New technique for analysing conjugated bile acids in gastric juice

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Abstract

A new technique of high performance liquid chromatography (HPLC) was developed for the analysis of conjugated bile acids in gastric juice. The assay is rapid, sensitive, and highly specific for bile acid conjugates over the range 30–10 000 $\mu\text{mol/l}$ and is not affected by the presence of food. Ten patients with a variety of common upper gastrointestinal disorders underwent continuous gastric aspiration for 16 hours, including a fasting, post-prandial, and nocturnal period, and aliquots of aspirates were analysed every two hours by the HPLC technique for the six most prevalent bile acid conjugates present in human hepatic bile. Intragastric bile acid concentrations were lowest in the post-prandial period and highest in the early hours of the morning. Conjugated bile acid proportions, or profiles, varied considerably from patient to patient, but tended to remain uniform over time in individual patients.

It is concluded that HPLC is superior to enzymatic techniques for the analysis of conjugated bile acids in the upper gastrointestinal tract.

Bile acids have been implicated in a variety of gastric and oesophageal disorders.¹⁻³ The most commonly used analytical technique for measuring bile acids in aspirates from the upper gastrointestinal tract is the 3α -hydroxysteroid dehydrogenase (3α -HSD) assay devised by Iwata and Yamasaki,⁴ adapted for analysis of duodenal aspirates by Fausa and Skalhogg,⁵ and validated for gastric samples by Collins.⁶ Recent reports of cross reactivity of the assay enzyme with ingested foodstuffs highlight the non-specific nature of this assay technique and its unreliability when used for post-prandial gastric specimens.^{7,8} The technique is further limited by a low sensitivity^{3,8} and is unable to quantify individual bile acid conjugates.

High performance liquid chromatography

(HPLC) using a reversed phase column and ultraviolet detection has been used to quantify bile acids in a variety of biological fluids,⁹⁻¹³ and recent refinement using the principle of "ion-pairing" has improved resolution.¹⁴ HPLC has advantages over the 3α -HSD technique for bile acid analysis in the upper gastrointestinal tract: it is rapid and does not require derivatisation or time consuming preparation of samples. As far as we are aware a technique for HPLC analysis for bile acids in gastric juice or oesophageal refluxate has not yet been validated. Separate validation is necessary because gastric juice differs from other body fluids in its pH, and it remains to be established whether the technique is unaffected by the presence of ingested foodstuffs. We developed and validated a technique of HPLC suitable for the quantitation of individual bile acid conjugates in gastric and oesophageal aspirates.

Methods

VALIDATION STUDIES

Apparatus

A Gilson 302 high performance liquid chromatograph, equipped with a Rheodyne high pressure sampling valve (model 71-25, USA) and a 20 μl sample loop, was used. The chromatograph was fitted with a Gilson holochrome spectrophotometric ultraviolet detector at 210 nm and an Apple IIe microprocessor controller/programmer. The stationary phase comprised two columns in series (Altex Ultrasphere ODS 5 mU; 250 mm $\times$ 4.6 mm ID, USA; and Varian micropak SP C18-5 mU; 150 mm $\times$ 4 mm ID, USA). A Gilson Pre-column (SSI 05-0418) was used as a filter. All pH measurements were performed with an EIL 7030 pH meter and combined glass electrode.

Solvents and reagents

Tetrabutylammonium phosphate (TBAP) and the sodium salts of glycine and taurine conjugates of cholate, chenodeoxycholate, and deoxycholate were obtained from commercial sources (Sigma, USA). Methanol, acetonitrile

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GDC=Glycodeoxycholate
GCDC=Glycochenodeoxycholate
TCDC=Taurochenodeoxycholate
TDC=Taurodeoxycholate
GC=Glycocholate
TC=Taurocholate

Figure 1 Chromatogram of a standard solution of pure bile acids obtained using HPLC.

Table 1 Retention times and capacity factors for each of six bile acids assayed

Bile acid	Retention time (minutes)	Relative retention time (GCDC)	Capacity factor (k')	Relative capacity factor (rk')
Taurocholate (TC)	9.0	0.552	0.667	0.33
Glycocholate (GC)	9.4	0.577	0.741	0.37
Taurochenodeoxycholate (TCDC)	12.6	0.773	1.333	0.66
Taurodeoxycholate (TDC)	13.9	0.853	1.574	0.78
Glycochenodeoxycholate (GCDC)	16.3	1.0	2.019	1.0
Glycodeoxycholate (GDC)	18.3	1.123	2.389	1.18

Figure 2 Bile acid calibration curves.

