THE ANALYSIS OF BILE ACIDS AND NEUTRAL STEROLS IN HUMAN FAECES AND THE BILE SALTS IN HUMAN BILE USING SOME NEWLY DEVELOPED CHROMATOGRAPHIC TECHNIQUES

A thesis submitted for the degree of

Master of Philosophy

by

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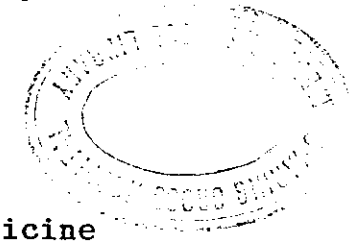

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A B S T R A C T

This study was undertaken to investigate bile acids and neutral sterols in the faeces of subjects taking an antiobesity agent fenfluramine; also separately, to examine the bile salts present in bile taken from gall bladders which did or did not contain cholesterol gallstones.

In a group of 16 subjects taking fenfluramine, total bile acid and neutral sterol excretion (mg/g dry weight of faeces; mean $\pm$ SD) increased during treatment from 26.0 $\pm$ 7.0 to 35.2 $\pm$ 10.7 ($N = 16$; $P<0.01$) and remained high for a time after stopping the drug. Qualitatively, bile acids remained unaltered except in 3 subjects with drug-induced diarrhoea. These 3 excreted chenodeoxycholic and cholic acids rather than deoxycholic and lithocholic acids. The excretion of coprostanol fell but cholesterol excretion increased in 15 subjects.

Qualitative and quantitative differences were found between the bile salts present in gall bladder bile taken from patients with gallstones and in bile salts present in samples of "fresh" post mortem bile taken from gall bladders without gallstones. The concentration of bile salts present in the bile of patients with gallstones was lower than the concentration found in post mortem bile. The percentage of cholic acid remained unchanged but the percentage of deoxycholic acid present in the bile of

patients with gallstones was significantly greater than the percentage of this bile acid found in bile without gallstones. The percentage of chenodeoxycholic acid in the bile of patients with gallstones was lower than that found in post mortem bile. In bile with gallstones, the percentage of bile salts conjugated with glycine was higher than that found in bile without gallstones.

Existing methods for estimating bile acids and neutral sterols using gas chromatography were improved. The important procedures developed were: semi-automated extraction, rapid freeze-drying of faeces, preparation of diazomethane, construction of chromatographic columns and evaluation of a new liquid phase.

Methods using 'high pressure liquid chromatography' have been developed for investigating the bile salts present in human bile. These methods have the advantages of simplicity, speed and reproducibility. They are preferred to thin layer chromatography or gas chromatography because artifacts which may occur during hydrolysis and the preparation of derivatives are avoided.

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A B B R E V I A T I O N S

The following abbreviations have been used:

		"Bile Acids"	
C	:	Cholic	Primary bile acids
CDC	:	Chenodeoxycholic	
DC	:	Deoxycholic	Secondary bile acids
LC	:	Lithocholic	
ILC	:	Isolithocholic	
UDC	:	Ursodeoxycholic	
		"Bile Salts"	
GLYC	:	Glycocholic	Primary bile salts
TC	:	Taurocholic	
GCDC	:	Glycochenodeoxycholic	
TCDC	:	Taurochenodeoxycholic	
GDC	:	Glycodeoxycholic	Secondary bile salts
TDC	:	Taurodeoxycholic	
		"Others"	
TLC	:	Thin layer chromatography	
GC	:	Gas chromatography	
HPLC	:	High pressure liquid chromatography	
R-P	:	Reverse-phase	
GC-MS	:	Gas Chromatography-Mass Spectrometry	
IR	:	infrared	
UV	:	ultraviolet	
NMR	:	Nuclear magnetic resonance	
RI	:	Refractive Index	
FID	:	Flame Ionisation Detector	
TMS	:	Trimethylsilyl	
TFA	:	Trifluoroacetate	
R_t	:	Retention time	
RR_t	:	Relative retention time	
min	:	minute	

nm	:	nanometer
mV	:	millivolt
µ	:	micro
µg	:	microgram
mg/ml	:	milligram per millilitre
mg/g	:	milligram per gram
w/v	:	weight for volume
v/v	:	volume for volume
SD	:	Standard deviation
BAP	:	Bile acid pool
EHC	:	Enterohepatic circulation
ml/min	:	millilitre per minute
o.d.	:	outside diameter
i.d.	:	inside diameter

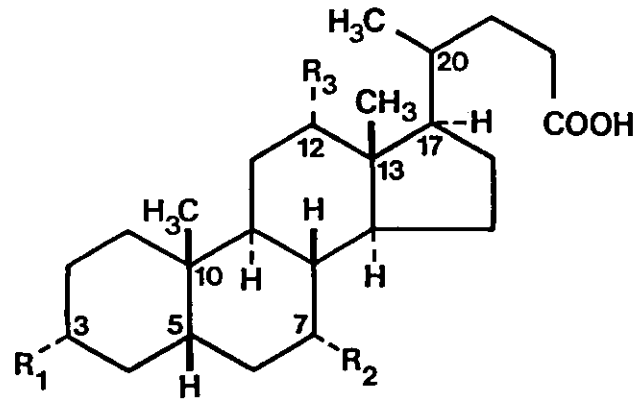
NOMENCLATURE

The nomenclature used in this thesis follows that recommended by the International Union of Pure and Applied Chemistry.

Commonly used names have been used in the body of the text, The term "bile acid" is used for the unconjugated materials: the term "bile salt" is used to describe bile acids conjugated with glycine and taurine. Cholic acid and chenodeoxycholic acid are regarded as "primary bile acids". These are converted into deoxycholic and lithocholic acids usually referred to as "secondary bile acids".

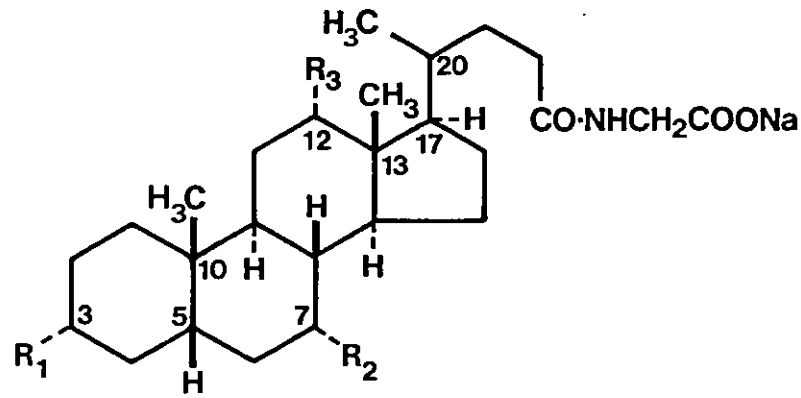
STRUCTURE OF BILE ACIDS

A) BILE ACIDS



COMMON FORMS	R_1	R_2	R_3
Cholic	OH	OH	OH
Chenodeoxycholic	OH	OH	H
Deoxycholic	OH	H	OH
Lithocholic	OH	H	H

B) BILE SALT (Glycoholic acid sodium salt)



R_1, R_2, R_3 = OH

Broken lines represent bonds to atoms or groups lying below the plane of the paper,(α, alpha configuration); and solid, thickened lines represent bonds to atoms of groups lying above the plane of the paper (β, beta configuration).

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INTRODUCTION

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1.1 GENERAL INTRODUCTION

In recent years considerable attention has been focused on the metabolism of bile acids in man in connection with hepato-biliary (Makino, Schinozaki, Yoshino and Nakagawa, 1975) and gastrointestinal disease (Bouchier, 1979).

There is increasing evidence that the physicochemical properties of bile acids are important in the formation of a "lithogenic bile" (i.e. bile that is supersaturated with cholesterol) and in the pathogenesis of cholesterol gallstones (Rains, 1964; Small, 1968; Heaton, 1972). It is also believed that certain bile acids are responsible for causing colonic disease (Hill, 1977). The relatively insoluble lithocholic (LC) may separate from bile and obstruct the biliary passage (Javit and Emerman, 1968). More recent work has shown that the decrease in cholesterol solubilising capacity of bile is due to increased deoxycholic (DC) in gallstone patients (McDougal, Walker and Thurston, 1976; Low-Beer, 1977). It is now well documented that gallstone formation occurs from either a relative excess of cholesterol or a relative lack of bile salts (Coyne, Marks and Schoenfield, 1977; Bouchier, 1979).

During the past few years, certain bile acids such as chenodeoxycholic (CDC) have been investigated as possible therapeutic agents to dissolve cholesterol gallstones in vivo (Thistle and Schoenfield, 1971). More recent reports describe the use of ursodeoxycholic (UDC), a bile acid structurally related to CDC and found in small amounts in human bile, to

dissolve cholesterol gallstones (Makino et al, 1975). It has a similar action to CDC but has the advantage that it can be given in smaller doses; it does not cause diarrhoea and changes in liver function are less apparent than those after CDC treatment (Maton, Murphy and Dowling, 1977).

