose glycoconjugate and the 20 minute peak detected in this incubation for example, is 19 minutes. The retention time difference is much too large for two compounds that have a high degree of similarity i.e. protoporphyrin-glucose and protoporphyrin-glucuronic acid glycoconjugates. This would exclude the
Fig. 4.5(a) Liver Homogenate Incubation with UDPX

Protoporphyrin IX

UDP GT

Absorbance (mAUFS)

Time (min)
Fig. 4.5(b) Liver Homogenate Incubation with UDPG
Fig. 4.5(c) UDPGT Enzyme Standard in Tris-HCl Buffer
Fig. 4.6(a) Protoporphyrin IX, UDPGA Incubation in the Presence of UDPGT

Absorbance (mAUFS)

Protoporphyrin IX

Time (min)

35 X

0 10 20 30 40 50 60 70 80

0 10 20 30 40 50 60 70 80

148
Fig. 4.6(b) Protoporphyrin IX, UDPGA Incubation in the Absence of UDPGT
possibility of these peaks being conjugates of glucuronic acid. A further indication as to the nature of these unknown peaks was obtained by the following experiment.

Investigation was carried out in order to ascertain whether the Harderian gland glycoconjugates could be obtained by the incubation of UDP and Xylose with UDPGT enzyme. Protoporphyrin and MgCl₂ was also added to the incubation mixture as with the UDPGA incubation. The HPLC results showed protoporphyrin eluting at 53 minutes (Figure 4.7a). As with the UDPGA incubation above, two further peaks were detected at 17 and 20 minutes which were absent in the controls (Figure 4.7b). These peaks were thought to be due to UDP, however, no other reaction had taken place.

4.3.5 Incubation of protoporphyrin with UDPG and UDPX in the presence of UDPGT – the use of TritonX-100

Protoporphyrin is very insoluble in aqueous solution. It was therefore entirely possible that the previous incubations carried out so far failed to yield any conclusive results due to the insolubility of protoporphyrin. In order to compensate for this insolubility TritonX-100 was used (10%) in Tris-HCl buffer (50mM, pH7.4). This was then used as the incubation medium for UDPG and UDPX incubations.

The resultant HPLC analysis showed the unreacted protoporphyrin peak eluting at 53 minutes. A number of small peaks were also seen before the main band however, they were also present in the controls and hence were disregarded. This would suggest that the lack of glycoconjugates was not due to the insolubility of protoporphyrin.
Fig. 4.7(a) Protoporphyrin IX, UDP and Xylose Incubation in the Presence of UDPGT
Fig. 4.7(b) Protoporphyrin IX, UDP and Xylose Incubation in the Absence of UDPGT
4.4 Conclusion

From the above results it is apparent that the synthesis of protoporphyrin glycoconjugates seems to take place in the Harderian gland. Attempts to synthesise these glycoconjugates in-vitro, non-enzymatically and enzymatically, using UDPGT as the enzyme, have been unsuccessful. This may imply that an enzyme other than UDPGT is used to carry out the synthesis in the Harderian gland. If this is indeed an enzymatic reaction, then this particular enzyme, which utilises protoporphyrin as the substrate, would seem to be absent in the liver and may be present in the Harderian gland. The identity of this enzyme is of great significance. If this enzyme is specific to the Harderian gland, which synthesises more porphyrins than any other tissue studied to date, then this would almost certainly indicate a dedicated function for the protoporphyrin glycoconjugates being synthesised.

What the function of these glycoconjugates may be, remains unclear. The purpose of their synthesis may only be to increase their hydrophilicity as an aid to excretion. However, since the reactions carried out in this study are by no means exhaustive, it would be premature to exclude a possible function for these glycoconjugates by reaction with other compounds not investigated in this study.
Chapter 5

Amino acids, peptides and protein-adducts of protoporphyrin: preparation and analysis by LC-MS and MALDI-TOFMS

5.1 Introduction

Protoporphyrin glycoconjugates have been detected in the Harderian gland of rats (Chapter 2). These glycoconjugates have also been detected in the urine of a patient with a human condition known as erythropoietic protoporphyria (Chapter 3), a disorder caused by the reduced activity of the ferrochelatase enzyme. The function of these conjugates is not known. These findings have prompted us to investigate the general reactivity of protoporphyrin and protoporphyrinogen with other compounds of biological origin, such as amino acids, peptides and protein. This investigation takes on added importance since porphyrin-peptide conjugates have been found in variegate porphyria (VP) and spectrofluorometric detection of these conjugates in plasma has been used for the diagnosis of VP (Elder et al, 1990). The conjugates are also excreted in the faeces and have been called ‘X’ porphyrin (Rimmington et al, 1968; Eales and Grosser, 1971). Small quantity of ‘X’ porphyrin is also detected in normal faeces. The porphyrin-peptide conjugates are thought to be porphyrin c-type compounds with cysteine containing peptides linked to the porphyrin by a thio-ether bond.

In 1961 Sano and Granick reported the effect of sulphhydryl compounds on porphyrinogens while investigating the properties of coproporphyrinogen oxidase
(COPRO-O). They found that, although beneficial in inhibiting the autooxidation of porphyrinogens, thioglycollate reacted with protoporphyrinogen during autooxidation by light in an acidic solution. They further reported reactions occurring with mercaptoethanol, cysteine and glutathione to form porphyrin c-type compounds. Moreover the authors reported a very slow reaction occurring between thioglycollate and protoporphyrin and a complete absence of any reaction between cysteine and protoporphyrin. Sano and co-workers investigated these results in 1964 where they reported these reactions occurring in acidic media (pH 3.5) when incubated overnight at 37°C in the dark. These reactions were also reported to occur in a neutral media (pH 7.2) albeit at a much lesser extent. Although these reactions were found to occur with protoporphyrinogen however, they occurred much more readily during the autooxidation of protoporphyrinogen.

