Determination of Porphyrins in Bile
Using High Performance Liquid Chromatography
and Some Clinical Applications

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Summary: A simple and fast HPLC method for the determination of porphyrins in bile without extraction is described. Porphyrins were determined in bile from control subjects and from patients after orthotopic liver transplantation, including three patients with erythropoietic protoporphyria. It was found that:

1) coproporphyrin I is the predominant porphyrin in bile of controls, accompanied by some coproporphyrin III and protoporphyrin, whereas protoporphyrin mostly but not always is the predominant porphyrin in the bile of erythropoietic protoporphyria patients. In two of the three erythropoietic protoporphyria patients, the bile contained a hundred times more protoporphyrin than that of non-porphyric orthotopic liver transplantation patients. The third erythropoietic protoporphyria patient remained cholestatic and was unable to excrete sufficient amounts of protoporphyrin.

2) All investigated bile samples contained no secondary porphyrins derived from protoporphyrin, i. e. no deutero-, pempto-, or mesoporphyrin. Even when extracts of bile and serum were concentrated fifty to a hundred times, no traces of deutero-, pempto- and mesoporphyrin were detected. This complete absence of secondary porphyrins suggests that an enterohepatic circulation of dicarboxylic porphyrins from the distal gastrointestinal tract does not exist.

3) The HPLC chromatograms contain peaks from unknown compounds. No correlation between porphyrins and these compounds was found.

Porphyrin profiles were followed in the bile of some orthotopic liver transplantation patients. Three episodes are recognizable. During the first three days after orthotopic liver transplantation there is a very high coproporphyrin excretion. There is then a lag of one to three weeks, in which no or very low porphyrin concentrations are detectable, followed by the restoration of normal biliary porphyrin patterns.

Introduction

Determination of porphyrin profiles in biological samples by means of HPLC has proved valuable in the diagnosis and treatment of patients suffering from currently known forms of porphyria, all caused by a deficiency or inhibition of enzymes involved in the synthesis of haem.

The porphyric diseases can be identified by profiling of urine, faeces or blood (1—3), and detecting the porphyrins that are greatly elevated. Thus, together with their clinical presentation, congenital erythropoietic porphyria, porphyria cutanea tarda and attacks of acute intermittent porphyria can be identified by analysis of urine, whereas stool analysis is needed to identify at-

tacks of hereditary coproporphyria and variegate por-
phyria. Erythropoietic protoporphyria can be confirmed
by analysis of the protoporphyrin content of erythro-
cytes. In many clinical laboratories, such determinations
are presently performed on a routine basis.

So far, not much attention has been paid to porphyrin
profiles in human bile, since the diagnosis and follow
up of porphyria can be satisfactorily carried out by ana-
lysing urine, faeces or blood. However, for asymptom-
atic patients with porphyria those methods are not al-
ways sufficiently discriminatory. Bile was recently suc-
cessfully used for the evaluation of asymptomatic pa-
tients with variegate porphyria (4). For some weeks
following orthotopic liver transplantation, which is a
regular operation in our hospital, bile is removed via a
T-drain. For the following reasons, we decided to exploit
this ready source, in order to determine the porphyrin
composition of bile. First, the HPLC method, practised
in our laboratory for the specific determination of por-
phyrins, analyses a wide variety of biological samples,
using simple and essentially similar purification steps.
Therefore we expected no problems in adapting the
method for the analysis of bile. Secondly, three of the
many patients underwent transplantation following liver
failure due to longstanding erythropoietic protopor-
phyria. In this disease protoporphyrin is produced in ab-
normal amounts, mainly in erythrocytes and also in
liver, due to ferrochelatase deficiency. The hydrophobic
protoporphyrin molecules accumulate in the liver and
have to be removed by excretion in bile. Although proto-
porphyrin is potentially toxic, most erythropoietic proto-
porphyria patients manage to preserve a reasonable liver
function, because the raised quantity of produced proto-
porphyrin can still be adequately excreted in the bile.
However, after the gradual accumulation of protopor-
phyrin, some erythropoietic protoporphyria patients suf-
fer from irreversible liver damage and finally liver fail-
ure, requiring orthotopic liver transplantation in order to
survive (5—10). After transplantation, the patients have
to be monitored regularly for the ability of the liver to
adequately excrete the continuing excessive production
of protoporphyrin from bone marrow, because of the risk
of renewed liver failure (9). For these reasons we con-
sidered it of clinical interest to compare the biliary
excretion of porphyrins of erythropoietic protoporphyria
patients in the weeks after orthotopic liver transplanta-
tion with that of orthotopic liver transplantation patients
operated for liver failure from other diseases.