The recent revival of interest both in the pathophysiology of bile acids and their possible therapeutic use stresses the importance of detailed analysis and characterisation of individual bile acids and their distribution in pathological states.

Although the development of methods available for the analysis of bile acids has progressed in recent years (Grundy, Ahrens and Miettinen, 1965; Eneroth and Sjovall, 1971) all are complex and time consuming, and the methods suitable for routine application were still lacking when this work was started. The methods then currently available were based on a preliminary hydrolysis of conjugates. It was therefore not possible to accurately measure the bile salts as they occurred in bile. Further, hydrolysis was frequently incomplete and artifacts could be found when making derivatives (Okishio, Nair and Gordon, 1967; Shioda, Wood, and Kinsell, 1969). Methods using 'high pressure liquid chromatography' (HPLC) have therefore been developed and used to investigate bile salts as they exist naturally in bile.

1.1 A AIMS OF THE PROJECT: HYPOTHESIS: SCOPE OF THESIS

Patients receiving fenfluramine (an antiobesity agent) sometimes have diarrhoea. The cause of this diarrhoea is not known. It is suggested that fenfluramine may alter the amount and the kind of bile acids or bile salts present in the ileum. To test this suggestion attempts have been made to develop qualitative and quantitative assay methods for bile acids and bile salts present in the faeces following the administration of fenfluramine.

Excessive faecal loss of bile acids may increase the risk of gallstone disease by decreasing the amount of bile salts present in bile (Mok, von Bergmann and Grundy, 1977). The bile salt composition of bile taken from gall bladders with and without gallstones has therefore been investigated.

Since the analytical procedures then available were inadequate the development of suitable methods forms the main part of this thesis.

1.1 B HISTORICAL BACKGROUND

The composition of bile has been regarded important throughout the history of man (Horall, 1938). Bile salts have been implicated in, a) fat digestion and absorption (Sabotka, 1937), b) liver disease (Budd, 1845; Rous, 1925), and c) colonic carcinogenesis (Hill, Drasar and Aries, 1971). The early history of the investigations into the components of bile is well reviewed (Sabotka, 1937 and 1938; Haslewood, 1955).

Bile acid studies were stimulated when the researches of Windaus (1932) and Wieland and Dane (1932) on the structure of cholesterol and bile acids were brought to fruition by the proposal of new structural formula for cholesterol by Rosenheim and King (1932). The 'modern era' may be thought to have begun when Wieland, Kraus, Keller and Ottawa, (1936) used adsorption chromatography on aluminium oxide to separate bile acids. The use of partition chromatography on silica gel columns was described by Martin and Synge (1941) and three years later, the silica gel support was replaced with paper (Consden, Gordon and Martin, 1944). Paper chromatography was extensively used for separating bile salts (Kritchevsky and Kirk, 1952) but its application was limited because of low resolution, lack of specificity and long analysis time. The use of 'reverse-phase' (R-P) partition chromatography for separating bile salts was described by Bergstrom and Sjovall (1951), but the detection of bile salts in the column effluent was a problem.

A significant advance was made when James and Martin (1952) demonstrated the separation of volatile fatty acids by gas chromatography (GC), but a serious limitation in its application was the lack of sensitive detectors. In the mean time, thin layer chromatography (TLC) was introduced (Stahl, 1956) but lack of resolution, specific means of detection and quantification posed problems.

GC entered a new phase when the 'flame ionisation detector' (FID) became available (McWilliam and Dewar, 1958) and in

the same year, mass spectrometry (MS) was applied to the characterisation of bile acids (Bergstrom, Ryhage and Stenhagen, 1958). Within two years of these developments, GC was successfully used to separate steroids without their decomposition (Vanden-Heuvel, Sweely & Horning, 1960). A major advance in this field came when then group working at Karolinska Institute (Eneroth, Gordon & Ryhage, 1966) developed and applied 'gas chromatography mass spectrometry' (GC-MS) to analyse and identify bile acids in complex biological mixtures.

Another technique introduced into the bile acid field was 'spectrophotometry' using ultraviolet (UV) absorbing chromogens (Mosbach, Kalinsky, Halpern & Kendall, 1954). These methods lack specificity for different bile acids and most procedures described require some form of chromatography prior to spectrophotometry. The development of 'isotope methods' (Lindstedt, 1957) has made possible the measurement of bile acid pool (BAP), the hepatic synthesis of bile acids from cholesterol and the 'enterohepatic circulation' (EHC) of bile acids. However, these techniques suffer from limitations such as, they are technically complex, time consuming, expensive and therefore unsuitable for routine application.

A non-destructive 'enzymatic method' using 3α-hydroxysteriod dehydrogenase (3α-HSDH) was developed for estimating bile acids (Iwata & Yamasaki, 1964); and its sensitivity increased by Murphy, Billing and Baron (1970). The use of a second enzyme, 7α-hydroxysteriod dehydrogenase (7α-HSDH)

has been described (Haslewood & Haslewood, 1974) but despite their potential value 'enzymatic methods' are regarded by some as complicated, expensive (Tiselius & Heuman, 1978) and non-specific (Ende, Radecker & Starmans, 1974). The latter claim that 'enzymatic methods' show a lower precision than methods using TLC or GC. Furthermore, extraction and purification of bile acids prior to enzymatic determination is still required. Evidence presented recently suggests that the lower results obtained when using 'enzymatic methods' are due to the lack of highly purified enzyme preparations (Javit, Budai, Shan, Siskos & Cahill, 1979). The use of 'enzymatic method' may increase as cheaper, highly purified and specific enzyme preparations become available.

Radioimmunoassay (RIA) has been claimed to have a high specificity and sensitivity for GLYC and GCDC (Spenney, Johnson, Hirschowitz, Mihas & Gibson, 1977) but RIA for DC is not yet established (Becket, Hunter & Percy-Robb, 1978); furthermore, we have found no reports describing the use of RIA for UDC. Despite their diagnostic value in liver disease, RIA has not received a wide application (Mihas, Spenney, Hirschowitz & Gibson, 1977). This may be due to the expensive equipment which is not readily available in all laboratories.

Therefore, despite the more recent developments of infrared (IR), nuclear magnetic resonance (NMR), enzymatic methods, and radioimmunoassay (RIA), GC still has many attractions for the simultaneous separation and estimation of bile acids and bile acid derivatives. However, for some special

applications GC suffers from two major disadvantages in that it involves hydrolysis and the preparation of derivatives. In view of this, laboratories working in this field investigated ways to bring 'liquid chromatography' to an analytical scale. Towards the end of 1960's there was the emergence of what is known as 'high pressure liquid chromatography' or 'high performance liquid chromatography' (Siggia & Dishman, 1970; Done & Knox, 1972). Some of the advantages of HPLC are, a) speed, b) resolution, c) sensitivity, d) reusable columns and e) small sample size. Furthermore, certain compounds can be analysed without hydrolysis; and the preparation of derivatives is not necessary. In 1974, work from this laboratory demonstrated the separation of LC, DC, CDC and C by HPLC on Whatman's Partisil-10 columns connected to a refractive index (RI) detector, but the problems were, a) lack of strong UV absorbance by bile acids, b) lack of sensitive detectors and c) lack of suitable HPLC columns for separating bile acids. From the time these became available, several laboratories managed to separate bile acids by HPLC (Okuyama, 1976; Jefferson & Chang, 1976; Shaw & Elliott. 1976; Shaw, Smith & Elliott (1978).

1.2 REVIEW OF METHODS

In this section are reviewed some of the more important methods currently used for the analysis of bile acids and neutral sterols found in human faeces and bile salts present in human bile:

i) Analysis of faecal bile acids by gas chromatography

Bile acids present in the faeces are the end products of cholesterol metabolism (Danielsson & Sjovall, 1975). The amount of bile acids found in the faeces is believed to represent the amount synthesised daily in the liver (Grundy et al. 1965; Dowling Mack & Small, 1970). The detailed knowledge of the kind and quantity of these compounds is important in the diagnosis and treatment of gastrointestinal disease.