Sano and co-workers also investigated the reactivity of cysteine with haematoporphyrinogen in acidic and neutral solutions. They found the reactivity of cysteine was much greater with haematoporphyrinogen than with protoporphyrinogen in both acidic and neutral solutions. Further, they found that the reaction could also take place in anaerobic conditions. Their investigation of the reactivity of mercaptans with porphyrins such as haematoporphyrin, protohaem and mesoporphyrin, deuteroporphyrin, coproporphyrin, uroporphyrin failed to show positive results. Based on these studies and by using modern analytical techniques such as HPLC/ESI-MS, tandem MS and MALDI-TOFMS, the reactions of amino acids, peptides and proteins with protoporphyrin and protoporphyrinogen have been re-investigated.
5.2 Experimental Section

5.2.1 Materials

Protoporphyrin IX, uroporphyrin I and III, coproporphyrin I, deuteroporphyrin IX, mesoporphyrin IX, lyophilized horse heart myoglobin (90% purity), glutathione and all amino acids were purchased from Sigma Chemical Co. (Dorset, U.K.). Globin was extracted in-house from human red blood cells. The polypeptides consisting of 4 (Cys-Val-3-[2-Naphthyl]Ala-Met, Figure 5.1) and 13 amino acid residues (CGYGPKKKRKVGG, Figure 5.2) were purchased from Sigma Chemicals Co. (Dorset, U.K.).

Acetonitrile (CH$_3$CN, HPLC grade), methanol (MeOH, HPLC grade) dimethyl sulphoxide (DMSO, analytical reagent grade), sodium hydroxide (analytical reagent grade), potassium hydroxide (analytical reagent grade), sodium acetate (analytical reagent grade), EDTA (disodium salt), sodium hydrogen carbonate (analytical reagent grade) and concentrated (conc.) HCl (S.G. 1.18, analytical reagent grade) were obtained from Fisher Scientific, Loughborough, UK.

Anhydrous trifluoroacetic acid (TFA, protein sequencing grade) was obtained from Pierce and Warriner (Chester, UK). A 0.1% v/v solution was made by dissolving 1ml (one ampule) in a litre of distilled water.

5.2.2 Incubation of porphyrin(ogen)s with cysteine, serine, peptides and globin

Porphyrins (0.3 mg) were dissolved in HCl (20µl, 6M) and the resultant HCl solution was diluted with water (1:63 v/v) to obtain 0.24mg/ml of porphyrin solution in 0.09M HCl. The porphyrins were then reduced to their corresponding porphyrin-
Fig. 5.1 Structure of the tetrapeptide used in the present study

Fig. 5.2 Structure of the polypeptide containing 13 amino acid residues
ogens as described below or reacted directly as follows. The diluted porphyrin solution (600μl) was added to an equal volume of 1M sodium acetate buffer (pH 5) containing 1mM EDTA. The solution was vortex mixed for 5 seconds and 600μl was added to an equal volume of the compound of interest dissolved in either 0.09M HCl or water, depending on the solubility of the compound. The mixture was vortex mixed for 30 seconds and incubated overnight at 37°C in the dark. The mixture was diluted with 600μl of CH₃CN/DMSO (3:1 v/v) and analysed by HPLC and on-line HPLC/ESI-MS.

Porphyrins were effectively reduced to their corresponding porphyrinogens by using a modification of a previously published method by Luo and Lim (1995) using sodium amalgam. The porphyrins, dissolved in 0.09M HCl, were vortex mixed with sodium amalgam (5%) for 15 minutes under nitrogen in the dark. The vortex mixing was continued until no fluorescence was detected under a UV lamp. The reaction of porphyrinogens with biological compounds was carried out as described above and analysed by HPLC or on-line HPLC/ESI-MS.

5.2.3 HPLC of porphyrin conjugates

All HPLC separations were carried out on a Varian Model 9012 liquid chromatograph (Walton-on-Thames, Surrey, UK) and a Varian Model 9050 UV/Vis detector. The HPLC conditions used depended on the compound being analysed as outlined below.

5.2.3.1 Porphyrin – amino acid conjugates

The preliminary analyses of protoporphyrin-cysteine conjugates was carried
out on an Aquapore Butyl column (220mm x 4.6mm I.D.; 7μm particle size; Brownlee column, Applied Biosystem) with 0.1% TFA in water (v/v, A) and 0.1% TFA in acetonitrile (v/v, B) as the mobile phase at a flow rate of 1ml/min with the following elution programme: from 0 to 20 minutes, 100%A, 0%B to 50%A, 50%B; from 20 to 25 minutes, 50%A, 50%B to 10%A, 90%B; from 25 to 30 minutes, isocratic elution at 90%B.

The subsequent analysis and conjugate resolution of protoporphyrin-cysteine, as well as all other porphyrin-amino acid reactions, was carried out with a Flypersil BDS column (C18, 250mm x 4.6mm I.D.; 5μm particle size; Hypersil, Runcorn, Cheshire, UK) with 9% acetonitrile in 1M ammonium acetate (pH5.16) (v/v, A) and 10% acetonitrile in methanol (v/v, B) as the mobile phase at a flow rate of 1ml/min. The elution programme was as follows: from 0 to 20 minutes, isocratic elution at 55%B; from 20 to 25 minutes, 45%A, 55%B to 10%A, 90%B; from 25 to 40 minutes, isocratic elution at 90%B.