Further we had at our disposal a number of samples
from different patients after duodenal intubation, i. e. A
bile (fasting duodenal content), B (gall bladder) bile and
C (hepatic) bile. The porphyrin profiles of these samples
were investigated and compared with bile from or-
thotopic liver transplantation patients.

Since none of the bile samples contained traces of por-
phyrins derived from the bacterial conversion of endoge-
nous porphyrins in the gut (i. e. secondary porphyrins
like meso-, pempto- and deuteroporphyrin (3)), we rein-
vestigated the controversial issue of the existence of an
enterohepatic circulation for porphyrins (11—14). For
this purpose large amounts of bile and serum were puri-
fied for analysis of porphyrins. These concentrated ex-
tracts were investigated for the presence of secondary
porphyrins, but these appeared to be absent. As dis-
cussed in this paper, this does not support the hypothesis
that dicarboxylic porphyrins, including protoporphyrin,
are subject to enterohepatic circulation from the lower
gastrointestinal tract. Altogether the results of HPLC
porphyrin profiling in human bile described in this paper
confirm that such determinations constitute a useful ex-
tension of the available procedures for porphyrin profil-
ing in clinical laboratories in certain clinical conditions.

Materials and Methods

Patients and samples

Porphyrins were studied in the bile of two different groups of pa-
tients. The first group of six subjects had undergone an orthotopic
liver transplantation, three of them on account of erythropoietic
protoporphyria. The second group consisted of 11 subjects who for
different reasons were intubated for gall bladder investigations. The
orthotopic liver transplantation procedure in our hospital has been
described earlier (15). The biliary procedure was an end-to-end
choledochocholedochostomy over a bile-tube and removal of the
gall bladder. After 3—4 weeks, or later if indicated, the tube was
clamped, then removed after some days.

The intubation procedure to obtain A-, B-, and C-bile was de-
scribed previously (16). The intubation was performed in patients
who had fasted for at least 14 hours; a plastic tube with a metallic
end was positioned in the third part of the duodenum, using fluo-
roscopic monitoring. A-bile (fasting duodenal contents, consisting
of secretions from the pancreas, bile ducts and duodenum) was
aspirated, whereafter contraction of the gall bladder was stimulated
by intravenous administration of cholecystokinin. Within 10—15
minutes after the injection, dark gall bladder bile was aspirated (B-
bile). After 45 to 60 minutes a third sample of light-coloured aspi-
rate was drawn (C-bile). This C-bile consists mainly of hepatic bile
with pancreatic and duodenal secretions (16).

Bile samples of the patients were obtained with regard to the rules
of the Medical Ethical Committee of our hospital. The samples
were protected from light as much as possible and were stored
frozen without preservation prior to analysis.

Porphyrin analysis

Bile

Two-hundred µl of bile, 200 µl of 3 mol/l HCl and 100 µl of an
internal standard solution (500 nmol/l deuteroporphyrin (Porphyrin
Products, Logan, USA) in a 0.8 mol/l sulphosalicylic acid solution)
were mixed in an Eppendorf cup. After centrifugation, 40 µl of the
clear supernatant was injected onto the HPLC column.