The analysis of bile acids in the past has been a 'difficult technical problem' (Horning, Brooks & Vanden Heuvel, 1968), complicated, time consuming and tedious (Nakayama, 1969) and unsuitable for routine work. Since the publication of the first GC separation of bile acids (Vanden-Heuvel, Sweeley Horning, 1960) several other techniques have been developed (Eneroth & Sjovall, 1971) but GC has remained the most sensitive method for estimating bile acids (Heaton, 1972). Some of the more popular GC method for estimating these compounds in human faeces are reviewed below:

A method which has been repeatedly used includes a double saponification lasting for 4 hours followed by column chromatography to separate bile acids contaminants. Further purification is carried out by TLC after methylation of bile acids. For quantification, the trimethylsilyl (TMS) derivatives of bile acids are analysed by GC on SE-30, F-60 , QF-1 or XE-60 liquid

phases. (Grundy et al. 1965). This method is very lengthy and tedious. Ali, Kuksis & Beveridge, (1966) extracted faeces with methanol/ethylene chloride (1:3) v/v for 48 hours before saponification at 120°C at 15 psi for 4 hours; but the methyl esters formed required purification by column chromatography and TLC. GC on QF-1 columns was used to identify and quantitate methyl ester trifluoroacetate (TFA) derivatives of bile acids. Similarly, Eneroth Hellstrom & Sjovall, (1968) extracted homogenized faeces with chloroform/methanol (1:1; v/v) for 48 hours before saponification for 3 hours, but the extraction of bile acids took 16 hours and silicic acid chromatography was required for separating bile acids from fatty acids and neutral sterols in the faecal mixture prior to GC on QF-1 columns as methyl ester TFA derivatives. Evrard & Janssen (1968) reported a rapid method on which our modified method is based but their procedure did not analyse the individual bile acids in faeces and the extraction was tedious. We have analysed the individual bile acids to estimate the bile acid content and semi-automated the extraction procedure making it suitable for routine use.

ii) Analysis of faecal neutral sterols by gas chromatography

The isolation, identification and quantification of neutral sterols present in human faeces has also been a difficult problem (Miettinen et al. 1965). Eneroth, Hellstrom & Ryhage, (1964) extracted neutral sterols present in the faeces with chlorofrom/methanol (1:1;v/v)

for 48 hours prior to saponification for 1 hour. The neutral sterols were extracted with petroleum ether (b.p. 40-60^{0}C) and these compounds were identified by GC on QF-1 and SE-30 columns before and after the preparation of TMS derivatives or dimethylhydrazone derivatives. Miettinen et al (1965) described a method in which the neutral sterols in faecal homogenates were extracted into petroleum ether after mild saponification for one hour, but their procedure required TLC on Florasil for separating the neutral sterols into groups before quantification by GC as TMS derivatives. In our modified method these procedures have been simplified and the extraction semi-automated. Thus the method is suitable for routine estimation of neutral sterols in faeces. Furthermore, TLC prior to GC is rendered unnecessary and the sterols are estimated using the underivatised extract.

iii) Analysis of bile salts present in bile

The analysis of bile salts in bile presents less problems than the analysis of these compounds in faeces. The main reason is that the mixture of bile salts in bile is less complex with only six major bile salts: GLYC, GCDC, GDC, TC, TCDC and TDC which are present in much higher concentrations than in faeces. Other bile acids such as LC and UDC have also been identified but these are present only in trace amounts (Kuksis, 1969).

The majority of the methods available use ethanol for extracting bile salts from bile. After brief boiling and cooling the proteins are removed by filtration and the extract purified by TLC or column chromatography. Other solvents such as chloroform/methanol and methanol/acetone have also been used. Methods using paper chromatography and TLC have been reviewed (Eneroth & Sjovall, 1971). These procedures have the limitations of: a) inadequate resolution; b) difficulties of identification and quantification; c) low specificity and d) long analysis time. Some of these problems have been overcome by turning to GC after hydrolysis and derivisation of bile salts. Unfortunately, the disadvantages are: a) GC is unsuitable for direct analysis because bile salts have low volatility; b) individual conjugates can not be differentiated; c) time is lost in hydrolysis and derivatisation (Shioda et al. 1969; Goto, Hasegawa, Kato & Nambara, 1978).

1.3 Limitations of present methods for estimating bile acids and bile salts present in faeces and in bile

The review of methods shows that there are limitations at major stages in the estimation of faecal bile acids and bile salts present in bile. Some of these problems are discussed briefly:

a) Extraction of faecal bile acid and neutral sterols: the objective is to obtain quantitative extract, but the procedures currently available are either too long (48 hours) or too

complicated. A rapid method on which our present method is based (Evrard & Janssen, 1968) has been developed making it possible to extract these compounds in a shorter time using a semi-automated procedure.

b) Sample preparation: analysis of bile acids and neutral sterols present in faeces involves handling of large amounts of stools. The existing method of freeze-drying faeces was found unsatisfactory, this has been improved.

c) Isolation of faecal neutral sterols: previous procedures use TLC or column chromatography prior to GC but these procedures are time consuming and may increase bile acid losses. A semi-automated extraction is introduced making it suitable for routine use.

d) Separation of bile acids by GC: the resolution of some isomeric bile acids was inadequate; a new GC liquid phase was tested and found to provide improved separations.

e) Quantification of bile acids and neutral sterols in faeces and bile salts present in bile: this has been a problem because of the varying 'flame ionisation detector' (FID) response to different bile acids and neutral sterols. The linearity and the daily variation in response was examined. For estimating bile salts present in bile, the use of 'refractive index' (RI) and UV detector was investigated.

f) Hydrolysis of bile salts - most methods use high temperatures and strong alkaline conditions which may degrade bile acids (Eneroth & Sjovall, 1971); this stage was examined.

g) Preparation of derivatives: majority of the methods use methyl ester derivatives but the methods available for preparing the methylating reagent, diazomethane, were unsatisfactory. A simplified and safer method was developed for routine use.

h) Are there any 'new bile acids'? In 1973, Carey listed some 25 bile acids in human faeces; it is possible there are other yet undiscovered bile acids. In this respect the mixture of bile salts found in gall bladder bile was much simpler.

1.4. CONCLUSION

This review shows that the methods available for separating and estimating bile acids and neutral sterols in faeces were complex, technically difficult and had certain limitations. Similarly, the methods commonly used for estimating bile salts present in gall bladder bile were inadequate in terms of separation, specificity and the difficulty of quantification. GC has partly overcome some of these limitations (Chapter 2) but it is unsuitable for the analysis of bile salts. Hydrolysis and the preparation of derivatives is still necessary. Since the information of the kind and quantity of glycine and taurine conjugates may be important in gallstone disease, methods for the analysis of these compounds using HPLC were developed (Chapter 4).

CHAPTER 2

THE DEVELOPMENT OF GAS CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF BILE ACIDS AND NEUTRAL STEROLS PRESENT IN HUMAN FAECES

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2.13 Comment on a new stationary phase SP 2250

2.14 Discussion

2.1 Introduction

The problems reviewed in the Introduction have been examined and the improved procedure which conforms to the usual criteria of being accurate, reproducible, sensitive and specific is described below.

2.2 The improved method used for estimating bile acids and neutral sterols in faeces

a) Extraction: An aliquot of freeze-dried powdered faeces (200-300 mg) was weighed into a 'Pyrex' test tube (16 X 170 mm, Quickfit) and 3 ml of glacial acetic acid containing 900 ug of 23-Nor-DC as an internal standard was added. The tube was stoppered and shaken in an oil bath at 120± 2°C for 30 min using a modified Gallenkamp shaker (Figure 2.1. The tube was then removed from the shaker and allowed to cool before adding 6ml of toluene and mixing vigorously for 1 min on a 'Whirlmixer' (Fisons). The clear brown supernatant was used for bile acid and neutral sterol estimations.

b) Hydrolysis: 3ml of the toluene layer was evaporated in a 'Pyrex' test tube (16 x 170 mm) using a water bath at 45°C and a rotary evaporator. The residue was dissolved in 1ml of 20% KOH in ethylene glycol (w/v). The mixture was refluxed in the test tube for 20 min at 220 ± 2°C using a heating block (Isopad-Isomantle). The upper part of the open test tube served as an air condenser.

c) Removal of neutral sterols from the extract: The hydrolysate was diluted with 2 ml of 20% aqueous NaCl