**5.2.3.2 Protoporphyrin – peptide conjugates**

All protoporphyrin-peptide conjugates were analysed using a Hypersil BDS column (C18, 250mm x 4.6mm I.D.; 5μm particle size; Hypersil, Runcorn, Cheshire, UK). The mobile phase used was 9% acetonitrile in 1M ammonium acetate (pH5.16) (v/v, A) and 10% acetonitrile in methanol (v/v, B) with a flow rate of 1ml/min. The conjugate separation was achieved with a gradient elution programme of 10 to 90%B in 40 minutes followed by isocratic elution at 90%B for a further 20 minutes. The UV/Vis detection was carried out at 405 nm.
5.2.3.3 Protoporphyrin - protein conjugates

An Aquapore butyl column (220mm x 4.6mm I.D.; 7μm particle size; Brownlee column, Applied Biosystem) with 0.1% TFA in water (v/v, A) and 0.08% TFA in 80% acetonitrile (v/v, B) as the mobile phase at a flow rate of 1.5ml/min was used for the separation of protoporphyrin – globin conjugates. The elution programme employed was as follows: from 0 to 30 minutes, 65%A, 35%B to 35%A, 65%B; from 30 to 35 minutes, 35%A, 65%B to 10%A, 90%B; from 35 to 45 minutes, isocratic elution at 90%B. The wavelength of 405 and 215 nm was used for detection.

The protoporphyrin – globin conjugates were also analysed using a Perkin-Elmer LS3 (Buckinghamshire, U.K.) fluorescence detector set at excitation and emission wavelengths of 405 and 600 nm, respectively. The peaks of interest were collected and the purified fractions were evaporated under reduced pressure to dryness for spectrophotometric analysis.

The protoporphyrin – myoglobin reaction was also analysed using an Aquapore Butyl column. The solvent system used however, was 0.1% TFA in water (v/v, A) and acetonitrile (B) with a gradient elution programme of 10 to 90%B in 40 minutes followed by isocratic elution at 90%B for a further 20 minutes. The flow rate was kept at 1ml/min. The UV/Vis detector was set to 405 nm.

5.2.4 Stability of the conjugates

The stability of the protoporphyrin-cysteine conjugates was tested over four weeks by storing the reaction mixture at -20°C in the dark. The mixture was allowed to thaw to room temperature immediately prior to HPLC analysis and the areas of the conjugate peaks compared. The remaining mixture was refrozen to -20°C for future
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analyses.

5.2.5 On-line HPLC/ESI-MS and ESI-MS/MS

On-line HPLC - electrospray ionisation mass spectrometry (ESI-MS) was carried out on a VG Quattro quadrupole instrument (Micromass Ltd., Altrincham, U.K.) as outlined in Chapter 2 with the exception that the cone voltage of 90 or 35V was used, as indicated. The results were acquired either as full-scan continuum data over a mass range of 100-1300Da or selected ion recording (SIR) and processed using VG Masslynx data system.

Argon was used as the collision gas. for tandem ESI-MS, with the gas cell pressure of $1.3 \times 10^{-3}$ mBar and the collision energy of 40 or 20eV, depending on the compound being analysed. Product ions were scanned over a mass range of 200-750Da and the spectra were collected in the form of continuum data.

5.2.6 MALDI-TOFMS

Mass spectra were acquired on a Voyager DE STR MALDI-TOF mass spectrometer (PE-Biosystems, Framingham, MA, USA) equipped with a delayed extraction source and 337nm pulsed (3ns) nitrogen laser. The accelerating voltage was $20kV$. Saturated solution of $\alpha$-cyano-4-hydroxycinnamic acid in CH$_3$CN/0.1%TFA (1:1 v/v) was used as the matrix. The sample (0.5μl) dropped onto the MALDI sample plate was overlayed with the matrix solution (0.5μl) and allowed to dry at room temperature. The mass spectra was acquired over a mass range of 250-5000Da.
5.2.7 Spectrophotometric analysis of porphyrins

5.2.7.1 UV/Visible Spectrophotometry

UV/Visible spectra of conjugates was obtained using a Perkin-Elmer Lambda 2S UV/Vis spectrophotometer (Perkin-Elmer Ltd, Buckinghamshire, U.K.) in a 1ml glass cuvette with a 1cm pathlength. The spectra were processed using Perkin-Elmer Lambda2 software on an IBM compatible 486 PC. The spectra of the porphyrins in CH$_3$CN /DMSO (3:1 v/v) were recorded from $\lambda$ 300 to 800 nm. Acetonitrile/DMSO (3:1 v/v) was used as blank.

5.2.7.2 Fluorescence Spectrophotometry

Fluorescence spectra of protoporphyrin-globin conjugate was acquired using a Perkin-Elmer LS50B luminescence spectrofluorometer (Norwalk, CT, USA) with the Perkin-Elmer Fluorescence Data Manager software on an IBM compatible Pentium 75 computer. The protoporphyrin-globin reaction mixture was analysed directly in a 3ml quartz cuvette with a 1cm pathlength using an excitation wavelength of 405 nm. The fluorescence spectra were recorded from 550 to 750 nm.

5.3 Results and Discussion

5.3.1 Protoporphyrin-cysteine conjugates

In the present study the reaction of protoporphyrinogen with cysteine in acidic conditions, showed two peaks eluting at 9 and 19.5 minutes (Figure 5.3) upon analysis by HPLC using a C4 column. The analysis of the reaction mixture by on-line HPLC/ESI-MS showed two peaks at m/z 563. The peak eluting at 19.5 minutes was
confirmed to be the MH⁺ ion of free acid protoporphyrin as compared to the authentic standard (Figure 5.4, peak 2). Peak 1 eluting at 9 minutes was the product ion of the protoporphyrin-cysteine conjugate with an MH⁺ ion at m/z 805 (Figure 5.4, peak 4) due to in-source collision-induced dissociation. The new conjugate also produced one further product ion with an m/z 684 (Figure 5.4, peak 3).

