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Evaluation and Clinical Application of the Enterotest® for the Determination of Human Biliary Porphyrin Composition

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Summary: The Enterotest® string test is an easy and non-invasive method for sampling duodenal fluid, which has been successfully used for the analysis of duodenal microflora, as well as biliary bile acid and lipid composition. The method was evaluated for determination of porphyrins in duodenal bile in normal subjects and subjects with porphyria, following cholecystokinin induced gall bladder contraction; it is known that analysis of biliary porphyrins is more discriminatory for the diagnosis of asymptomatic porphyria than their analysis in faeces or urine. Moreover, serial analysis of bile from patients with erythropoietic protoporphyria may help in establishing their ability to secrete protoporphyryn in bile and to assess effects of treatment.

The binding of various porphyrins to Enterotest® strings was investigated by incubating pieces of the string in different human bile samples with low to very high porphyrin concentrations, followed by HPLC analysis of porphyrins both in the native bile and in extracts obtained from the strings. No differences between porphyrin composition in native bile and extracts were observed. Duodenal fluid obtained by means of the Enterotest® from volunteers not receiving cholecystokinin showed large variations in porphyrin patterns not resembling those of native bile. Mesoporphyrin, a secondary porphyrin derived from protoporphyryn by bacteria, was often detectable. These data indicate that the duodenal content without cholecystokinin injection does not reflect biliary porphyrin composition. The presence of mesoporphyrin in the whole intestinal tract, but not in serum and bile, suggests that there is no enterohepatic circulation of secondary porphyrins.

There was close agreement between the porphyrin ratios found with the standard duodenal intubation technique and the Enterotest®, performed simultaneously in one healthy volunteer after induction of gall bladder contraction by cholecystokinin. From these experiments, it was concluded that fluid adsorbed to the Enterotest® string after gall-bladder contraction can be used to determine biliary porphyrin composition.

Since duodenal bile is diluted gall bladder bile, variable porphyrin concentrations were found when applying the Enterotest® in combination with cholecystokinin in the same subject on successive days. However, porphyrin ratios, such as the protoporphyryn to coproporphyrin I ratio, were relatively constant.

In subjects with symptomatic variegate porphyria, the Enterotest® showed highly aberrant porphyrin patterns, with increased protoporphyryn to coproporphyrin I ratios and, in addition, the presence of some unknown porphyrins. A deviating biliary protoporphyryn/coproporphyrin I ratio in one patient appeared to be a useful diagnostic index for the presence of latent variegate porphyria (or variegate porphyria in remission).

Finally, the Enterotest® method is also useful for monitoring patients with erythropoietic protoporphyria for their ability to secrete protoporphyryn via bile, evaluated by measurement of protoporphyryn/coproporphyrin I and III ratios in Enterotest® strings.

We conclude that the Enterotest® is a valuable tool for investigating porphyrin metabolism and biliary secretion in humans.

Introduction

Porphyrias comprise a group of autosomal inherited disorders of haem synthesis (1). Variegate porphyria is clinically characterized by attacks leading to photosensitivity and neurologic dysfunction. This variant is usually symptomless in periods between attacks, which can be evoked by drugs, alcohol, hormones, stress, infection or starvation (2). During attacks, porphyrin levels in urine and faeces are very high, and enable diagnosis of the disease (3). Identification of porphyric carriers is important because potentially fatal attacks can be prevented by avoiding porphyrogenic drugs and circumstances (4). However, in the latent phase only increased porphyrins in bile are detectable, because food, bacteria and haemorrhage in the gut blur the borderline between normal and abnormal porphyrin patterns in faeces, but not in bile (5).

Erythropoietic protoporphyria is a disease with severe photosensitivity and multiple cutaneous eruptions after sun exposure. It can develop into liver cirrhosis and fulminant liver failure, due to hepatic protoporphyrin accumulation (6–10). Biliary protoporphyrin excretion can be useful for evaluating the capacity of the liver to excrete this substance in this condition (11).

Thus, examination of porphyrins in bile has potential clinical value in certain situations. Although gall bladder bile is an excellent medium for this purpose, its collection is elaborate and extremely unpleasant for patients. This is probably the main reason that the porphyrin composition of bile is rarely investigated for diagnostic purposes (11–13). At present, the duodenal intubation technique is the only non-surgical method for obtaining duodenal bile (5). Duodenal bile can be considered as diluted gall bladder bile, consisting of a mixture of duodenal, gastric and pancreatic secretions. In addition, biliary porphyrin concentrations strongly depend on the retention time of bile in the gall bladder. In principle, interpretation of results obtained with duodenal fluid is not necessarily hampered by these facts, as dilution and concentration effects can be eliminated by using ratios instead of absolute concentrations. For instance, the protoporphyrin/bile acid ratio has been used for diagnostic purposes (5, 11). In this study, we demonstrate that porphyrin ratios can fulfil a similar role in the bile of subjects with porphyria.