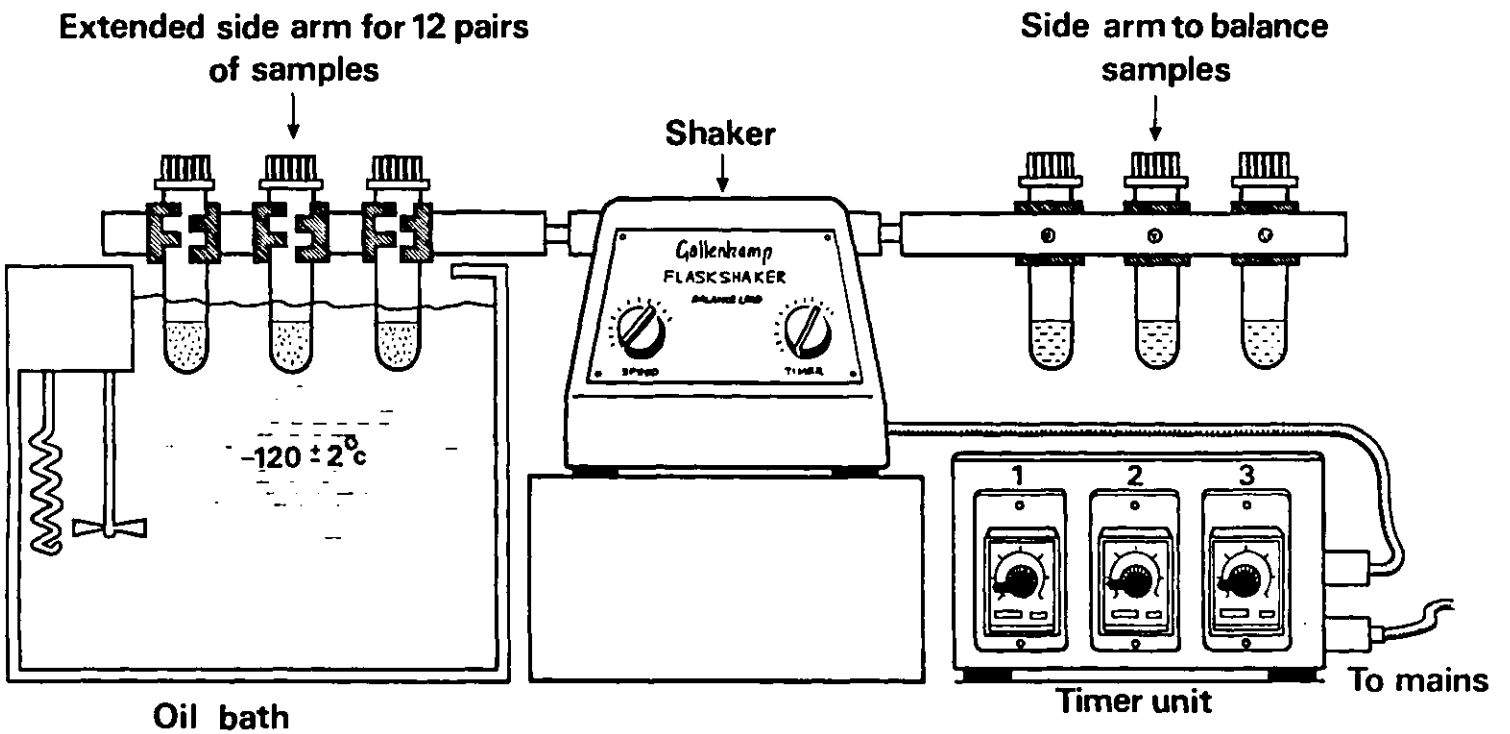


Figure 2.1 Apparatus for extracting bile acids and neutral sterols from freeze-dried faeces. The details of the materials and the supplier are included in Appendix B.

(w/v) and 1ml of methanol added. Neutral sterols were extracted three times with 5ml of petroleum ether (b.p. 40-60°C) the shaker being set for 2 min each time. The pooled petroleum ether extracts were evaporated to dryness and the residue dissolved in 1ml of chloroform before gas chromatography of neutral sterols on JXR columns.

d) Recovery of bile acids from the neutral sterol extract: After removing the neutral sterols, the aqueous alcohol was diluted with 8ml of 20% aqueous NaCl (w/v) acidified to pH 1-2 with 6N HCl; using thymol blue as external indicator. The bile acids were taken into three lots of 5ml of di-ethyl ether using the shaker set for 2 min. The pooled ether extracts were evaporated to dryness. The residue was used for preparing bile acid methyl esters.

e) Preparation of ethereal diazomethane : Ethereal diazomethane was prepared from nitrosomethylurea. The details are to be found in Appendix **D**. For methylating the terminal carboxyl group of bile acids, the residue obtained in (2.d) was dissolved in 2ml of freshly distillled di-ethyl ether containing 0.2ml of freshly distilled methanol. 3-4ml of ethereal diazomethane was added using the apparatus designed for this purpose (Figure 2.2). After 15 min the excess reagent was removed at the water pump before taking to dryness under vacuum. The bile acid methyl esters were dissolved in 1ml of methanol and 1-2 µl of this solution was used for

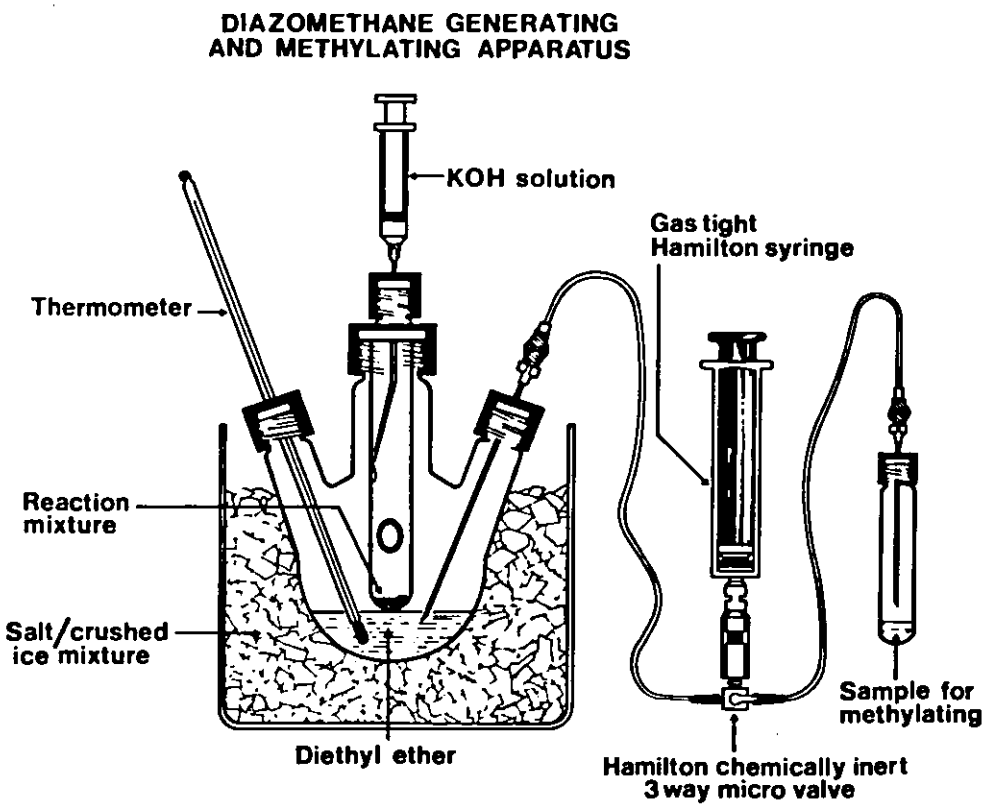


Figure 2.2 Apparatus for generating diazomethane and for methylating bile acids. The details of the procedure are included in Appendix D.

gas chromatography on QF-1 columns.

f) Reference mixture of standards: With each series of faecal samples analysed, duplicate portions of 1ml of a standard solution in acetone containing 200 ug each of: cholesterol, coprostanol and 5α-cholestane were evaporated treated with KOH, and prepared for GC in the same manner as the faecal samples. In the same way, duplicate aliquots (1ml) of a standard solution in methanol containing 300 ug each of: LC, 23-Nor-DC, DC, CDC and C acids were evaporated, treated with KOH, and prepared for GC of the methyl esters in the same manner as the faecal samples.

g) Quantification: All quantitative results were referred to calibration curves prepared daily for the main neutral sterols and bile acids present in faecal extracts. The ratio of the peak area of the internal standard in the reference mixture to that in the faecal sample was used as a 'correction factor' to make allowance for losses during the whole procedure.

h) Qualitative analysis: For tentative identification of neutral sterols and bile acids present in faecal extracts only the major neutral sterols and bile acids were identified. For all samples analysed, the standard mixtures were run either just before or immediately after the faecal sample run. The retention times (R_t) of neutral sterols were calculated relative to the retention time of cholesterol, and the retention times

of bile acids were calculated relative to that of DC.

2.3 Comment on sample preparation:

i) Freeze-drying: Because of the advantages of working with dried faecal samples, the freeze-drying method of Evrard & Janssen (1968) was tested. Their freeze-drying technique took approximately 14 hours and required the use of 'celite'. The procedure now used completes the freeze-drying in 4-6 hours without the addition of a 'filter aid'. Homogenised faeces (40-50g) were transferred to a 500ml flask and the sample frozen as a thin film using the apparatus shown in Figure 2.3. The flask was disconnected and placed in the main chamber of an Edward's Model EF03 freeze dryer. The dried sample was powdered, weighed and stored in a dark bottle.

ii) Homogeniety of freeze-dried powder: This was tested using reproducibility experiments on samples taken from different parts of a well mixed 3-day stool collection. The mean and standard deviation of the apparent bile acid content (mg/g dry weight) of six samples analysed in duplicate was 10.6 $\pm$ 0.5 (N=12 estimates). The standard deviation calculated from the difference between duplicate estimated was small and the coefficient of variation less than 5% (Appendix Table 2.1).

iii) The effect of sample storage: Because it is not always possible to analyse faeces immediately, the bile acid content of freshly collected faeces was compared with the amount found in the same samples when these samples