The mass difference of 242 Da between the MH⁺ ion at m/z 805 and the molecular mass of cysteine (121 Da) indicated the addition of two cysteines to protoporphyrin to form a diconjugate. Similarly, the mass difference of 121 Da between the ion at m/z 684 and cysteine indicated the addition of one cysteine to form a monoconjugate. Tandem ESI-MS results confirmed that the ions at m/z 684 and 563 are indeed product ions of MH⁺ ion at m/z 805 (Figure 5.5). The presence of porphyrin conjugates bound to cysteine by thio-ether linkage has been suggested before by Pilloud and co-workers in 1998. It has also been observed previously that protoporphyrin is degraded in the presence of thiol-containing compounds and peroxidase enzymes in both plants and mammals (Jacobs 1999, Dayan 1999).

There are three possible sites where protoporphyrin can form a conjugate with cysteine. These are (a) N-substitution of the hydrogen atom within the centre of the tetrapyrrolic macrostructure, (b) thiol ester formation with the carboxyl groups of the propionic acid side chains and (c) reaction of cysteine with vinyl groups.

It has been previously shown (Smith and Farmer, 1982) that N-substitution of porphyrins shifts the Soret band significantly towards higher wavelengths. As for the Harderian gland glycoconjugates, the UV-visible spectra of these conjugates also
Fig. 5.3 HPLC analysis of the Protoporphyrinogen-cysteine reaction mixture
Fig. 5.4 On-line HPLC/ESI-MS analysis of Protoporphyrinogen-cysteine reaction mixture

m/z 563
m/z 684
m/z 805

TIC
Fig. 5.5 On-line HPLC/ESI-MS/MS analysis of Protoporphyrinogen-cysteine reaction mixture (MS/MS of m/z 805 ion eluting at 9 minutes)
Fig. 5.6 UV-Visible absorption spectra of (a), protoporphyrin-cysteine conjugate in chloroform and (b), protoporphyrin IX standard in chloroform.
Fig. 5.7 Fluorescence spectra of (a), protoporphyrin-cysteine conjugate in chloroform and (b), protoporphyrin IX standard in chloroform
showed the absorption maxima of the Soret peak to be almost identical to that of the unmodified protoporphyrin standard (Figure 5.6) and hence N-substitution was ruled out. Similarly the fluorescence spectra of the protoporphyrin standard and conjugates were also identical (Figure 5.7). Furthermore, our attempts at transesterification of the protoporphyrin-cysteine adducts were unsuccessful and hence eliminated the possibility of the cysteine being bound to the carboxyl groups of the propionic acids.

The nature of this reaction and reaction products have been investigated by Sano and co-workers in 1964 and in 1971 from their work on the synthesis of porphyrin c-type compounds. The above results are in agreement with those obtained by Sano and colleagues in that an addition reaction is taking place between the cysteine and protoporphyrinogen.

Although the tandem ESI-MS results showed the fragmentation of the \( \text{MH}^+ \) ion at \( m/z \) 805 to yield \( \text{MH}^+ \) ions at \( m/z \) 684 and 563 by the loss of one and two cysteine groups, respectively, however, this does not preclude the synthesis of diastereoisomers which were not separated by the HPLC column and conditions being used. In order to establish whether the peak obtained at 9 minutes (Figure 5.3) was a single conjugate or consisted of co-eluting isomeric conjugates, a C_{18} BDS column was employed with the other HPLC conditions being kept the same. The resulting chromatogram showed a partially resolved peak consisting of 3 components eluting at 25, 25.5 and 26 minutes, with protoporphyrin eluting at 47 minutes (Figure 5.8). The corresponding on-line HPLC/ESI-MS analysis showed three peaks eluting at the same retention time with \( \text{MH}^+ \) ion at \( m/z \) 805 (Figure 5.9, peak 3a, b and c). The in-source collision-induced dissociation again yielded product ions of \( m/z \) 684 and 563 for all three peaks. These results would suggest that the three peaks are isomers of the
protoporphyrin-cysteine conjugate. Further optimisation using 45% (v/v) 1M ammonium acetate buffer (pH 5.16) in 55% (10% acetonitrile in methanol) as mobile phase resulted in complete resolution of the three peaks eluting at 13.5, 15 and 17 minutes, respectively (Figure 5.10, peak 1, 2 and 3).

The investigations carried out by Sano and colleagues in 1964 concluded that the addition of the thiol-containing compounds occurred at the α-carbon atoms of the vinyl side chains of protoporphyrinogen. The reaction of cysteine with protoporphyrinogen was also enhanced with increasing light intensity and acidity strongly suggesting the involvement of thiol radicals (Sano and Granick, 1961). A possible reaction mechanism for the formation of protoporphyrin-dicysteine conjugate is outlined in Figure 5.11.

The protoporphyrin-dicysteine conjugate formed by α-addition would have only 2 asymmetric carbons resulting in 4 diastereoisomers. From the results obtained in Figure 5.8 it can be observed that the area of the three closely eluting peaks, peaks 1, 2 and 3, are in the ratio of 1:2:1, respectively. This correlates with the type of isomers of protoporphyrin-dicysteine possible (Figure 5.12) with co-elution of two of the isomers which have a greater similarity. These two isomers, namely the 1S,2R and 1R,2S (Figure 5.12, Isomer a and b), are similar in structure and have not been separated by the HPLC conditions used.