Although advances in the intubation technique for obtaining duodenal bile have been made, such as speed of performance and control of the zone from which the sample is obtained (5), patient discomfort, radiation burden, and equipment requirements for tube placement and control of position remain the principal drawbacks of this technique. We therefore looked for a more conve-

nient sampling method to obtain duodenal bile. The Enterotest® is an accurate, simple and non-invasive diagnostic tool, so far successfully used for sampling duodenal fluid for detection of microorganisms and analysis of bile acids and lipid composition (15–18). We anticipated that the Enterotest® would also permit an easier and more routine measurement of porphyrins in duodenal bile, than is at present possible with the standard duodenal sondage techniques. Therefore a study was performed to investigate the usefulness of the Enterotest® as a new diagnostic tool for evaluation of porphyrin secretion in subjects with porphyria, using both duodenal fluid (duodenal contents sampled without gall bladder contraction) or duodenal bile (duodenal content sampled 15 minutes after contraction of the gall bladder).

Subjects, Materials and Methods

Subjects

The participants in this study were 7 healthy volunteers (20–60 years, 3 females and 4 males), 5 females (49–66 years) and 3 males (47–53 years) with proven variegate porphyria, one female (50 years) suspected of latent variegate porphyria, and two females (34–39 years) with erythropoietic protoporphyria, who had undergone orthotopic liver transplantation. More details of the latter two subjects are given elsewhere (13, 19). A number of participants underwent the Enterotest® more than once.

Materials

Porphyrin standards were obtained from Porphyrin Products, Logan, USA.

Cholecystokinin octapeptide was purchased from Ferring Pharmaceuticals, Malmö, Sweden. The Enterotest® was from Health Development Corp., Mountain View, CA, USA.

Enterotest®

The Enterotest® consists of a gelatine-encapsulated nylon string, which has to be swallowed with water while one end is taped at the corner of the mouth. The gelatine capsule dissolves in the stomach, and the string, which is heavier at its distal end, enters the duodenum by peristalsis in the following few hours or less. The upper intestinal fluid is adsorbed on the string, which is then recovered by withdrawing from the mouth. Patients and volunteers were in a fasting state, otherwise no special instructions were given concerning their behaviour during the test (18).

After pulling out, the pH of the string was checked with the pH stick supplied with the Enterotest®. The string part with a pH > 7 was cut into lengths of 10 cm, which were placed in preweighed Eppendorf cups, then weighed. Weight differences without corrections for the weight of the string itself were used for the calculation of the amount of fluid adsorbed on the string. This enables the investigation of the porphyrin content of different parts of the string. The pieces were eluted with an acid solution and a solution containing the internal standard (500 nmol/l deuteroporphyrin in a 0.8 mol/l sulphosalicylic acid solution), or they were extracted with a freshly prepared solution of diethyl ether/acetic acid, also after addition of the internal standard solution (20).

Incubation of duodenal sondage samples or native bile with pieces of Enterotest® string

A, B and C bile samples used in this study were obtained by duodenal sondage techniques as described previously (21), whereas bile of erythropoietic protoporphyria patients, who had undergone orthotopic liver transplantation, was collected by means of a temporary implanted T-tube as described previously (22).

Two 10 cm pieces of the Enterotest were incubated in 2.0 ml aliquots of these bile samples for 1 hour. In this way, several bile samples with different porphyrin concentrations were incubated. These strings were investigated for their porphyrin content, using both HCl elution and ether extraction, and the results were compared with those obtained from direct investigation of porphyrins in the corresponding native bile samples.

Unstimulated duodenal fluid

Four hours after being swallowed by a subject in a fasting state, the Enterotest® string was retrieved and stored at -20 °C until analysis of porphyrins.

Stimulated duodenal bile

The Enterotest® string was swallowed by preference at night at home. The next morning at hospital, or in some cases at home, gall bladder contraction was induced by intramuscular administration of cholecystokinin at a dose of 20 ng/kg of body weight given as bolus injection in the upperleg, or given intravenously as a dilution with 9 g/l saline, according to the instructions of the manufacturer. Exactly 15 minutes after injection, the Enterotest® string was retrieved for porphyrin analysis, which was performed on 10 cm pieces of the yellow and white parts of the string.

Comparison of duodenal sondage samples and Enterotest® strings

Results from the standard duodenal intubation technique for sampling bile were compared with those of the Enterotest®. For this purpose, a string was applied to the pharynx, and the location of the tube was determined by X-ray. By means of vacuum drainage a portion of 1 ml of unstimulated duodenal fluid was sampled, followed by induction of gall bladder contraction with cholecystokinin (intramuscularly). Immediately after sampling a portion of 2 ml of dark yellow „B“ bile, both the string and tube were retrieved. The distal dark yellow coloured end and the proximal uncoloured part of the string were investigated for porphyrins with the HCl elution method.

Determination of porphyrins in urine, faeces, bile and duodenal sondage samples

Porphyrins in urine, faeces and bile were analysed according to previously described methods (13, 20, 23). Duodenal sondage samples were treated as bile samples.

Determination of porphyrins adsorbed on the string using the HCl elution method

In Eppendorf cups, 10 cm lengths of Enterotest® string were eluted with 100 µl of 3 mol/l and 50 µl of the internal standard solution, thoroughly mixed with a vortex mixer and centrifuged at 13 000 g for 5 min at room temperature. Aliquots of 50 µl of the clear supernatant were injected onto the HPLC column.