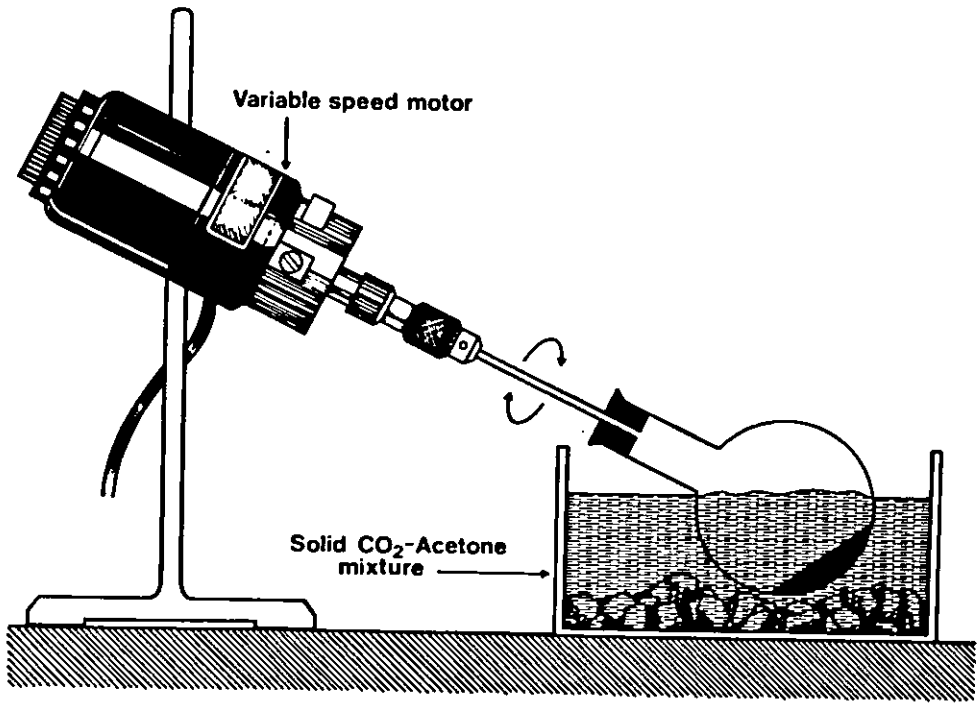


Figure 2.3 Apparatus for 'freezing' samples of faecal homogenate. The details of the materials and the supplier are included in Appendix B.

were stored in different ways and varying periods. Freshly collected faeces were stored at -20°C and analysed after 9 weeks and also after 1 year. The apparent bile acid content (mg/g dry weight;mean ± SD) did not change from the amount found in the same samples when these were analysed immediately (fresh samples = 9.1 ± 0.4; N=6 vs. 9.3 ± 0.4 after 9 weeks at -20°C; and after 1 year at -20°C = 9.0± 0.4).

Neither was any change found if the same samples were freeze-dried and stored at + 4°C and analysed after 3 years (9.3 ± 0.4 at 9 weeks vs. 9.2 ± 0.4 after 3 years; N=6; P>0.05 not significant; Appendix Table 2.2.

2.4 Comment on semi-automated extraction of bile acids and neutral sterols present in faeces

The method of Evrard & Janssen (1968) was modified. A Gallenkamp shaker and an oil bath replaced manual shaking and the heating mantle. Preliminary experiments showed that intermittent shaking was more convenient and the shaker was modified to permit this (Appendix Figure 2.4)

i) Duration of extraction:

In order to test the completeness of extraction by the modified method, faeces were extracted for periods between 0.25 hours and 6 hours and the bile acid content estimated by GC. The amount of bile acids extracted in 0.5 hour was nearly the same as that found

after extracting for 6 hours (0.5 hour = 8.4; 6 hours = 8.6 mg/g dry weight; Table 2.3). To increase speed of analysis the shorter extraction was used.

Table: 2.3 The effect of the duration of extraction on the yield of bile acids

Duration of Extraction (hours)	Apparent Bile Acid Content of Faeces (mg/g dry weight) Duplicate Estimates		
	A	B	mean
0.25	7.4	7.6	7.5
0.50	8.6	8.2	8.4
1.00	8.5	8.1	8.3
2.00	8.0	8.4	8.2
4.00	8.7	8.4	8.5
6.00	8.2	8.8	8.6

Bile acids were estimated as 'oxo' derivatives on JXR columns. The yield at 0.5 hour does not differ from that after 6 hours.

ii) Vigor of extraction: Two batches of faecal samples obtained from the same 3-day stool collection were extracted for 0.5 hour, one with the shaker set at 100 $\pm$ 25 shakes/min (Method A) and the second 200 shakes/min (Method B). The mean and standard deviation of the apparent bile acid content of faeces (mg/g dry wt.) estimated by the two methods showed no difference (7.4 $\pm$ 0.7 by method A, compared with 7.6 $\pm$ 0.8 by Method B; $P>0.05$ Not significant; Table 2.4). To avoid

excessive vibrations the lower speed was preferred.

Table 2.4 The effect of vigor of extraction

Sample	Bile Acid Content of faeces (mg/g dry weight) Method A (100 shakes/min)	Method B (200 shakes/min)
1	6.6	7.0
2	7.2	8.9
3	8.6	7.1
4	7.9	6.9
5	7.4	8.1
6	6.8	7.3
Mean =	7.4 ± 0.7 (SD)	7.6 ± 0.8 SD
Coefficient of variation =	9.9%	10.5%

Bile acid content of faeces was estimated as for Table 2.3

iii) Tests on semi-automated extraction method

a) Recovery experiments

The recovery of 23-Nor-DC (internal standard) added to the freeze-dried faeces before extraction was used to test the efficiency of extractions. The overall recovery after complete procedure for amounts between 0.1, to 1.0 mg was in the range 80÷98% (Mean ± SD = 85 ± 6; Appendix Table 2.5).

b) Comparison with manual method :

There was close agreement between the bile acid content (mg/g dry weight) estimated by the semi-automated method proposed and the manual method of

Evrard & Janssen (mean ± S.D. of 'semi-automated' method = 8.4 ± 1.0 compared with 8.0 ± 1.2 by Evrard & Janssen's method; N = 8; P > 0.05, not significant). A higher yield was expected by the semi-automated method but this series only shows a small increase (Appendix Table 2.6).

c) Reproducibility of semi-automated extractions: The results of eight replicate analyses made on samples taken from a single stool collection are shown in Appendix Table 2.7. The percentage differences from the mean are small (± 5.3%) and the coefficient of variation less than 4%, suggesting good reproducibility.

2.5. Comment on evaporation of faecal extract:

The evaporation of faecal extract has been a difficult problem. This is mainly due to the 'foaming effect' of bile salts. Of a number of techniques tried, evaporation using a rotary evaporator (Buchi, Model Rotavapor-EL, Orme Scientific Instrument, Manchester) was found most suitable. A special feature of this evaporator is a built-in vapour duct and a control valve to regulate the rate of evaporation and the flow of distillate.

i) Comment on hydrolysis of bile salts

Hydrolysis of bile salts is a necessary stage in all GC methods. The procedure of Evrard & Janssen (1968) was selected even though Okishio, Nair & Gordon (1967) suggested that drastic hydrolytic conditions may result

in some losses.

The results of recovery experiments showed that between 81 and 95% of the bile salts were recovered after hydrolysis by this method (Appendix Table 2.8). In order to reduce operator time, a timer (Smiths Industries) was used which allowed the temperature of the heating mantle to be maintained at 220 $\pm$ 2^{o}C for 20 mins.

2.6. Comment on the preparation of derivatives

i) Methyl Derivatives of bile acids: Prior to gas chromatography, the terminal carboxyl group of bile acids must be protected. Firstly, to increase the volatility of these compounds and secondly, to guard against decomposition. Methylation with diazomethane is usually used. However, diazomethane and its starting materials are extremely toxic (Arndt, 1950; Spinks, 1980). Further, explosions have been reported to occur during the preparation and codistillation of diazomethane into ether (de Boer & Backer, 1963).

In order to reduce the risks associated with the preparation of diazomethane a method not using codistillation has been developed. The diazomethane so prepared was used to methylate bile acids. The identity of methyl esters was checked using gas chromatography-mass spectrometry.

ii) 'Oxo' derivatives of bile acids and neutral sterols: These derivatives were prepared by the method of Anderson, Haslewood & Wootton (1957). The derivatives

were usually dissolved in a suitable amount of acetone and aliquots (1-2 µl) were used for gas chromatography.

iii) Trifluoroacetate and Di-methylhydrazone derivatives: These derivatives for bile acids were prepared according to the method of Eneroth & Sjovall (1971) with small modifications.

2.7 Gas chromatography and modifications

The gas chromatograph used was a Pye, Series 104, Model 64 fitted with dual flame ionisation detector (FID) and dual columns. This was connected to a flat bed, 1 mV Hitachi-Perkin Elmer chart recorder and a Varian, Model CDS III A integrator.

The basic GC instrument was found inadequate for bile acid analysis at high temperatures and was modified in a number of ways to provide accurate temperature control, strict carrier gas flow control, better injections and flow switching for Nitrogen, Hydrogen and Air supply lines. In this way the performance of GC and the repeatability of chromatography from one day to the next was improved.

i) Preparation of GC columns: All GC columns were packed with Gas Chrom-Q AW-DMCS (acid washed dimethyl chlorosilane) treated support material coated by the 'slurry' method (Horning, Brooks & Vanden-Heuvel, (1968). The columns were formed by applying suction at one end and conditioned for at least 48 hours at a temperature of 10^{o}C above the maximum operating

temperature.

ii) Regenerating GC columns During the course of this work a method for improving column performance was found by treating columns with trimethylchlorosilane (TMS) injected to the column *in situ* (2 x 5 µl) at 100°C with the detector end disconnected. The column was used after 1 hour.

iii) GC Liquid Phases used: All quantitative information on samples obtained from patients has been obtained using the columns of either QF-1 for methyl ester derivatives of bile acids or JXR columns for estimating the neutral sterols or the 'oxo' derivatives of both neutral sterols and bile acids.