The porphyrins of bile were eluted under the same conditions as
porphyrins of urine, blood and faeces. Details of these methods
including the use of internal standard were described previously
(2, 3).

During the development of the determination of porphyrins in bile the sample volume and its predilutions were chosen so that normal bile samples give peak heights comparable to those seen in normal urine, blood and faecal samples.

Traces in bile

For the determination of traces of porphyrins in bile the following procedure was used. A bile solution of 100 ml, obtained from fractions of bile of different subjects, was divided into two equal portions. To one portion the internal standard deuteroporphyrin was added. Porphyrins were extracted twice with 100 ml of a freshly prepared ether — glacial acetic acid solution (3 + 1, by vol.). In a waterbath set at 37 °C the extracts were evaporated to dryness by means of a nitrogen stream. The porphyrins were dissolved in 1 ml of 6 mol/l HCl solution, and 40 µl of the acid solution was injected onto the HPLC column.

Traces in serum

Due to the low porphyrin and high protein concentrations in serum compared with bile, the porphyrin extraction procedure for serum completely differs from that of bile. The porphyrins were extracted according to a combination of methods described by *Falk* (17). Briefly, a portion of 200 ml serum, pooled from more than 50 different subjects, was divided into two equal portions. To one portion the internal standard deuteroporphyrin was added. To the sera a mixture of 500 ml of ethyl acetate and glacial acetic acid (3 + 1, by vol.) was added slowly with vigorous stirring and the mixtures were allowed to stand for three hours. The precipitated proteins were removed on a *Büchner* funnel, and washed well two times with 100 ml of an ethyl acetate — acetic acid mixture (3 + 1, by vol.). The protein residues may still contain considerable amounts of uroporphyrins, but since we were concerned only with tetra- and dicarboxylic porphyrins no further attention was paid to the uroporphyrins. The combined filtrates were transferred to a separating funnel, and washed twice with 300 ml of a saturated sodium acetate solution. The washings were extracted twice with 100 ml of ethyl acetate. The ethyl acetate layers were added to the main ethyl acetate — acetic acid layers. The combined ethyl acetate layers were washed once with 100 ml of 0.2 mol/l sodium acetate solution. The porphyrins were extracted completely from the ethyl acetate — acetic acid solution with 2 portions of 50 ml of a 4 mol/l HCl solution. In a separating funnel the porphyrins were transferred from the combined HCl extract into 500 ml ether by adjusting the pH to about 4 with a solution of 4 mol/l NaOH and 50 ml of a saturated sodium acetate solution. The mixture was shaken frequently thus preventing precipitation of porphyrins. The aqueous layers were re-extracted twice with fresh ether, and the ether fractions were combined. The combined ether layers were washed once with 100 ml of 0.2 mol/l sodium acetate solution, and their volumes were reduced by 95% under a nitrogen stream. The porphyrins were extracted in 500 µl of 6 mol/l HCl, and 200 µl of this solution was injected onto the HPLC column.

Reproducibility, linearity and recovery

The intra-assay coefficient of variation of the method was determined with bile containing normal coproporphyrin I and III concentrations and a high content of protoporphyrin (2.5 µmol/l). This sample was assayed 10 times in the same run. The linearity of the method was determined by analysing two biles with a high coproporphyrin I and protoporphyrin content, respectively, in increasing volumes (10, 20, 50, 100, 200, 300, and 400 µl) to which 100 µl of internal standard and HCl solution (3 mol/l) were added to an end volume of 600 µl. The recoveries of coproporphyrin I and protoporphyrin were determined in normal bile fortified with 100 and 30 nmol/l, respectively.