Determination of porphyrins adsorbed on the string using the ether extraction method

In glass stoppered tubes, 50 µl of the internal standard solution were added to 10 cm lengths of string, and the porphyrins were

extracted twice with 2.0 ml of a freshly prepared ether-glacial acetic acid solution (3 : 1, by vol.). The ether layers were combined and evaporated under a stream of nitrogen in a heater set at 30 °C. The porphyrins were dissolved in a 300 µl solution of 3 mol/l HCl, and 50 µl of the dissolved residue were injected onto the HPLC column.

Statistical calculations

Group means were compared by the *Student* t-test. Differences were considered significant if p values were less than or equal to 0.05.

Results and Discussion

Validation of the Enterotest®

We started our investigation on the usefulness of the Enterotest® procedure with “in vitro” experiments. Results of porphyrin analysis on pieces of string incubated in 2.0 ml portions of bile, with low to high concentrations of coproporphyrin I, coproporphyrin III and protoporphyrin, were compared with those obtained from direct porphyrin analyses in the same (native) bile samples. Moreover, we compared porphyrin profiles in native bile samples before and after incubation with Enterotest® string. In this way, we aimed to establish whether porphyrin profiles, determined after adsorption of biliary porphyrins on string, differ from those of native samples, e. g. due to preferential binding of porphyrins to the string or to incomplete desorption of the adsorbed porphyrins from the string. For the analysis of porphyrins adsorbed on pieces of string, both the “HCl elution method” and the “ether extraction method” were employed.

In figure 1, four porphyrin profiles from the same bile sample (of a non-porphyrin subject) are shown: one in native bile, one in native bile after incubation with string, one in incubated string analysed by the HCl elution method and one in incubated string analysed by the ether extraction method. This figure clearly demonstrates a good qualitative correspondence between the profiles. The same applies when quantitative results, obtained for the three main biliary porphyrins, coproporphyrin I, coproporphyrin III and protoporphyrin, were compared by linear regression analysis, using ten different bile samples. All possible pairs of analytical methods were considered. The derived slopes, intercepts and squared correlation coefficients (R^2) are given in table 1. Paired t-tests showed no statistical differences between the corresponding samples. The correlation coefficients for the porphyrins found in native bile and determined with the two string methods (HCl elution and ether extraction), are good, the HCl elution method showing a somewhat better score. The latter method gives slopes of approximately 1 for the coproporphyrins,

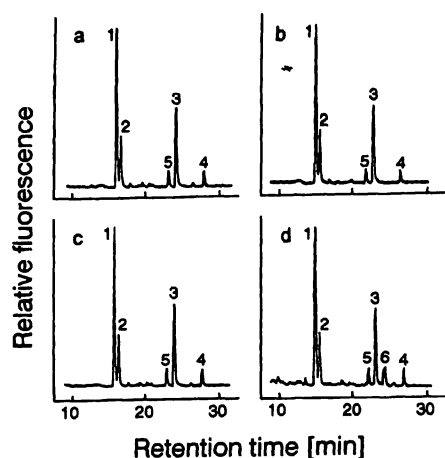


Fig. 1 Chromatograms of porphyrins in bile obtained by different methods, showing absence of preferential binding of porphyrins to the Enterotest® string.

a) native bile sample;
 b) bile recovered from 10 cm string and processed according to the "HCl elution" method;
 c) bile after incubation with two pieces of 10 cm string;
 d) bile recovered by 10 cm string and processed according to the "ether extraction" method.

Peak numbers: 1: coproporphyrin I; 2: coproporphyrin III; 3: internal standard deuteroporphyrin; 4: protoporphyrin; 5 and 6 are unknown compounds.

with intercepts smaller than those of the extraction method. However, the recovery (slope) of protoporphyrin is somewhat higher for the ether extraction method than for the HCl elution method. Apparently, there is some loss of protoporphyrin in the HCl elution method, but the found concentrations are still fairly comparable

to those found in native bile. The correlation coefficients found by comparison of the results obtained for two string methods with those obtained for native or incubated bile suggest that Enterotest® results for all porphyrins correspond well with those found in native bile. Altogether, from figure 1 and the results shown in table 1, we conclude that the Enterotest® string does not preferentially bind specific porphyrin species. The ether extraction method is more laborious than the HCl elution method, and, in addition, the extracts contain more non-porphyrinic compounds (see fig. 1d). Furthermore, if uroporphyrin is present in bile (normally very low to absent), this will not be recovered by the ether extraction method. Therefore the HCl method was used in subsequent studies.

The Enterotest® applied to normal volunteers without and with gall bladder contraction

We investigated porphyrin profiles from Enterotest® strings swallowed by seven healthy volunteers to see whether the porphyrin profiles corresponded with those reported for normal human bile (either gall bladder bile or duodenal aspirates). Human bile is characterized by relatively high concentrations of coproporphyrin I, lower concentrations of coproporphyrin III and a relatively low concentration of protoporphyrin (6–20 times lower than coproporphyrin I) (12, 13). Secondary porphyrins, like deuter-, meso-, and pemptoporphyrin are absent from normal human bile (13). Our reference val-

Tab. 1 Linear regression values for methods of porphyrin analyses in bile for in-vitro validation of non-preferential binding of porphyrins by Enterotest® strings. The concentrations for different biliary porphyrins ranged from 0–4000 nmol/l (coproporphyrin I), from 0–3800 nmol/l (coproporphyrin III) and from 0–7200 nmol/l (protoporphyrin).

R²: squared coefficient of correlation.