2.8. Comment of the variation of detector response and the preparation of calibration curves

The variation in the flame ionisation detector (FID) response to known quantities of coprostanol injected to GC columns was checked in two ways, a) during the same day between 10 am and 4 pm and b) at approximately the same time on different days.

The detector response to neutral sterols analysed on JXR columns did not vary significantly between 10 am and 4 pm each day (Appendix, Table 2.9) but did vary from day to day. Similar consideration applied to the methyl esters of bile acids analysed on QF-1 columns. The calibration curves for both neutral sterols and bile acids were prepared daily.

The internal standard was used to make allowance for losses occuring during the whole procedure. Typical calibration curves are shown in Appendix Figures 2.5A - 2.5H.

2.9 Examples of separation of neutral sterols found in human faeces

i) QF-1 Columns

Figure 2.6A shows the typical tracing obtained for pure neutral steroids most commonly found in human faeces. Figure 2.6B shows a similar trace obtained using a faecal extract.

ii) JXR Columns

Figure 2.7A and 2.7B show similar separations obtained for neutral steroids analysed on JXR columns.

The use of JXR columns was preferred since this liquid phase provided more qualititative information of neutral steroids present in faecal extracts. The retention times of neutral sterols analysed on both QF-1 and JXR columns without preparing derivatives are listed in Appendix Table 2.10.

2.10 Examples of separation of bile acid methyl esters found in human faeces

i) QF-1 Columns

Figure 2.8A shows the typical tracing obtained for pure methyl esters of bile acids normally found in human faeces. Figure 2.8B shows the tracing obtained for methyl esters of bile acids found in a faecal

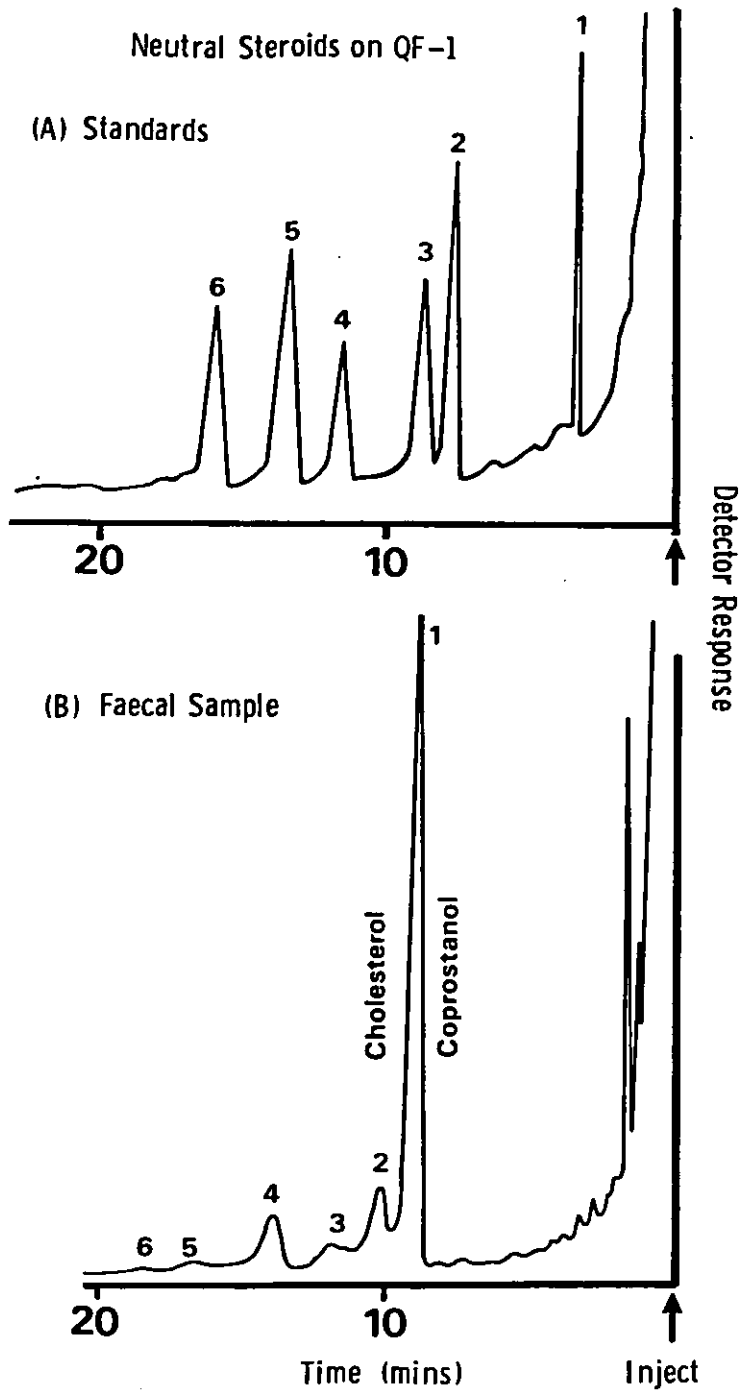


Figure 2.6A & B Tracings obtained for neutral steroids present in a standard mixture (upper trace, A) and neutral sterols found in a faecal extract (lower trace, B). Peak identity, upper trace: 1) 5α-cholestane, 2) coprostanol, 3) cholesterol, 4) stigmasterol, 5) β-sitosterol, 6) coprostanone; lower trace: 1) coprostanol, 2) cholesterol; 3,4,5 & 6) plant sterols. GC conditions for QF-1 column as in Appendix F.

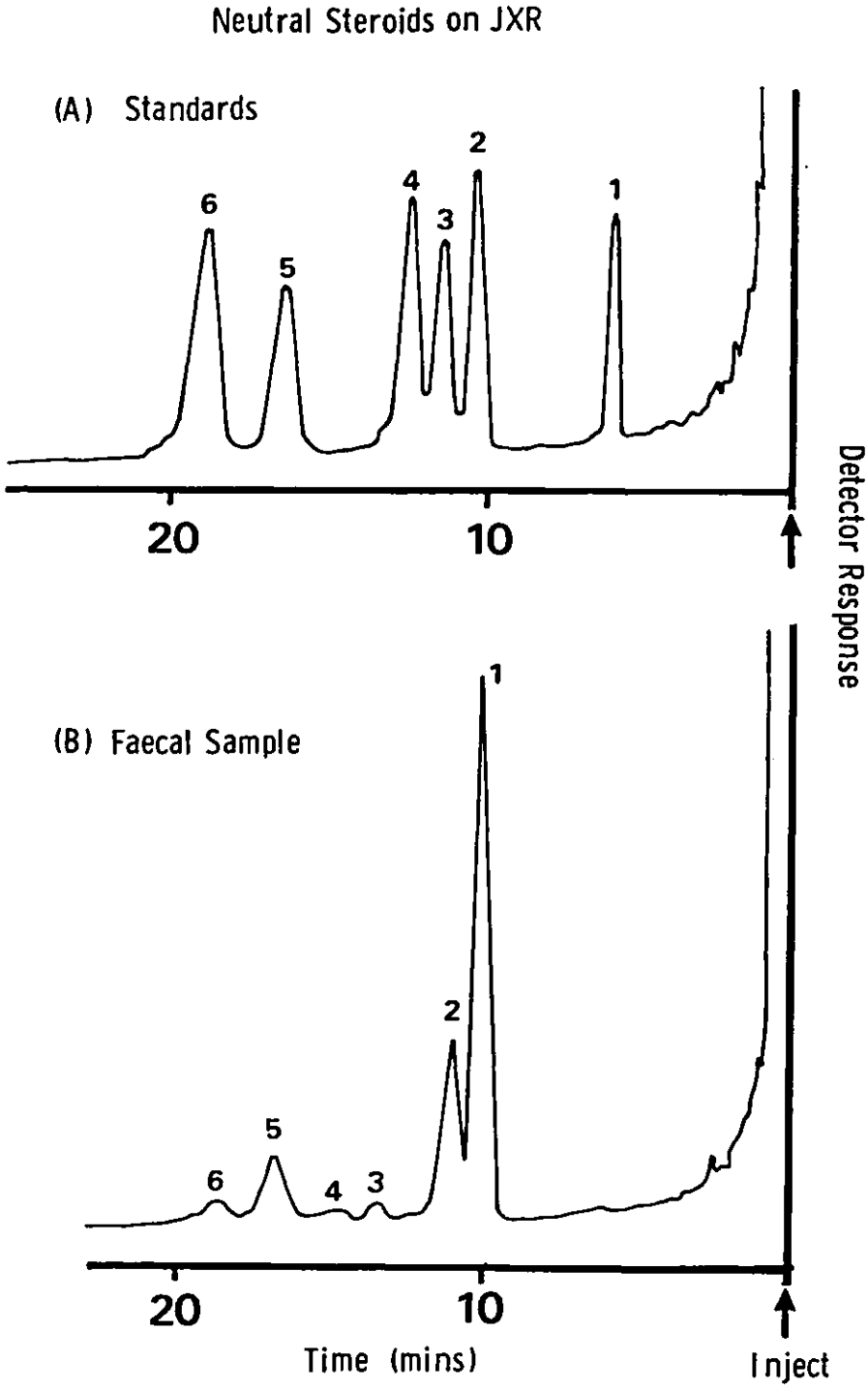


Figure 2.7A & B Tracings obtained for neutral steroids present in a standard mixture (upper trace, A) and neutral sterols found in a faecal extract (lower trace, B). Peak identity, upper trace: 1) 5α-cholestane, 2) coprostanol, 3) cholesterol, 4) cholestanone, 5) stigmasterol, 6) β-sitosterol; lower trace: 1) coprostanol, 2) cholesterol; 3,4,5 & 6) plant sterols. GC conditions for JXR column as in Appendix F.