Results

Chromatograms of bile porphyrins after orthotopic liver transplantation are shown in figure 1 (1a and 1c, non-erythropoietic protoporphyria patients; 1b and 1d, a single patient at different times after orthotopic liver transplantation). The following porphyrins were found to be present in bile: coproporphyrin I and III (peaks numbered 1 and 2) and protoporphyrin (peak 4). Clearly the erythropoietic protoporphyria patient excretes considerably more protoporphyrin in bile. From figure 1, one can further deduce that bile contains no pempto-, and mesoporphyrin (peaks numbered 7 and 8), while deuteroporphyrin (peak 3) also appeared to be absent when omitted as an internal standard (not shown in fig. 1). In order to ascertain that bile and serum contained not even traces of these porphyrins, we concentrated normal bile and serum extracts about fifty to two hundred times (compared with routine procedures) and injected these extracts onto the HPLC column. The results of these experiments are shown in figure 2 (2a: bile with, 2b: bile without internal standard, 2c: serum with internal standard, 2d: plain serum) and they demonstrate again that no traces of deutero-, pempto-, and mesoporphyrin are present in bile or serum. There is therefore no objection taking deuteroporphyrin as the internal standard for the determination of biliary porphyrins. Figures 1 and 2 show that peaks (numbered 5 and 6) of unknown compounds are also found, eluting between copro- and deuteroporphyrin. Investigation of many other bile samples revealed that the peak heights of these unknown compounds did not correlate with those of protoporphyrin (see for example the chromatograms shown in fig. 1).
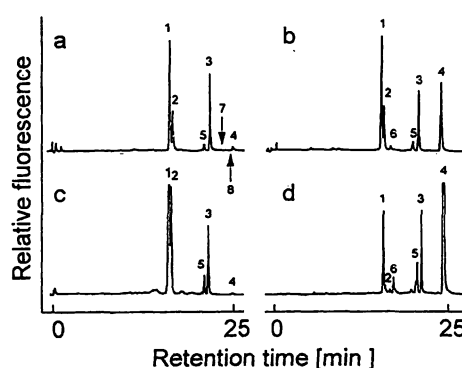

Fig. 1 Chromatograms of porphyrins in bile of patients who underwent an orthotopic liver transplantation. Chromatograms 1a and 1c are obtained from patients not suffering from a porphyric disease. Depicted are porphyrins in bile two days (1c) and three weeks (1a) after orthotopic liver transplantation. Chromatogram 1b and 1d are from one patient with erythropoietic protoporphyria, 50 and 56 days after orthotopic liver transplantation, respectively. Bile for 1d was collected during a surgical operation for peptic ulceration. Numbering:

1 = coproporphyrin I, 2 = coproporphyrin III, 3 = internal standard deuteroporphyrin, 4 = protoporphyrin, 5 and 6 unknown compounds, 7 = pemptoporphyrin, 8 = mesoporphyrin.

Because of their porphyric-like fluorescence it is tempting to speculate that these unknown compounds possess a porphyrin structure. Although of potential clinical interest, we have not attempted to study further the nature of the compounds represented by these peaks.

Figure 3 shows data for the time course of concentration changes of coproporphyrin I and protoporphyrin in bile of different patients after orthotopic liver transplantation. Figures 3a and 3b represent results of three erythropoietic protoporphyria patients (one of them died half a year after orthotopic liver transplantation, the other two
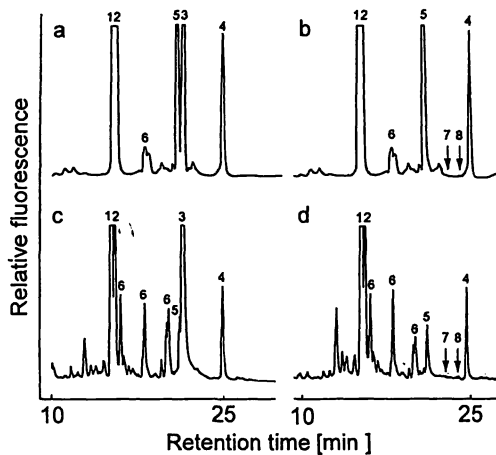

Fig. 2 Chromatograms of porphyrins in extracts of bile and serum, showing the absence of secondary porphyrins in these body fluids. Bile (2a and 2b) is concentrated fifty, and serum (2c and 2d) two hundred times. To the extracts 2a and 2c, the internal standard deuteroporphyrin was added, to final concentrations of 50 and 75 nmol/l, respectively.