"Native": native bile samples; "incubated": bile samples after incubation with pieces of string; "elution": incubated pieces of string processed by the "HCl elution" method; "extraction": incubated pieces of string processed by the "ether extraction" method.

Independent: x-axis; Dependent: y-axis.

Porphyrin species	Independent	Dependent	Intercept	Slope	R ²
Coproporphyrin I	Native	Incubated	11.5	0.960	0.9890
	Native	Elution	-10.5	0.984	0.9845
	Native	Extraction	39.8	0.884	0.9452
	Incubated	Elution	0.6	1.040	0.9669
	Incubated	Extraction	46.7	0.842	0.9347
	Elution	Extraction	53.7	0.788	0.9142
Coproporphyrin III	Native	Incubated	14.0	0.871	0.9973
	Native	Elution	-11.9	1.141	0.9905
	Native	Extraction	17.3	0.795	0.8565
	Incubated	Elution	-3.4	1.042	0.9143
	Incubated	Extraction	29.1	0.676	0.7506
	Elution	Extraction	32.0	0.644	0.8073
Protoporphyrin	Native	Incubated	-3.8	1.089	0.9984
	Native	Elution	-0.1	0.658	0.9974
	Native	Extraction	11.5	0.707	0.9532
	Incubated	Elution	-6.0	0.870	0.9737
	Incubated	Extraction	3.1	1.009	0.9482
	Elution	Extraction	8.9	1.170	0.9901

ues are: coproporphyrin I: < 400 nmol/l, coproporphyrin III: < 400 nmol/l and protoporphyrin: < 40 nmol/l bile.

After withdrawing strings from the upper intestine of healthy volunteers without injection of cholecystokinin, it was noted that the strings were not homogeneously coloured and contained white, brown, and yellow areas. Therefore, we divided the strings into 10 cm pieces regardless of the colour. Porphyrin profiles in the first and fifth piece of string are depicted in figure 2a and 2b and the derived concentrations of the observed porphyrins in all eight pieces of string in figure 2c and 2d, demonstrating a non-homogeneous distribution of porphyrins along the length of the string. Furthermore, these porphyrin profiles do not resemble at all the characteristic patterns obtained from normal human bile as shown in figure 1. Protoporphyrin is the predominant porphyrin in this duodenal fluid and mesoporphyrin is frequently detectable. Mesoporphyrin is a dicarboxylic porphyrin derived from protoporphyrin by bacterial conversion (23). Levels of uroporphyrin in unstimulated duodenal fluid ranged from undetectable to levels of 36 nmol/l. The coproporphyrin levels (coproporphyrin I often being absent) were always lower than the protoporphyrin levels, and low to high protoporphyrin concentrations were detected from day to day when the Enterotest[®] was per-

formed on consecutive days in the same volunteers. Porphyrins in these samples presumably originate largely from food products and/or are produced by bacteria in the gut (23, 24). This experiment indicates that duodenal fluid without induction of gall bladder contraction cannot be used for the analysis of biliary porphyrin composition. On the other hand, the results permit conclusions as to the presumed presence of an enterohepatic circulation of porphyrins, as proposed by *Pimstone* et al. (25). In a previous study from our laboratory, no traces of dicarboxylic porphyrins like meso-, pempto-, and deuteroporphyrin could be demonstrated in serum and bile, whereas these porphyrins are found in huge amounts in faeces (13, 23). From these results were concluded that there is no enterohepatic circulation of dicarboxylic porphyrins at the distal part of the intestinal tract (13). In the present study we found mesoporphyrin in the duodenum and upper bowel. We now conclude that there is no, or at most a negligible, enterohepatic circulation of dicarboxylic porphyrins in the whole intestinal tract. A third conclusion is that porphyrins are not homogeneously distributed in duodenum and upper bowel.

Although the Enterotest[®] used in this way did not yield porphyrin patterns representative of human bile, we reasoned that these could possibly be obtained after injection of cholecystokinin to enforce gall bladder con-