We have also investigated the reaction of protoporphyrin, as well as protoporphyrinogen, with various amino acids. Protoporphyrin failed to react with any of the compounds tested. Protoporphyrinogen failed to react with serine, methionine and S-methyl-L-cysteine, and histidine. However, as with cysteine, the reaction of glutathione with protoporphyrinogen also formed conjugates.
Fig. 5.8 HPLC analysis of Protoporphyrinogen-cysteine reaction mixture with a C_{18} column
Fig. 5.9 On-line HPLC/ESI-MS analysis of Protoporphyrinogen-cysteine reaction mixture with a C<sub>18</sub> column

- **m/z 563**: Protoporphyrin
  - 1a, 1b, 1c

- **m/z 684**:
  - 2a, 2b, 2c

- **m/z 805**:
  - 3a, 3b, 3c

Time (min)
Fig. 5.10 Optimised HPLC method for the analysis of Protoporphyrinogen-cysteine reaction mixture with a C$_{18}$ column
Fig. 5.11 A free radical mediated mechanism for the formation of protoporphyrin-dicysteine conjugate

\[ 2x \left( R_1 - S - H \right) \rightarrow 2x \left( R_1 - S' + H \right) + \text{Cysteine radical} \]

\[ \text{Protoporphyrinogen} \]

\[ \text{R}_1 = \text{-CH}_2\text{CH(NH}_2\text{COOH)} \]

Key:

\[ \text{37 deg C} \]

\[ \text{O}_2/\text{H}^+ \]
Fig. 5.12 Stereoisomers of protoporphyrin-dicysteine conjugate

a) 1S,2R  

b) 1R,2S

c) 1R,2R  
d) 1S,2S
Fig. 5.13 Stability of protoporphyrin-cysteine adduct over four weeks in acetonitrile/DMSO (3:1 v/v)
5.3.1.1 Stability of Protoporphyrin-cysteine conjugates

Isomer 1 of protoporphyrin-cysteine conjugate (Figure 5.8) was found to be stable over four weeks with little or no change in peak area over this time (Figure 5.13). Isomer 2 of the protoporphyrin-cysteine conjugate, which may be composed of more than one isomer co-eluting, showed a marked degradation after 13 days. Some peak broadening also occurred. Isomer 3 was also stable for 13 days after which time some peak broadening occurred leading to an apparent increase in peak area.

5.3.2 Protoporphyrin-glutathione conjugates

The HPLC chromatogram of the reaction of protoporphyrinogen with glutathione (MW 307) showed a similar profile to that of protoporphyrinogen-cysteine reaction. The HPLC chromatogram showed a set of fully resolved triplet peaks eluting at 31, 32 and 32.5 minutes (Figure 5.14, peak 1, 2, and 3). Due to the identical number of conjugates as well as the similarity of the peak profile to proto-cysteine conjugates (Figure 5.8), the presence of isomers of protoporphyrin-glutathione was indicated.

On-line HPLC/ESI-MS analysis of the protoporphyrin-glutathione reaction mixture, with cone voltage set to 30, showed the presence of two peaks with an m/z of 589 (Figure 5.15, peaks 1 and 2) corresponding to the [M+2H]^{2+} ions of protoporphyrin-glutathione di-conjugate. The detection of two peaks, as opposed to the triplet by HPLC, were due to the formic acid being used in the mobile phase instead of TFA in order to increase sensitivity. This lead to the loss of resolution as compared to the HPLC chromatogram.
Fig. 5.14 HPLC analysis of Protoporphyrinogen-glutathione reaction mixture
Fig. 5.15 On-line HPLC/ESI-MS analysis of Protoporphyrinogen-glutathione reaction mixture with short scan ranges as indicated (Cone voltage 30)

Protoporphyrin-glutathione diconjugate (m/z 589)
Scan range m/z = 582-592

Protoporphyrin (m/z 563)
Scan range m/z = 558-568
Fig. 5.16 On-line HPLC/ESI-MS analysis of Protoporphyrinogen-glutathione reaction mixture with short scan ranges as indicated (Cone voltage 90)

- Protoporphyrin-glutathione diconjugate (m/z 589)
  Scan range m/z = 582-592

- Protoporphyrin (m/z 563)
  Scan range m/z = 558-568
Fig. 5.17 MALDI-TOFMS analysis of Protoporphyrinogen-glutathione reaction mixture (* = calibration standard)
The analysis of the protoporphyrin-glutathione mixture at cone voltage 90, the optimum for protoporphyrin analysis on our system, showed two peaks at m/z 563 eluting at 16.1 and 16.3 minutes (Figure 5.16, peaks 3 and 4). The m/z of 563 corresponds to that of protoporphyrin obtained by the in-source collision-induced dissociation of the protoporphyrin-glutathione di-conjugates due to the higher cone voltage, thus confirming the presence of protoporphyrin-glutathione conjugates.

The analysis of protoporphyrin-glutathione reaction mixture by MALDI-TOFMS yielded two ions of interest (Figure 5.17). The MH⁺ ion at m/z 1177.4 (Figure 5.17) corresponding to protoporphyrin-glutathione diconjugate (562 + (2x307) = 1176) confirms the reaction to be an addition reaction. The ion at m/z 870 (Figure 5.17) corresponds to protoporphyrin-glutathione monoconjugate fragment obtained from the protoporphyrin-glutathione diconjugate (m/z 1177). It is also of note that an ion at m/z 565 is observed, which may be obtained by the loss of the glutathione from protoporphyrin leaving the hydrolysed vinyl group, to which it was bound.

The failure of protoporphyrinogen to react with amino acids except those with a thiol group such as cysteine and glutathione suggests that the presence of a thiol group is necessary for the reaction. It was decided therefore, to investigate if similar reactions occur with peptides containing cysteine.

5.3.3 Protoporphyrin-peptide conjugates

The reaction of protoporphyrinogen with two small peptides yielded similar results to that of protoporphyrin-glutathione reaction. However, separate analytical conditions had to be used for each of the peptides due to the size difference as described in the Experimental Section.
Fig. 5.18 HPLC analysis of Protoporphyrinogen-peptide (4 mer) reaction mixture
Fig. 5.19 On-line HPLC/ESI-MS analysis of Protoporphyrinogen-peptide (4 mer) reaction mixture with selected ion recording (SIR) (Cone voltage 30)

- Protoporphyrin (m/z 563)
- Protoporphyrin-peptide (4 mer) diconjugate (m/z = 830 [M+2H]^2+)
- Total ion current (TIC)

Time (min):
- 36.00
- 38.00
- 40.00
- 42.00
- 44.00
- 46.00
- 48.00
- 50.00
Fig. 5.20 On-line HPLC/ESI-MS analysis of Protoporphyrinogen-peptide (4 mer) reaction mixture with selected ion recording (SIR) (Cone voltage 90)

Protoporphyrin (m/z 563)

Protoporphyrin-peptide (4 mer) diconjugate
m/z = 830 [M+2H]^{2+}

Total ion current (TIC)
Chapter 5

The reaction of protoporphyrinogen with the tetrapeptide (MW 548) produced a set of four peaks eluting at 38.4, 39.5, 39.9 and 40.3 minutes (Figure 5.18, peaks 1, 2, 3 and 4). The HPLC chromatogram also shows other unidentified but much smaller peaks with an increased retention time, with the unreacted protoporphyrin eluting at 48 minutes. Due to the low levels of the protoporphyrin-peptide conjugates present selected ion recording (SIR) was used for their analysis by on-line HPLC/ESI-MS.