For explanations of the numbers see figure 1.

In both serum and bile, coproporphyrin I is the most prominent porphyrin. the copro I/proto ratio in this serum pool is 4.4. The estimated protoporphyrin concentration in the serum pool is about 70 pmol/l.

are still alive three years after orthotopic liver transplantation). Figures 3c and 3d depict results of three orthotopic liver transplantation subjects, who were transplanted because of primary biliary cirrhosis (n = 1), chronic active (n = 1) and inactive cirrhosis (n = 1).

Table 1 presents a survey of the results of porphyrin concentrations in A-, B-, and C-bile of a number of patients.

The coefficients of variation for intra-run precisions measured for coproporphyrin I, coproporphyrin III and protoporphyrin were 2.1, 5.3 and 1.1% (n = 10), respectively. The method was linear up to 4 μmol/l for all porphyrins, while recoveries for coproporphyrin I and protoporphyrin were > 95%.

Discussion

Reports of determinations of porphyrins in bile are scanty, partly due to the fact that bile is not easily available and partly because analysis of bile is not essential for the diagnosis and follow up of porphyric patients. However, in a recent report, the analysis of porphyrins in bile proved to be superior to the analysis in faeces in establishing the diagnosis of variegate porphyria, as the difference between normal and pathological porphyrin excretions in bile was greater than in faeces (4). Moreover, investigation of biliary porphyrins provides information on the ability of the liver to excrete the different porphyrins. In contrast to uro- and coproporphyrins, highly hydrophobic protoporphyrin can only be excreted in bile, hepatic secretion being rate limiting (19). In the case of erythropoietic protoporphyria, the increased blood protoporphyrin tends to accumulate in the liver, which firstly can lead to cholestasis and eventually to

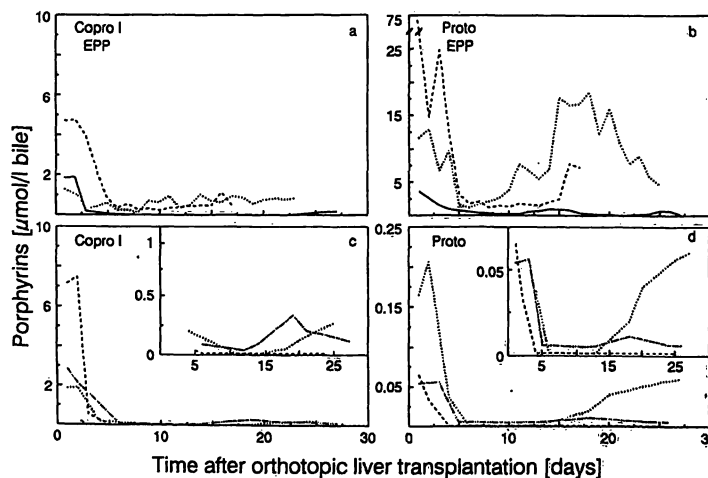Time after orthotopic liver transplantation [days]

Fig. 3 Time course profiles of coproporphyrin I (copro I) and protoporphyrin (proto) in bile of patients who underwent an orthotopic liver transplantation. In figure 3a and 3b the profiles of three erythropoietic protoporphyria patients (EPP) are shown, whereas in figure 3c and 3d profiles of three non-porphyric patients are depicted. The solid line in figure 3a and 3b represents the erythropoietic protoporphyria patient with a putative cholestatic liver.

Tab. 1 Survey of the porphyrin analyses of A-, B-, and C-bile from patients who underwent a duodenal intubation.