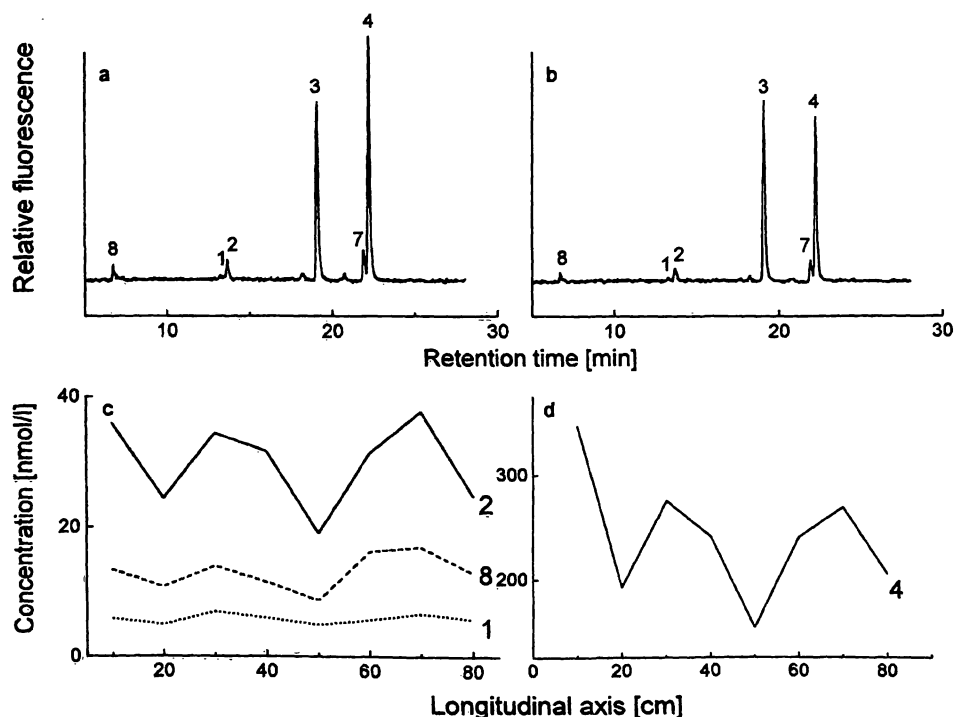


Fig. 2 Porphyrin profiles and concentrations in the longitudinal axis of an Enterotest[®] string from duodenal and upper bowel fluid of a healthy volunteer, performed under unstimulated conditions. The whole nylon string had a pH > 7. Therefore the string (80 cm) was cut into lengths of 10 cm. For explanation of the peak numbers see figure 1 and additionally peak number 7 (mesoporphyrin) and 8 (uroporphyrin).

a) porphyrin chromatogram from the first 10 cm of the string; b) porphyrin chromatogram from the fifth piece; c) coproporphyrin I, coproporphyrin III and uroporphyrin concentrations; d) protoporphyrin concentrations, in each of the obtained eight pieces of string.

traction. Therefore, a healthy volunteer swallowed an Enterotest® string at night, and received an intramuscular injection of cholecystokinin at the hospital the next morning. Fifteen minutes later the string was recovered. The proximal part of the string was uncoloured whereas the distal part was dark yellow. Porphyrin profiles of both parts are shown in figure 3a and 3b respectively. The porphyrin pattern in figure 3a (high protoporphyrin, but low coproporphyrins) does not look like the normal biliary pattern as shown in figure 1, in contrast to the closely similar pattern depicted in figure 3b (high coproporphyrins, low protoporphyrin). Apparently the colour of the strings is indicative of the presence of adsorbed duodenal bile. Yellow-coloured pieces of string after cholecystokinin injection yield porphyrin patterns characteristic for bile.

Definite proof that the Enterotest® after cholecystokinin injection is an excellent substitute for intubation was provided by the following "in vivo" experiment. This experiment included combined sampling of duodenal sondage fluid and the technique of the Enterotest® string in the same healthy volunteer as in figure 3a and 3b. Duodenal fluid was aspirated as usual before cholecystokinin injection and immediately thereafter. Again, after gall bladder contraction and withdrawal of tube and string, the proximal part of the string was white and the distal part of the string yellow-coloured. Both the yellow and white parts of the string as well as the two duo-

denal sondage samples were investigated for porphyrin composition; the resulting porphyrin profiles are shown in figure 3c–3f. Figure 3c, the porphyrin profile of duodenal sondage fluid before cholecystokinin, and figure 3d, the profile of a piece of white string, are practically identical; similarly figures 3e and 3f correspond very well, being the profiles of duodenal sondage fluid after cholecystokinin and a yellow piece of string respectively. However, it should be noted, by comparison of the peak heights of the internal standards (peaks numbered 3) in figure 3b and figure 3f, which are approximately the same, and those of the endogenous porphyrins (peaks numbered 1, 2 and 4), that the concentrations of the coproporphyrins and protoporphyrin in the string sample depicted in figure 3f are much higher than in the string sample depicted in figure 3b. Apparently the duodenal fluid in the latter sample (fig. 3b) is considerably more dilute than in the former sample (fig. 3f). This "dilution and concentration" problem has already been discussed in the Introduction. On the other hand, the relative concentrations of the porphyrins are essentially the same, suggesting that ratios of biliary porphyrins may be used to evaluate whether or not abnormalities in porphyrin excretion take place. We investigated this issue by calculating ratios of porphyrins in normal native (hepatic) bile, gall bladder bile and duodenal sondage fluid, using data published in the literature (12, 13) and data from the present study. The calculated ratios protoporphyrin/coproporphyrin 1, protoporphyrin/copropor-