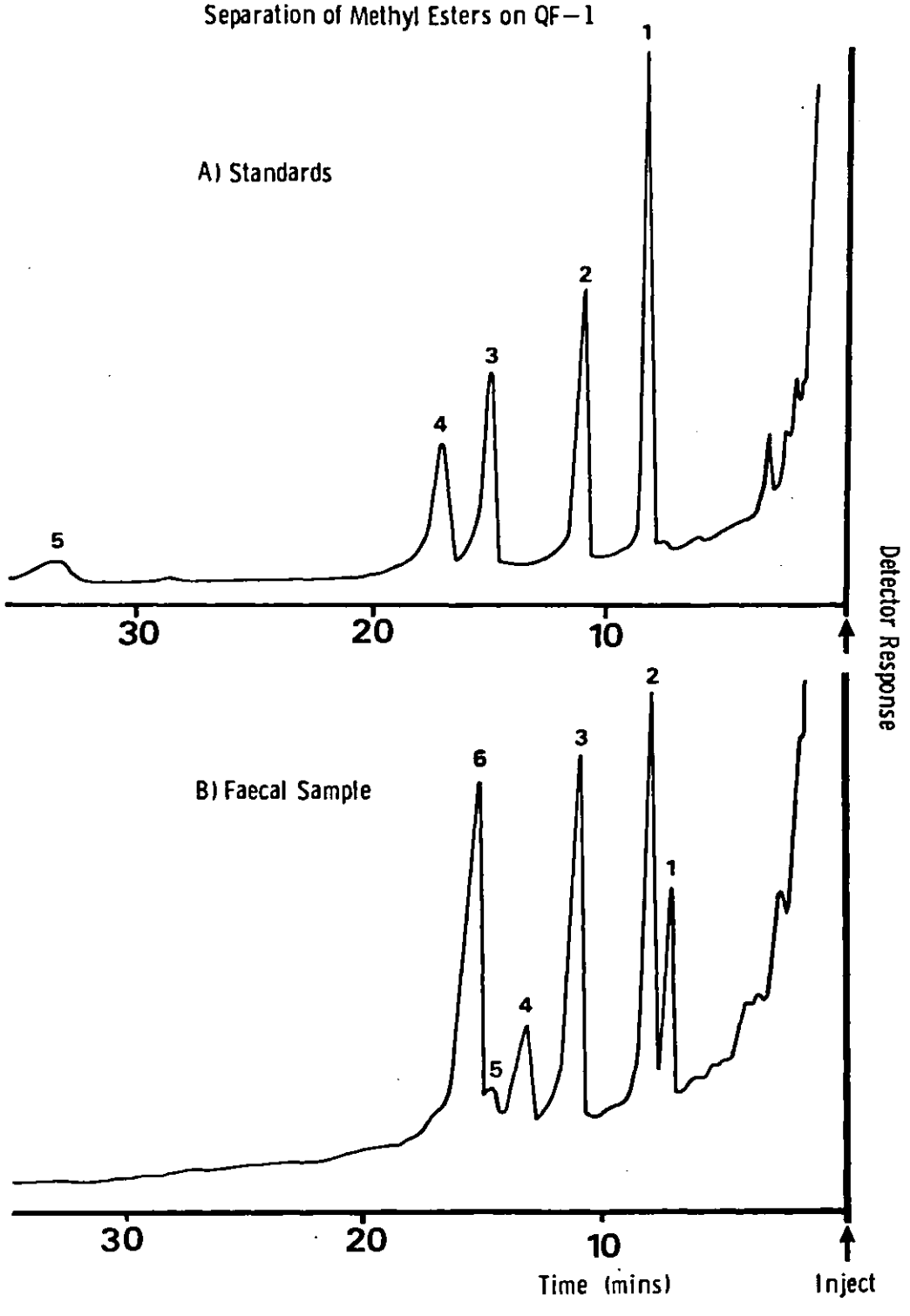


Figure 2.8A & B Tracings obtained for bile acid methyl esters present in a standard mixture (upper trace, A) and bile acids found in a faecal extract (lower trace, B) Peak identity, upper trace: 1) LC, 2) 23-Nor-DC, internal standard, 3) DC, 4) CDC, 5) C; lower trace: 1) 1LC 2) LC, 3) 23-Nor-DC, 4) 3β, 12α, 5) 3β, 12β, 6) DC. Sample: 2µl of faecal extract prepared for GC. GC conditions as in Appendix F.

extract.

ii) JXR Columns

JXR was found unsatisfactory liquid phase for the analysis of methyl esters of bile acidsobtained from faeces even though satisfactory separations could be obtained for the pure compounds.

The use of QF-1 was preferred since this liquid phase provided more information of the kinds of bile acids present in faeces. The retention times of methyl esters on QF-1 are listed in Appendix Table 2.11.

2.11 Examples of separations of methyl ester 'oxo' derivatives of bile acids

i) QF-1 Columns

This liquid phase was highly selective for methyl ester 'oxo' derivatives of bile acids. The analysis time was extensively long and impracticable.

ii) JXR Columns

Figure 2.9A shows the typical tracings obtained for pure methyl ester 'oxo' derivatives of bile acids whilst Figure 2.9B shows the separation of these compounds found in a faecal extract. Peaks,1, 3 and 4 (Figure 2.9B) represent bile acids which on oxidation give the corresponding mono-, di- and tri-ketocholanoate respectively. Peak 2 arises from the internal standard (23-Nor-DC).

The use of JXR columns was preferred since the analysis

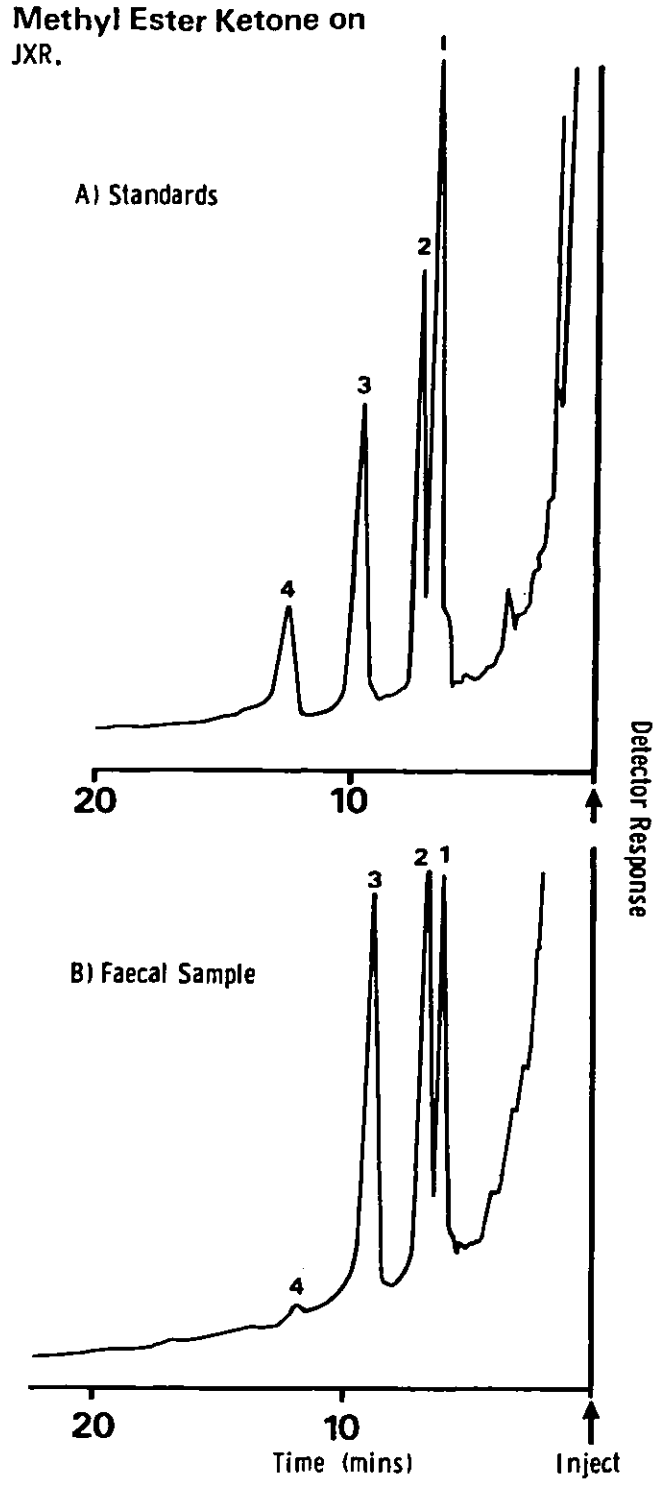


Figure 2.9A & B Tracings obtained for methyl ester ketone derivatives of bile acids present in a standard mixture (upper trace, A) and bile acids found in a faecal extract (lower trace, B). Peak identity, upper and lower trace: 1) LC, 2) 23-Nor-DC, internal standard, 3) DC, 4) C. GC conditions for JXR column as in Appendix F.