	Mean	Median	Minimal	Maximal
Coproporphyrin I (nmol/l)				
A-bile (n = 11)	158	103	31	378
B-bile (n = 9)	249	244	83	363
C-bile (n = 10)	139	111	27	309
Coproporphyrin III (nmol/l)				
A-bile	71	30	11	411
B-bile	77	87	33	147
C-bile	47	36	11	134
Protoporphyrin (nmol/l)				
A-bile	9	<0.5	<0.5	22
B-bile	11	6	<0.5	29
C-bile	12	6	<0.5	32
Copro I/copro I + III				
A-bile	0.76	0.79	0.48	0.86
B-bile	0.76	0.80	0.50	0.88
C-bile	0.75	0.79	0.47	0.82
Copro I/protoporphyrin				
A-bile	18	–	–	30
B-bile	26	13	–	48
C-bile	18	18	–	28

terminal liver failure, requiring orthotopic liver transplantation for survival. The fact that bile can be obtained from orthotopic liver transplantation patients during the first weeks after the operation enabled us to compare the biliary porphyrin excretion, in particular of orthotopic liver transplantation, of three transplanted erythropoietic protoporphyria patients with that of some non-erythropoietic protoporphyria patients. As shown by the chromatograms in figure 1 and the quantitative data in figure 3 (time course of porphyrin concentrations in the bile of 3 erythropoietic protoporphyria patients and 3 non-erythropoietic protoporphyria patients after orthotopic liver transplantation), biliary protoporphyrin is always much higher in erythropoietic protoporphyria patients than in non-erythropoietic protoporphyria patients (in quantitative terms more than a hundred fold), whereas biliary coproporphyrin I and III excretion in erythropoietic protoporphyria patients is only slightly higher. These results clearly demonstrate that in erythropoietic protoporphyria the high amounts of protoporphyrin mainly originate from the erythropoietic system in bone marrow. Figure 3 also shows that in the first 5 days after orthotopic liver transplantation both coproporphyrins and protoporphyrin are excreted in huge amounts, which then return to very low values, later tending to rise somewhat again. The reason for the strikingly raised biliary porphyrin excretion in the first days after orthotopic

liver transplantation cannot be given with certainty and is the subject of further study. It might be ascribed to the accumulation of porphyrins produced in the donor liver in the period between its removal and the time that transplantation took place and/or to the accumulation of porphyrins in the host during the operation phase. After transplantation these accumulated porphyrins could then be washed out. After approximately 5 days, a new steady state situation is attained, in which the production and excretion of porphyrins match again, and this washout would no longer occur. To our knowledge, porphyrins are the only compounds excreted in highly increased amounts immediately after orthotopic liver transplantation (15).

During the 5–15 days after orthotopic liver transplantation a temporary difficulty in excretion of porphyrins might be the case, or a low porphyrin de novo synthesis due to liver regeneration, thus explaining the relatively low biliary excretion of porphyrins in that period. However, in two of the three erythropoietic protoporphyria patients, 10–15 days after orthotopic liver transplantation, protoporphyrin values show a sustained rise to very high levels, which vary greatly in individual patients from day to day.

The third erythropoietic protoporphyria patient (see fig. 3) continuously excreted considerably less protoporphyrin than the other two erythropoietic protoporphyria patients, despite high protoporphyrin concentrations in blood (15 $\mu mol/l$, normal < 0.56 $\mu mol/l$). Also his protoporphyrin concentrations in serum were increased (data not shown), reflecting the absence of good liver function (18). He had severe cholesterasis and died half a year after orthotopic liver transplantation. Retrospectively it can be stated that even from the start of orthotopic liver transplantation he probably had difficulties in adequately excreting protoporphyrin, leading to an accumulation of protoporphyrin, which in turn contributed to early liver failure (18). Protoporphyrin was undetectable in the serum of the other orthotopic liver transplantation patients and the other two erythropoietic protoporphyria patients, using our method, which has a detection limit of < 0.5 nmol/l (data not shown).