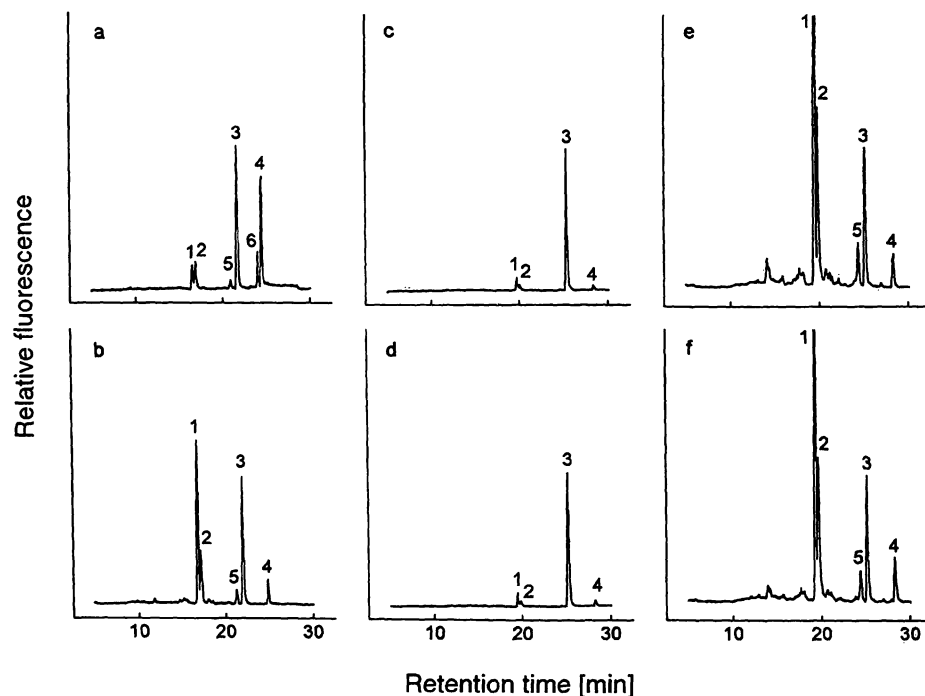


Fig. 3 Chromatograms of porphyrins in duodenal fluid (a) obtained without gall bladder stimulation, and in duodenal bile (b) after artificial gall bladder contraction. The subject was a healthy volunteer, and the results were obtained with the Enterotest®. Also shown are chromatograms of porphyrins in duodenal fluid (c and

d) and duodenal bile (e and f) of the same volunteer, obtained by simultaneously performed duodenal sondage technique (c and e) and the Enterotest (d and f). For identification of the peaks see figure 1.

phyrin III and coproporphyrin I/(coproporphyrin I + III) are shown in table 2. They demonstrate that, with the exception of the protoporphyrin/coproporphyrin III ratio, these ratios only fluctuate within relatively small margins in non-porphyrin subjects. Subsequent experiments in patients with porphyria variegata, as described below, made clear that abnormalities or changes in biliary porphyrin profiles can be conveniently represented by reporting ratios of porphyrin concentrations and comparing them with those in normal bile.

Enterotest® results in subjects with variegata porphyria

The Enterotest® was applied to eight patients known to have variegata porphyria. In addition a patient was investigated, who was suspected of suffering from porphyria variegata in remission, on the basis of occasional neurological problems, light sensitivity and abdominal pain in combination with persistently raised urinary excretion of coproporphyrins and δ -aminolaevulinic acid (coproporphyrin I: 6.8 μ mol/mol creatinine, copro-

porphyrin III: 137 μ mol/mol creatinine and δ -aminolaevulinic acid: 22 mmol/mol creatinine, normal: lower than 2.9, 19.0 and 2.2, respectively). However, porphobilinogen and faecal porphyrins were normal. All these patients swallowed an Enterotest® string at night and were given a cholecystokinin injection the following day in a fasting state; 15 minutes later the string was recovered and porphyrin profiles in yellow pieces of string were determined. The obtained profiles of three of the known variegata porphyria patients and the patient suspected of variegata porphyria are depicted in figure 4. Comparison with profiles of normal bile (see fig. 1, 3b, 3e and 3f) revealed typical differences, characteristic for the presence of variegata porphyria, in particular for the known variegata porphyria patients. The found concentrations of coproporphyrins and protoporphyrin in duodenal bile are shown in table 3 and the calculated ratios in table 2.

Most striking is the considerably raised protoporphyrin excretion in relation to those of coproporphyrins, reflected in abnormally high protoporphyrin/coproporphyrin I and protoporphyrin/coproporphyrin III ratios (see

Tab. 2 Mean values, standard deviations and ranges of three porphyrin ratios in (duodenal) bile, derived from results obtained by using the Enterotest® method, in normal subjects and porphyric patients. Ratios in normal subjects were also compared with ratios

reported previously (12, 13). Enterotest results are designated with the term "this paper", those from the literature with "others".

* Bile sampled during 21 consecutive days;

** Bile sampled during 17 consecutive days.

	Porphyrin ratios						
	Normal subjects			Porphyric subjects			
	Normal This paper n = 4 ³)	Normal Others ¹⁾ n = 8 ³)	Normal Others ²⁾ n = 31 ³)	Variegata porphyria		Erythropoietic protoporphyria	
			symptomatic This paper n = 8 ³)	in remission This paper n = 1 ³)	Subject A* This paper n = 21 ⁴)	Subject B** This paper n = 17 ⁴)	
Protoporphyrin/Coproporphyrin I							
Mean	0.14	0.13	0.06	2.14	0.60	17.3	5.7
S. D.	0.04	0.03	0.03	0.70		12.5	2.7
Minimum	0.08	0.10	0.0	0.9		1.7	3.2
Maximum	0.20	0.18	0.11	7.6 ⁵⁾		52	15
Protoporphyrin/Coproporphyrin III							
Mean	0.53	0.97	0.20	1.28	1.32	716	36
S. D.	0.15	0.46	0.11	0.64		560	32
Minimum	0.39	0.51	0.09	0.63		30	6
Maximum	0.75	1.37	0.32	2.60		1784	110
Coproporphyrin I/Coproporphyrin I + III							
Mean	0.76	0.87	0.76	0.33	0.68	0.84	0.81
S. D.	0.03	0.03	0.10	0.11		0.08	0.13
Minimum	0.73	0.80	0.47	0.22		0.60	0.54
Maximum	0.81	0.91	0.86	0.55		0.91	0.91

¹⁾ Data derived from l. c. (12).