The on-line HPLC/ESI-MS analysis of the reaction mixture, using cone voltage of 30, shows four peaks with m/z of 830 (Figure 5.19, peaks 1, 2, 3 and 4). This mass-to-charge ratio corresponds to the [M+2H]^{2+} ions of protoporphyrin-peptide diconjugate. The repeat analysis of the same reaction mixture with cone voltage set to 90 shows the protoporphyrin product ions obtained from each of the di-conjugate isomers (Figure 5.20, peaks 1, 2, 3 and 4). As with protoporphyrin-cysteine reaction the reaction of protoporphyrinogen with the tetrapeptide also appears to be an addition reaction. However, the slightly bigger peptide groups confer bigger structural differences on the four diastereoisomers leading to complete separation.

HPLC analysis of protoporphyrin-peptide reaction (13-mer, MW 1376.8) showed the presence of three partially resolved peaks eluting at 14 minutes with the unreacted protoporphyrin eluting at 38 minutes (Figure 5.21). For this, much larger peptide, two of the diastereoisomers could not be separated under the conditions used.

On-line HPLC/ESI-MS with SIR detection of the protoporphyrin-peptide (13-mer) reaction mixture clearly showed a partially resolved triplet peak of m/z 1106 and 830, corresponding to [M+3H]^{3+} and [M+4H]^{4+} ions, respectively (Figure 5.22b and c) of protoporphyrin-peptide diconjugate eluting at 14 minutes. The protoporphyrin
Fig. 5.21 HPLC analysis of Protoporphyrinogen-peptide (13 mer) reaction mixture
Fig. 5.22 On-line HPLC/ESI-MS analysis of Protoporphyrinogen-peptide (13 mer) reaction mixture with selected ion recording (SIR) (Cone voltage 90)

a) Protoporphyrin (m/z 563)

b) Protoporphyrin-peptide (13 mer) diconjugate
   m/z = 830 [M+4H]^{4+}

c) Protoporphyrin-peptide (13 mer) diconjugate
   m/z = 1106 [M+3H]^{3+}

d) Total ion current (TIC)
Fig. 5.23 MALDI-TOFMS analysis of Protoporphyrinogen-peptide (13 mer) reaction mixture
product ion (m/z 563) of the conjugates could also be detected (Figure 5.22a).

Analysis by MALDI-TOFMS yielded an MH$^+$ ion at m/z 3318.8, corresponding to the molecular ion of protoporphyrin-peptide diconjugate. Although the ion at m/z 3318.8 itself does not correspond to the molecular ion of protoporphyrin-peptide diconjugate, however, closer inspection (Figure 23, Inset) reveals an ion at m/z 3316.7 which does correspond to the molecular ion of the dipeptide. The ion at m/z 3318.8 is observed due to the various isotopes of the atoms present in the peptide which distort the ion distribution. Similarly the ion at m/z 1940.0 (seen as m/z 1942) observed in Figure 23 corresponds to the protoporphyrin-peptide monoconjugate, a possible product ion obtained from the diconjugate.

### 5.3.4 Protoporphyrin-protein conjugates

Having established the reactivity of protoporphyrinogen with cysteine and cysteine containing peptides it was reasonable to conclude that the reaction would also take place with larger naturally occurring proteins such as globin.

The reaction of protoporphyrinogen with globin was analysed with the C$_4$ column at 215nm detection wavelength. With the conditions used, unreacted protoporphyrin eluted at 13.5 minutes (not seen at 215nm) with the beta and alpha chains of globin eluting at 21 and 22 minutes, respectively (Figure 5.24a). Only partial resolution of the alpha and beta chains of the globin standard could be achieved. Further attempts to increase resolution were unsuccessful probably due to the age of the column being used.

The analysis of the reaction mixture failed to show any extra peaks, either with
405nm or 215nm detection, but nevertheless the shape of the globin peak, and in particular the beta chain, changed considerably (Figure 5.24b). This observation is in agreement with previous findings where the higher reactivity of the beta chain of globin has been reported (Birt et al, 1998; Nguyen and Peterson, 1986). The lack of resolution could be due to the fact that whereas all compounds so far investigated were small with molecular weight of between 121 Da (cysteine) and 1376.8 Da (13-mer peptide), globin however, is a much larger molecule with an average molecular weight of 15500 Da. The addition of a single protoporphyrin, therefore, would not change the molecular weight sufficiently in order to resolve the conjugate from the unreacted globin. Similar lack of resolution has also been reported previously (Birt et al. 1998).

The analysis of the reaction mixture by HPLC using UV/Vis detection at 405nm showed the doublet peak at 21 and 22 minutes (Figure 5.25b). Since the absorbance of globin at this wavelength is negligible the positive result suggested the presence of protoporphyrin. However, the presence of protoporphyrin cannot be ascertained conclusively from the UV/Visible absorbance alone, since other compounds such as heme also absorbs at this wavelength. Thus, the analysis of the reaction mixture with HPLC using fluorescence detection, with excitation wavelength of 405 and emission wavelength of 620, was carried out. These results also showed a doublet peak eluting at 21 and 22 minutes thus supporting the presence of protoporphyrin (Figure 5.25a).