Table 2 Sensitivity of HPLC assay (62.5 micromolar standard solution was assayed 10 times)

Bile acid	Mean ($n = 10$) ($\mu\text{mol/l}$)	SD (SEM)	% Error from mean
Taurocholate	64.6	4.8 (1.53)	2.3
Glycocholate	61.2	5.3 (1.68)	2.7
Taurochenodeoxycholate	59.6	12.2 (3.85)	6.5
Taurodeoxycholate	62.0	6.8 (2.14)	3.5
Glycochenodeoxycholate	53.6	16.0 (5.07)	9.5
Glycodeoxycholate	34.8	17.8 (5.64)	16.2

Table 3 Recovery of bile acid standards added to bile contaminated gastric juice

Bile acid	Present	Added	Expected	Found	% Recovery
Taurocholate	970.3	1200	2170.3	2145.9	98
	877.2	857	1734.2	1719.5	98.3
Glycocholate	5694.3	1200	6894.3	7034.1	117
	227.1	857	1084.1	1067	98
Taurochenodeoxycholate	265.7	1200	1465.7	1411.6	95.5
	159.8	857	1016.9	1089.2	107
Taurodeoxycholate	200	1200	1400	1467	105.6
	9.9	857	866.9	864.7	99.7
Glycochenodeoxycholate	3439.7	1200	4639.7	4799.5	113
	0	857	857	805	94
Glycodeoxycholate	0	1200	1200	1110	92.5
	0	857	857	833.3	97
Total	10568.0	7200	17768	17968	101
	1274	5142	6416	6378.4	99.4

Table 4 Recovery of synthetic bile acids from three standard solutions

Expected	Found mean ($n = 5$)	SD (SEM)	Coefficient of variation	% Recovery
7200	7162.28	20.08 (8.98)	0.28	99.5
5143	5128.06	11.00 (4.92)	0.21	99.7
2571	2385.52	67.98 (30.40)	2.85	92.8

Table 5 Reproducibility (500 micromolar standard solution of bile acids was assayed on 14 consecutive days)

Bile acid conjugate	Mean (SD) ($\mu\text{mol/l}$) ($n = 14$)	% Deviation from mean
Taurocholate	500 (40.3)	8.0
Glycocholate	500 (35.6)	7.1
Taurochenodeoxycholate	500 (28.5)	5.7
Taurodeoxycholate	500 (24.9)	5.0
Glycochenodeoxycholate	500 (17.4)	3.5
Glycodeoxycholate	500 (29.7)	5.9

Table 6 Intra-assay variation (one sample was assayed 14 times)

Bile acid conjugate	Mean (SD) ($\mu\text{mol/l}$) ($n = 14$)	% Deviation from mean
Taurocholate	171.0 (11.2)	6.6
Glycocholate	525.0 (14.2)	2.7
Taurochenodeoxycholate	95.52 (2.67)	2.8
Taurodeoxycholate	67.7 (0.9)	1.35
Glycochenodeoxycholate	457.0 (14.9)	3.26
Glycodeoxycholate	586.5 (18.3)	3.11
Total conjugates	1852.7 (24.0)	1.26

trile, and hypersolve were obtained from BDH Ltd, Poole (UK). Double distilled water was provided by our own distillation plant (Fi-Stream, Fisons). Solvents and reagents were used without further purification.

Preparation of samples

Samples were thawed and 1 ml was combined with ice cold methanol (1:2 v/v). The sample was then centrifuged at $8 \times g$ for 10 minutes, passed through a $0.22 \mu\text{m}$ Millex filter (Millipore, Molsheim, France), and $20 \mu\text{l}$ were then injected directly into the sample loop.

High performance liquid chromatography

The mobile phase was prepared by mixing acetonitrile and double distilled water (54:46), and 0.4 M tetrabutylammonium phosphate (TBAP) was added as the counter-ion. The pH was adjusted to 2.5 by the addition of orthophosphoric acid (specific gravity 1.75; Analar; BDH, UK). The mobile phase flow rate was 0.5 ml/min at 1500 psi and was used at an ambient temperature of $22\text{--}23^\circ\text{C}$. The ultraviolet detector was set at 210 nm with a range of 0.01 absorbance units for range. Elution time was 18 minutes per sample. Columns were occasionally backflushed with double distilled water at 50°C , followed by methanol, acetonitrile, and finally the mobile phase. This prolongs column life and has allowed the processing of over 2000 samples without deterioration in column function.

PATIENT STUDY

Ten patients with a variety of upper gastro-intestinal disorders were studied. Four had reflux oesophagitis, four non-ulcer dyspepsia, and two had a healed gastric ulcer. The median age was 58 years (range 33–83 years); there was an equal sex distribution. No patient had had previous gastric surgery or vagotomy, and three had undergone cholecystectomy. The aim of the study was to determine the bile acid profiles in gastric aspirates from these patients and to ascertain whether they varied from person to person, and diurnally and nocturnally.