The complete absence of deutero-, pempto- and mesoporphyrins in the bile of orthotopic liver transplantation patients (see fig. 1) as well as in A-, B- and C-bile (chromatograms not shown) motivated us to investigate the question of whether or not an enterohepatic circulation of porphyrins operates in humans. These latter porphyrins are produced in the gut as a result of reductive conversion of protoporphyrin by anaerobic bacteria and are markedly present in faeces (3, 19, 20). If an enterohepatic circulation exists, there should be evidence of these "secondary" porphyrins in serum and bile, analogous to

the presence of secondary bile acids (deoxycholic acid and lithocholic acid) in serum and bile, produced in the gut by bacterial conversion of primary bile acids (cholic acid and chenodeoxycholic acid). In the literature the enterohepatic circulation of porphyrins constitutes a controversial issue. Some reports state that such a circulation exists (11−13), whereas others state the reverse (14). It might be that secondary porphyrins are present in bile or serum in trace amounts. To exclude this possibility we worked up portions of 50 ml of collected bile and 100 ml of pooled serum, each with and without internal standard (deuteroporphyrin). As figures 2a−d demonstrate, even in the concentrated extracts of bile and serum no traces of secondary porphyrins could be detected, which does not support the hypothesis that porphyrins are subject to enterohepatic circulation at the lower part of the gastrointestinal tract. This finding does not exclude the presence of an enterohepatic circulation of copro- and uroporphyrins nor the absorption of protoporphyrin at the upper part of the gut. In one of our previous studies it was demonstrated that in normal subjects more than 90% of the primary porphyrins in faeces (protoporphyrin and coproporphyrins) and all of the secondary porphyrins are synthesized by bacteria in the lower part of the small bowel and colon (3). Since dicarboxylic porphyrins are not absorbed in these parts, the total enterohepatic circulation of porphyrins at the upper part of the gastrointestinal tract, if existent, would be relatively small under normal conditions, due to the low biliary concentrations of porphyrins.

Finally, we analysed the porphyrin composition of A-, B- and C-bile from various patients not suffering from any of the porphyrias (see tab. 1). First of all, it is obvi-

ous that the concentration of porphyrins in these bile samples varies widely. The patterns of A-, B- and C-bile, which show close resemblance with those of bile obtained from (non-erythropoietic protoporphyria) orthotopic liver transplantation patients, are not essentially different, although the mean concentrations in B-bile are higher than in A- and C-bile. Apart from two unknown compounds ("peaks 5 and 6") coproporphyrins I and III, and protoporphyrin are present. Coproporphyrin I is almost always the prominent biliary porphyrin and its concentration far exceeds that of protoporphyrin, the latter not exceeding a value of approximately 30 nmol/l. The overall mean ratios coproporphyrin I/coproporphyrin I and III (copro I ratio) and coproporphyrin I/protoporphyrin are 0.76 and 21, respectively. The same results in bile have been previously reported (21, 22). Similar copro I ratios also are detected in faeces (2, 22). Only in one case was a copro I ratio of less than 0.50 (coproporphyrin III concentration exceeding that of coproporphyrin I) found. In contrast to bile, normal faecal protoporphyrin concentrations (and its derivatives deutero-, pempto- and mesoporphyrin) often markedly exceed those of coproporphyrin I. This can be attributed to both bacterial production and conversion of protoporphyrin in the gut (3, 19, 20).

To summarize, the profiling of porphyrins in bile reveals interesting aspects of their hepatic clearance, and shows that their quantitative determination can be of value in some clinical situations, e. g. the follow up of protoporphyrin excretion in erythropoietic protoporphyria patients after orthotopic liver transplantation. The absence of secondary porphyrins in bile (and serum) suggests the absence of an enterohepatic circulation for dicarboxylic porphyrins.

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