The analysis of the reaction mixture by on-line HPLC/ESI-MS, although showing globin (MW α-chain 15129, β-chain 15869), failed to show the presence of a
Fig. 5.24a HPLC analysis of Globin standard (detection wavelength 215nm)
Fig. 5.24b  HPLC analysis of Protoporphyrinogen-Globin reaction mixture
(detection wavelength 215nm)
Fig. 5.25 HPLC analysis of Protoporphyrinogen-Globin reaction mixture with UV-Visible and Fluorescence detection

a) Fluorescence detection
$(\lambda_{ex/em} = 405/620\text{nm})$

b) UV/Visible detection
$(\lambda = 405\text{nm})$
Fig. 5.26 On-line HPLC/ESI-MS analysis of (a) Globin standard and (b) Protoporphyrinogen-Globin reaction mixture.
protoporphyrin-globin adduct upon deconvolution of the spectra. This is probably due to the low resolution of the two globin chains, particularly after adduction with protoporphyrin. However, it can be seen from Figure 5.26a and b that, as for HPLC analysis, the shape and intensity of globin peak differs markedly before and after the reaction with protoporphyrinogen, with the β-chain showing the greater change.

5.4 Conclusion

The present study has established that protoporphyrinogen reacts with cysteine and any ‘thiol containing’ compounds non-enzymatically. As far as the cysteine is concerned, the results are in agreement with previously published data in that cysteine is a very reactive amino acid (Pilloud et al., 1998, Sano, 1971). All of the reactions occur with the thiol group of the cysteine and this seems to be the case with protoporphyrinogen. This is supported by the fact that serine, an amino acid similar in all respects to cysteine with the exception of a lack of a thiol group (Figure 5.27), failed to react with protoporphyrinogen. The reaction site on the protoporphyrinogen seems neither to be the carboxyl groups on the two propionic acid side chains nor the nitrogen atoms of the protoporphyrinogen. This is proved by the fact that firstly, the

Fig. 5.27 Similarity between Cysteine and Serine

\[
\begin{align*}
\text{Cysteine} & \quad \text{Serine} \\
\begin{array}{c}
\text{H}_2\text{N} & \quad \text{H}_2\text{N} \\
\text{COOH} & \quad \text{COOH} \\
\text{CH} & \quad \text{CH} \\
\text{H}_2\text{C} & \quad \text{H}_2\text{C} \\
\text{SH} & \quad \text{OH}
\end{array}
\end{align*}
\]
protoporphyrin-cysteine conjugate failed to transmethylate and secondly, our attempts in the present study to react uroporphyrinogen (eight carboxyl groups) and coproporphyrinogen (four carboxyl groups) with cysteine were unsuccessful. These two facts combined would eliminate any possibility of an esterification type reaction taking place. The UV/Visible absorption spectrum of the conjugate is almost identical to protoporphyrin hence also eliminating the possibility of N-substitution occurring.

An attempt has also been made to react deuteroporphyrin and mesoporphyrin with cysteine. These porphyrins are characterised by the absence of vinyl groups and have hydrogen atoms or ethyl groups, respectively. The failure of these porphyrins to react would indicate the reaction is taking place with the vinyl groups of protoporphyrin. An alternative site of reaction would be the meso positions of the protoporphyrin. In this reaction the resultant product would be in agreement with all the results obtained including more than two isomers, inability to transesterify, the ability of the conjugate(s) to fragment back to protoporphyrin with tandem LC/MS, and the similarity of the UV absorbance spectra to the parent compound. However the molecular weights of the conjugates obtained in this reaction would not be in agreement with those obtained in this study, namely, the loss of 2 hydrogen atoms from the protoporphyrin-peptide and protoporphyrin-glutathione diconjugates.

The additional nature of the reaction in the formation of protoporphyrin conjugates with thiol containing peptides and amino acids has been confirmed by MALDI-TOFMS which clearly shows a singly charged ion of the protoporphyrin-peptide diconjugates.
Chapter 6

Concluding Discussion and Future Studies

6.1 Protoporphyrin glycoconjugates in the Harderian gland

In this study we have discovered and fully characterised novel protoporphyrin-glycoconjugates present within the Harderian gland. A variety of analytical techniques have been utilised to achieve this. These include techniques which are well established i.e. HPLC to those which are relatively modern i.e. CE. Mass spectrometry has been used as a detector in line with HPLC. LC-MS provides details of the molecular weight of the compound in question. It may also give some structural information due to in-source fragmentation. In order to determine the structure of any unknown compound, tandem MS is normally utilised. In general the porphyrin macrocyclic structure is extremely stable to fragmentation as observed by the high cone voltage required for its ionisation. However, given sufficient collision energy fragmentation of protoporphyrin can be achieved. Tandem MS analysis is a powerful technique, which gives extremely useful structural information and has also been utilised in this study. These techniques have been combined effectively to yield structures of the unknown glycoconjugates.

The existence of the Harderian gland has been known for over 300 years, with intensive research of its possible function taking place for over 50 years, but we have yet to conclusively define its function. The huge amount of protoporphyrin present within the Harderian gland almost certainly serves a purpose. Many researchers have carried out studies to define this purpose. The conclusions they have reached are wide
and varied, from sex attractant (Payne, 1979) to involvement in diurnal rhythms (Pevet et al. 1984). However, it is evident that the extraction procedures used for the extraction of porphyrins from the Harderian gland for the past decades were too severe. These methods did not give an accurate picture of the type of porphyrins present in the gland. The majority of the protoporphyrin present has been shown to be in the form of glycoconjugates. This may provide us with additional clues as to the purpose of protoporphyrin and, if the function of these glycoconjugates can be found, may well give an indication of the function of the Harderian gland itself.