²⁾ Data derived from l. c. (13).

³⁾ Number of different patients, from whom a single sample was determined.

⁴⁾ Number of different samples determined from one single patient.

⁵⁾ Outlier, omitted in the calculations of mean and S. D.

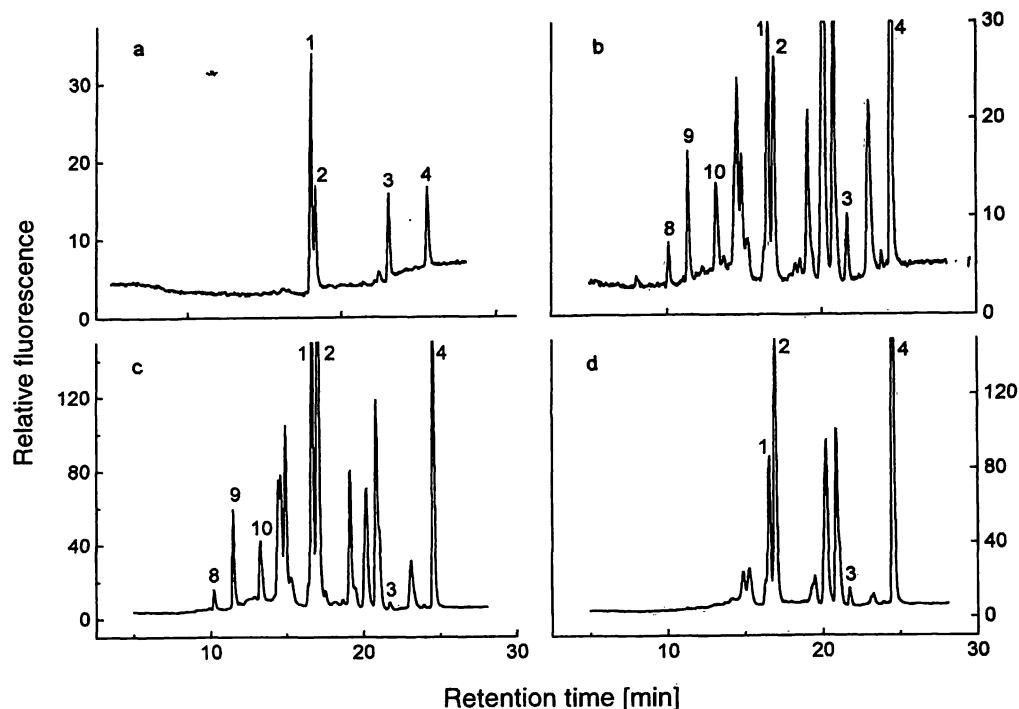


Fig. 4 Chromatograms of porphyrins in duodenal bile of four subjects with variegate porphyria, obtained by means of the Enterotest® and artificial gallbladder contraction.

a) subject with variegate porphyria in remission; b)–d) subjects with symptomatic variegate porphyria.

Peak number 9: heptacarboxylate porphyrin; 10: hexacarboxylate porphyrin.

For further identification of the numbered peaks see figure 1. Peaks which are not numbered are unknown (porphyrin) compounds.

tab. 2). Coproporphyrin I/(coproporphyrin I + III) ratios are lower, due to relatively increased coproporphyrin III levels, while the predominant excretion of coproporphyrin I, occurring in normal bile, is less pronounced. In some patients, coproporphyrin III was even higher than coproporphyrin I (figure 4c and 4d). In the patient with variegate porphyria in remission (fig. 4a) the porphyrin pattern differed less from normal, but the calculated protoporphyrin/coproporphyrin I ratio (see tab. 2) was markedly above the upper normal limit. In combination with other observed data we concluded that this patient very likely represented another case of variegate por-

phyria. The latter case clearly demonstrates that asymptomatic forms of variegate porphyria may be discovered by analysis of biliary porphyrins, as previously reported (5) for duodenal sondage aspirates. The Enterotest® has the advantage that it offers a more convenient means of collecting bile for this purpose. An other possible way to detect asymptomatic carriers of variegate porphyria is to demonstrate the presence of a putative dicarboxylic porphyrin peptide complex, only present in subjects with variegate porphyria, by investigation of fluorescence emission spectra in plasma (26–29). In our opinion, however, this method harbours a number of shortcomings, such as lack of sensitivity (5, 29) and the need of sensitive equipment. In addition, haemolysed plasma cannot be used, due to interference by Zn-protoporphyrin. Another drawback is that no correlations exist between the severity of the disease and the levels of the porphyrin-peptide complexes in blood (28).

Tab. 3 Mean values, standard deviations and ranges of porphyrin concentrations (nmol/l), in duodenal bile of 8 subjects with variegate porphyria, and porphyrin concentrations in bile of one subject with variegate porphyria in remission, all obtained with the Enterotest®.