After a six hour fast each patient had a size 14 Salem sump tube passed transnasally and positioned with its tip 12 cm below the lower oesophageal sphincter, as determined manometrically. Continuous aspiration was provided by an electric pump, and aspirate was collected in a flask over ice. A 16 hour study (starting at 1700 hours) was divided into two hourly periods (giving eight specimens for each patient), allowing fasting, post-prandial, and nocturnal periods to be assessed. A standard meal was given at 1900 hours, which consisted of an egg salad sandwich, canned peaches, and 250 ml of milk, providing 595 calories.⁸ A pause of 30 minutes during the meal was taken before sampling was started. Each two hour aliquot was centrifuged at 3000 rpm for 20 minutes, decanted, and stored at -70°C for later analysis.

Table 7 Effect of food on bile acid assay: detection response in three solutions

Standard solution	Total bile acid conjugates (mean $n = 5$)	SD (SEM)	95% CI	p value (t test)
285.7 μ M				
Saline	10.16	0.22 (0.13)	9.90–10.42	
Food	10.33	0.27 (0.15)	10.03–10.63	0.3
333.3 μ M				
Saline	11.59	0.29 (0.10)	11.39–11.79	
Food	11.32	0.44 (0.20)	10.92–11.72	0.5
571.4 μ M				
Saline	19.93	0.28 (0.16)	19.61–20.25	
Food	19.65	0.14 (0.08)	19.49–19.81	0.7

Figure 3 Bile acid concentrations in gastric aspirates during eight time periods.

Results

Separation of a standard mixture of glycine and taurine conjugated bile acids is shown in fig 1. Column retention behaviour is described by the capacity factor (k') and relative capacity factor (rk' —relative to glycochenodeoxycholic acid) (table 1).

Quantitative analysis

Known amounts of conjugated bile acids (62.5–1000 μ mol/l) were assayed to determine detection response. Calibration curves were calculated with these data (fig 2). The accuracy of the HPLC method is reflected in the small deviation of the plotted points from linearity, and sensitivity at 62.5 μ mol/l was acceptable (table 2). Linearity was maintained from a minimum bile acid concentration of 30 μ mol/l to at least 10 mmol/l.

Figure 4 Proportions of the six different bile acid conjugates in gastric aspirates.

Recovery

Test samples were prepared by adding known amounts of synthetic bile acids to bile contaminated gastric aspirates. Two gastric specimens were each analysed in triplicate, and the mean values are presented in table 3. Three standard solutions were prepared, and each was analysed five times. Recovery of total bile acid conjugates from standard solutions is shown in table 4.

Reproducibility

Reproducibility of the assay was assessed by repeated analysis of the same standard solution of synthetic bile acid conjugates on 14 different days (table 5). Intra-assay variability within the overall technique was tested by analysing a 20 μ l aliquot on 14 different days taken from the same sample of bile stained gastric aspirate. The results are shown in table 6. Deviation from the mean ranged from 1.35 to 6.6%.

Effect of food

To assess the effect of food on the assay, three solutions of synthetic bile acids were prepared. An aliquot of each solution was mixed with either 0.9% (physiological) saline or with food substitute (1:1 v/v). The food substitute was Fortison (Cow & Gate Ltd, Trowbridge, Wiltshire, England), which is a balanced enteral feeding solution containing 4 g protein, 4 g fat, 12 g carbohydrates per 100 ml water and a variety of essential vitamins and minerals. Each solution was then assayed for individual conjugated bile acids five times and the results were expressed as mean (standard deviation) of total conjugated bile acids (table 7). Food substitute did not interfere with the HPLC assay ($p > 0.3$, t -test).

Gastric aspirates

Of 80 two hour periods, bile acids were detected in gastric aspirates obtained during 51 (64%) of them, and were quantifiable (> 30 μ mol/l) in 38 specimens. Seventy nine per cent of specimens with bile acids were obtained during nocturnal sleep, and higher intragastric bile acid concentrations were found during the nocturnal period ($p < 0.001$ Mann-Whitney U test) (fig 3). Only one of 10 post-prandial specimens contained bile acid (83 μ mol/l).

Relative conjugated bile acid profiles were studied. These varied widely among patients (figs 4 and 5). Variation in bile acid profiles within patients from hour to hour also occurred but was much less pronounced (fig 6).