It is also interesting to note that previous researchers have found the Harderian gland to contain Harderoporphyrin (a tricarboxylic porphyrin) which can account for up to 29% of the total porphyrins present in the case of Wistar-Han rats (Kennedy et al. 1970). In the present study however, where female Wistar-Han rats have been used, we have not been able detect any Harderoporphyrin. The gender of the rats studied by Kennedy et al (1970) however, has not been mentioned and since the porphyrins present in the Harderian gland are gender specific, direct comparison is not possible.

### 6.2 Protoporphyrin Glycoconjugates and their Formation in the Harderian gland and EPP

Protoporphyrin glycoconjugates found in the current study appear to be formed by UDPGT using UDPGA as the substrate. This is indicated by the increase of glycoconjugates found when UDPGA is incubated in a Harderian gland with the addition of UDPGT.
UDPGT is also present in liver, however, incubation of UDPGA in liver homogenate fails to yield any glycoconjugates. Further, incubation of UDPGT standard with UDPGA in Tris-HCl buffer also fails to produce any such glycoconjugates. The reasons for these observations are not clear but may indicate an additional step in the formation of these glycoconjugates and may also suggest the involvement of other enzymes in conjunction with UDPGT in the production of the conjugates. The additional enzyme may only be present in the Harderian gland but absent in the liver leading to the observed results in the current study. This needs to be investigated further before a definitive decision on the formation of protoporphyrin glycoconjugates can be made.

In some of the human porphyrias, namely EPP and VP, as in the Harderian gland protoporphyrin is also produced in excess (Moore et al, 1990). This similarity made it entirely possible that similar glycoconjugates could also exist in patients suffering from such disorders. This hypothesis has been proved correct in the present study. In the EPP patient studied, protoporphyrin glycoconjugates have been detected albeit in much smaller amounts. In this patient the protoporphyrin-xylose glycoconjugate has been detected. The mechanism of formation of the glycoconjugates in EPP may well be of a similar type as found in the Harderian gland. However, in-vitro incubations in the current studies have shown no indication of the formation of glycoconjugates in the liver. This would suggest either the glycoconjugates in the EPP patient are being formed elsewhere or they are formed by a different mechanism which does not involve the additional enzyme. A thorough investigation is required to obtain a decisive explanation.
6.3 Amino acids, peptides and protein-adducts of protoporphyrin

In order to obtain an indication as to the mechanism of formation of the protoporphyrin-glycoconjugates it was necessary to establish the reactivity of protoporphyrin itself. We found protoporphyrin to be relatively unreactive to the compounds investigated which ranged from monosaccharides to proteins.

Protoporphyrinogen however, the reduced form of protoporphyrin, was found to react. From the compounds studied the reaction of protoporphyrinogen was limited to cysteine and thiol containing amino acids, peptides and proteins. The reaction of cysteine and all thiol containing compounds that were studied occurred at the vinyl groups of protoporphyrin. The mechanism of the reaction of cysteine and thiol containing peptides and proteins appears to be the same.

The reaction of these biological compounds occurs without any loss of hydrogen. This apparent anomaly can be entertained if the reaction occurs with protoporphyrin vinyl groups by a radical mechanism as explained in Chapter 5.

From the study of the reactivity of protoporphyrin and protoporphyrinogen it is apparent that similar glycoconjugates were not formed as those found in the Harderian gland and EPP patient. This would further reinforce the view that the mechanism of formation of these glycoconjugates is enzyme mediated. The fluorescence spectrum of protoporphyrin-polypeptide conjugates showed a maxima at 626nm. This is similar to that of the protoporphyrin-protein conjugates found in the plasma of patients with variegate porphyria. The reactions of protoporphyrinogen with cysteine, glutathione, peptides and proteins are therefore model reactions for the formation of protoporphyrin-protein conjugates in variegate porphyria.
6.4 Future Work

This study as any other scientific study has raised more questions than it has answered. Harderian gland is widely used by researchers all over the world as a model to study the heme biosynthetic pathway. However, it is evident that the conditions used to extract porphyrins from this gland, i.e. esterification, were unsuitable. The use of the conditions developed in this study for porphyrin extraction from the Harderian gland may lead to more as yet undiscovered conjugates. This gland seems to produce huge amounts of protoporphyrin but the purpose of this protoporphyrin is still a subject of hot debate. Our finding that most of this protoporphyrin is in the form of glycoconjugates may provide a further clue. It is essential to find out the reason behind the glycoconjugate synthesis. The answer to this question may solve the mystery of the function of Harderian gland itself.

The mechanism of formation of protoporphyrin glycoconjugates also needs further investigation. It is evident from our study that UDPGT and UDPGA are required, however the absence of glycoconjugates in liver incubations points towards a mechanism which may involve further steps, possibly a different enzyme. The discovery of this enzyme, if it is an enzymatic step, may well give new insights into the function of the Harderian gland. This discovery may also be of benefit in human porphyria. It is known that EPP has high amounts of protoporphyrin and that patients with this condition can suffer from fatal liver failure due to active chronic hepatitis with cirrhosis (Moore et al. 1990). The finding of these glycoconjugates may, therefore, be an indication as to the mechanism that causes cirrhosis. Further, the possible additional enzyme used in the production of the glycoconjugates, when found, may be used to increase the conjugation of excess protoporphyrin found in EPP
and VP patients. The increased conjugation will lead to an increased hydrophilicity of protoporphyrin thus leading to a decrease in the deposition of protoporphyrin in the liver thereby reducing the risk of liver failure.

It will also be useful to investigate the nature of protoporphyrin peptide and protein conjugates in VP, since protoporphyrinogen levels are also increased in this form of human porphyria. Since the link between VP and Hepatocellular cancer (HCC) is strongly suspected (Axelson, 1986), the finding and extent of glycoconjugates in this disorder may give us further clues as to the reason for the cancer and its possible prevention.
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