Symptomatic variegate porphyria (n = 8)			
	Copro-porphyrin I	Copro-porphyrin III	Proto-porphyrin
Mean	2790	5235	10744
S. D.	2184	3691	10830
Minimum	346	1082	1408
Maximum	6661	10156	27945
Variegate porphyria in remission (n = 1)			
	349	151	179

Longitudinal investigations of biliary porphyrins in patients with erythropoietic protoporphyria

Finally a feasibility study was performed as to the potential use of the Enterotest® for measuring biliary protoporphyrin excretion in subjects with erythropoietic protoporphyria. However, we did not have to subject patients to the Enterotest® procedure, as we had to our disposal a number of bile samples of two erythropoietic

protoporphyrin patients (A and B, tab. 2) who had undergone an orthotopic liver transplantation; during some weeks after this operation bile could be collected from the T-drain applied in these patients. With regard to the porphyrin pattern, this material has a composition comparable to duodenal bile, as already demonstrated above. Therefore, it may be assumed that these results are representative of those obtained with the Enterotest®.

During the first three weeks after orthotopic liver transplantation, the biliary porphyrin patterns of two female erythropoietic protoporphyria patients were determined; the results are depicted in figure 5 and relevant ratios in table 2. The results in this table show that in erythropoietic protoporphyria patients the protoporphyrin/coproporphyrin ratios both for coproporphyrin I and III are several times (sometimes more than a thousandfold) above normal. Figure 5 shows that the concentrations of protoporphyrin decrease in the first few days after orthotopic liver transplantation, and that coproporphyrins also decrease in patient B, but 10–15 days after

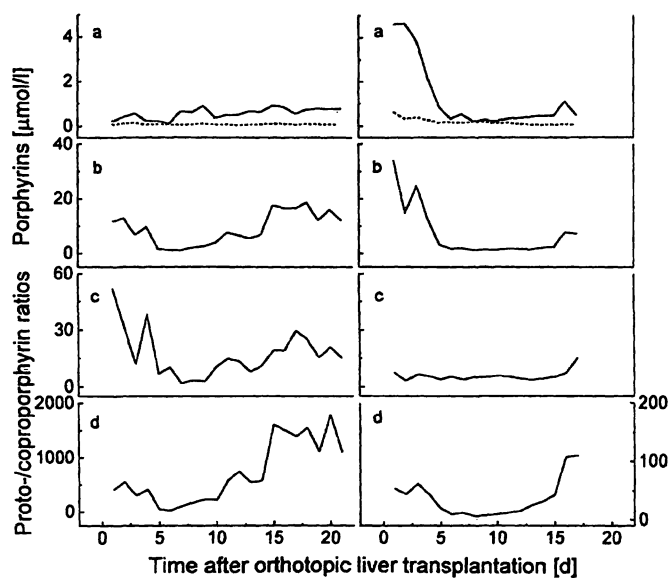


Fig. 5 Time course profiles of porphyrins in bile of two subjects with erythropoietic protoporphyria, who had undergone an orthotopic liver transplantation. Profiles on the left side are from subject A (see tab. 2), profiles on the right side from subject B.

a) coproporphyrin I (solid line) and coproporphyrin III (dashed line) concentrations;

b) protoporphyrin concentration;

c) ratio protoporphyrin/coproporphyrin I;

d) ratio protoporphyrin/coproporphyrin III.

As far as possible, the same ordinate scales were used for both subjects.

orthotopic liver transplantation the protoporphyrin concentrations rise again. The coproporphyrin III concentrations show fewer fluctuations than those of coproporphyrin I and protoporphyrin, from which we conclude that the protoporphyrin/coproporphyrin III ratio is perhaps the best quantity for monitoring the biliary protoporphyrin excretion. The increased protoporphyrin/coproporphyrin III ratios reflect the severity of the enzymatic block and demonstrate the capacity of the liver to secrete protoporphyrin adequately. The mean value for this quantity in an individual patient shows whether or not biliary protoporphyrin excretion is sufficient. It may be speculated that a severe drop in this ratio with no alteration in erythrocyte protoporphyrin concentration indicates an impaired capability of the liver to excrete protoporphyrin. Figure 5 clearly demonstrates that subject A synthesized and excreted considerably more protoporphyrin than subject B. However, more studies are needed to determine the precise diagnostic value of biliary porphyrin ratios in erythropoietic protoporphyria patients subjected to the Enterotest®.

At least, this experiment demonstrates the potential use of porphyrin ratios in bile, which can be obtained by means of the Enterotest®.

Conclusion

In summary, we have shown that the Enterotest® procedure constitutes a simple, non-invasive test for measuring the biliary porphyrin composition, enabling the detection of latent variegate porphyria and presumably also hereditary coproporphyrin. It is also potentially useful for measuring liver protoporphyrin excretion in erythropoietic protoporphyria patients. By measuring the porphyrin composition in bile instead of faeces, the contribution of bacteria to the porphyrin pattern can be eliminated. Another potential useful application (not investigated here) is the detection of the *Dubin-Johnson* syndrome, where the coproporphyrin I/III ratio is known to be abnormal in bile, as well as in urine (30). Our experiments further demonstrate that it is highly unlikely that an enterohepatic circulation of dicarboxylic porphyrins exists, since we detected secondary dicarboxylic porphyrins in duodenal fluid, and it has been recently reported that these are always completely absent in serum and bile (13).

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