Discussion

The most commonly used analytical technique for bile acids, the 3α -hydroxysteroid dehydrogenase assay,⁴ indirectly measures bile acid by relying on the reduction of nicotinamide-adenine dinucleotide (NAD) to NADH by the 3α -hydroxyl group, released from the bile acid molecule during the enzymatic reaction.¹⁵ This reaction is not specific because the α moiety occurs in a variety of other sterols¹⁵ and is the probable explanation for high "bile

Figure 5 Median bile acid proportions, or profiles, in eight patients.

acid-like reactivity" found in homogenised test meals known to be bile acid free,⁸ and in stool specimens from which bile acids have been extracted (R Owen, personal communication). Previous reports show an increase in gastric bile acid concentrations within patients following a test meal using the 3α -HSD assay,¹⁶⁻¹⁸ and studies on the role of bile acids in the pathogenesis of gastric and duodenal disorders have generally focused on the post-prandial period as a result.¹⁹ This may be responsible for previous conflicting results.²⁰ Our results show that HPLC is a sensitive, specific, and reproducible technique for the measurement of bile acid conjugates in gastric aspirates. We found bile acids in only one of 10 post-prandial gastric aspirates.

HPLC is specific for conjugated bile acids, relying on their individual ultraviolet absorbance. This provides an advantage over the 3α -HSD technique, which only measures total bile acid content. We validated the technique for the glycine and taurine conjugates of cholate, deoxycholate, and chenodeoxycholate which account for 98% of bile acids found in human bile.²¹ Because unconjugated bile acids are seldom found in gastric aspirates from patients with intact stomachs,²² the quantitation of individual and total bile acids depends almost entirely on the assay of these six bile acid conjugates. Conversely, up to 23% of bile acids in the stomachs of patients after gastrectomy may be unconjugated,^{23,24} and their presence

has been attributed to the action of bacteria.²⁵ HPLC is not suitable for the analysis of unconjugated bile acids, for which gas-liquid chromatography is more appropriate.²⁶

Another advantage of HPLC over 3α -HSD is its increased sensitivity. The lower limit of accuracy of the 3α -HSD method using spectrophotometry ranges from 62.5–300 $\mu\text{mol/l}$,^{3,8} which is the commonly encountered range of gastric bile acid concentrations.²² HPLC has a lower limit of detection of 2 $\mu\text{mol/l}$, and can accurately quantify bile acids at concentrations above 62.5 $\mu\text{mol/l}$.

Our HPLC method is based on that described by Wildgrube¹⁴ but differs in several important respects. Improved separation was obtained by acidification of the mobile phase to pH 2.5. Ionisation of bile acid conjugates is dependent on pH and results in a change in physicochemical behaviour. As the pK_a —that is, the pH at which the ionised:unionised molar ratio is equal—of glycine conjugates is around 2, and that of the taurine conjugates is around 4, retention times can be expected to be affected by a pH change from neutral to acid. The exact reason for the improved separation is not known, but it may be due to a combination of "ion pairing" and "ion suppression" effects.²⁷ Prolonged retention of bile acids on the columns at this pH has not been encountered, nor has there been a problem of reduced column life as predicted by Wildgrube.¹⁴

The second respect in which this technique differs from previous methods is the use of a pair of stationary phases (columns) in series. At pH 2.5 using the C_{18} Micropak column, all bile acid conjugates were separated except those of glycocholate and taurocholate. Addition of the Altex column separated these bile acids without affecting the elution profile or duration.

The patients in this study had a greater proportion of gastric aspirates contaminated by bile acid during the nocturnal period than during the diurnal period. Moreover, concentrations of bile acid conjugates were higher during nocturnal sleep. Indeed, some samples contained amounts of bile similar to those found in bile duct samples, and nocturnal duodenogastric reflux is the culprit according to the alkaline pH measured in these samples. This finding is not new, having been first described by James and Pickering in 1949 in patients with gastric ulcers.²⁸ Others have observed high bile acid concentrations in nocturnal gastric aspirates,^{22,29} and a large variation in bile acid contamination from hour to hour has been observed in individual patients.²⁹ This is reflected in the pattern of bile acid content found in the oesophagus of patients with reflux oesophagitis.³⁰ Bile acids were rarely found in post-prandial aspirates. These results suggest that previous studies of bile acid reflux in patients with oesophageal and gastric disease which have been limited to short term, day time, or post-prandial studies, may not have been of sufficient duration to permit valid conclusions about their role in the pathogenesis of such patient groups. Perhaps future studies of such patient groups should assess the whole nocturnal period.

Figure 6 An example of bile acid profiles during each time period in one patient.

HPLC permits the analysis of relative bile acid proportions or "profiles" in gastric samples. Bile acid profiles tended to remain similar in each patient throughout the study, but there were noticeable differences in profiles from patient to patient which did not seem to depend on pH. The clinical importance of this finding is not yet known.

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