time was much shorter than on QF-1 columns (R_t of cholic = 12 min on JXR compared with 60 min on QF-1 columns) and the peak shape better. The retention time of 'oxo' derivatives are listed in Appendix Table 2.12).

2.12 ASSESSMENT OF METHODS

A. NEUTRAL STEROLS

i) Accuracy

a) Comparison with other methods

The results obtained for neutral sterols estimated by ourselves and those reported by other authors are listed in Table 2.13.

b) Use of two different techniques

As a check on the accuracy of the final quantitation, the neutral sterol content of six extracts was estimated by the GC method described. The 'oxo' derivatives of neutral sterols were then prepared (2.6. ii) and the amount of these derivatives present estimated. There was no significant difference between results obtained using these different analytical techniques (Appendix Table 2.14).

ii) Reproducibility of method for neutral sterols

Reproducibility was examined in two ways, a) replicate analysis of a single stool collection and b) by examining the difference found when duplicate estimations were made on different samples of stools.

a) The results of nine replicate analyses performed during the course of one day on samples taken from a single stool collection are shown in Table 2.15 The standard deviation is small ($\pm$ 2.1 mg/g dry weight) and the coefficient of variation of 6.5% suggesting good reproducibility.

Table 2.13 Daily Neutral Sterol excretion in man (comparison with other methods)

Author	Method	Subjects	Excretion (mg/d) Range	Mean
Moore et al (1962)	Isotopic balance	5 Normals		(482)
Eneroth et al (1964)	GC	6 Normals	385-753	(527)
Grundy et al (1969)	Sterol-balance	9 Normals		(546)
Miettinen (1971)	Sterol-balance	10 Normals	375-1079	(634)
		10 Obese	861-1646	(1263)
		8 Normals	358-583	(464)
This study	GC	8 Obese	565-912	(766)

Table 2.15 Reproducibility of GC method for estimating neutral sterols present in faeces using replicate estimates.

Replicate Sample	Neutral Sterol Content (mg/g dry weight of faeces)	Difference From Mean	Difference From Mean %
1	31.2	1.2	3.7
2	32.4	0.0	0.0
3	33.6	1.2	3.7
4	29.8	2.6	8.0
5	34.7	2.3	7.1
6	34.4	2.0	6.2
7	29.6	2.8	8.6
8	30.8	1.6	4.9
9	34.6	2.2	6.8

Mean = 32.4 $\pm$ 2.1 (SD)

Coefficient of Variation = 6.5%

b) To assess more realistically the variation likely to occur during routine work, a method suggested by Snedecor (1952) was used. Duplicate estimations were made on six different days using six different samples. The results are shown in Table 2.16.

By both methods the standard deviation and the coefficient of variation appeared to be satisfactory.

iii) Sensitivity

The sensitivity of the estimates which may be defined as the least detectable quantity was calculated according to Brown, Bulbrook & Greenwood (1957). This was found to be approximately 0.12 mg/g dry weight of faeces.

iv) Specificity

This implies that the areas under the peaks correspond to the named neutral sterols. The identity of the main peaks in the tracing obtained for faecal extracts was checked by:

a) Retention times

The retention times and the elution pattern of the main peaks found in the faecal extracts agreed with those reported by Eneroth, Hellstrom & Ryhage (1964).

b) Separating components of faecal extracts before and after the preparation of 'oxo' derivatives

This method affected a 'peak shift' in the tracing this was found to agree with the results reported (Eneroth, Hellstrom & Ryhage, 1964).

Table 2.16 Reproducibility of GC method for estimating neutral sterols present in faeces using duplicate estimates

Subjects	Neutral Sterol Content (mg/g dry weight of faeces) Duplicate Estimates A	B	Difference (A-B) = D	Difference $(D)^2$
1	27.6	29.4	1.8	3.2
2	19.8	21.1	1.3	1.7
3	27.2	24.5	2.7	7.3
4	26.1	22.5	3.7	13.7
5	28.3	26.7	1.6	2.6
6	32.8	30.4	2.4	5.8

Mean = 26.3 $\pm$ 1.7 (SD) $\Sigma D^2 = 34.3$

Coefficient of Variation = 6.5%

c) By separating the components of faecal extracts on columns of different polarity (QF-1 and JXR).

d) Thin layer chromatography-gas chromatography

A number of faecal extracts were analysed by TLC. The identity of main spots checked by running standards and confirmed by GC after eluting the compounds from the silica gel.

2.12 B BILE ACIDS

i) Accuracy

a) Comparison with other methods:

The results obtained for bile acids estimated by ourselves and those reported by others are listed in Table 2.17.

b) Use of two different techniques:

As a check on the accuracy of the final quantitation, the apparent bile acid content of faeces was estimated by the method described. The 'oxo' derivatives were then prepared (2.6 ii) and the amount of these derivatives present estimated. There are no significant difference between the results obtained by these methods (Appendix Table 2.18).

ii) Reproducibility of method for Bile Acids

Reproducibility of the method was examined in a similar manner to that used for neutral sterols.

Table 2.17 Daily Faecal Bile Acid Excretion in Man (comparison with other methods)

Author	Method	Subjects	Excretion (mg/day) Range	Mean
Rosenfield et al (1962)	Isotopic balance	2		(290)
Grundy et al (1965)			120-225	(173)
Evrard & Janssen (1968)	GC	5 Normals	127-290	(208)
Nestel et al (1974)		8 Normals	170-352	(267)
		6 Obese	311-522	(429)
Miettinen (1971)	Isotopic balance	10 Normals	117-420	(235)
This study	GC	8 Normals	175-307	(230)
		8 Obese	270-524	(378)

a) The results of nine replicate analyses made during the course of one day on samples taken from a single stool collection are shown in Table 2.19. The standard deviation is small (± 0.5 mg/g dry weight) and the coefficient of variation of 5.4%.

b) When the variability of bile acid content of faeces was assessed by the method suggested by Snedecor (1952) the results obtained on six different days using six different samples are shown in Table 2.20.

By both methods the standard deviation and the coefficient of variation appeared to be satisfactory.

iii) Sensitivity

The sensitivity of the estimates was calculated as for neutral sterols. This was found to be approximately 54 ug/g dry weight of faeces.

iv) Specificity

This implies that the areas under the peaks correspond to the named bile acids. The identity of the main peaks in the tracings obtained for faecal extracts was checked by:

a) Retention times:

The retention times and the elution pattern of the main bile acids found in faecal extracts agreed with those reported (Eneroth et al 1966); Kuksis, 1969; Eneroth & Sjovall, 1971).

Table 2.19 Reproducibility of GC method for estimating bile acids using replicate estimates

Replicate Sample	Bile Acid Content of Faeces (mg/g dry weight)	Difference From Mean	%Difference From Mean
1	9.1	-0.1	-1.0
2	8.9	-0.3	-3.2
3	9.4	+0.2	+2.2
4	9.7	+0.5	+5.4
5	8.8	-0.4	-4.3
6	9.3	+0.1	+1.0
7	9.6	+0.4	+4.3
8	9.8	+0.6	+6.5
9	8.7	-0.5	-5.4

Mean = 9.2 $\pm$ 0.5 (SD)

Coefficient of Variation = 5.4%

b) Separating components of faecal extracts before and after the preparation of 'oxo' and trifluoroacetate derivatives:

These methods affected a 'peak shift' in the tracings The results obtained for the 'oxo' and the TFA derivatives of the main bile acid found in faecal extracts agreed with those reported (Kuksis, 1969; Eneroth & Sjovall, 1971).

Table 2.20 Reproducibility of GC method for estimating bile acids present in faecal extracts using duplicate estimates

Subject	Duplicate Estimates (mg/g dry weight of faeces) A	B	Difference D (A-B)	Difference D^2 $(A-B)^2$
1	9.3	9.1	0.2	0.04
2	8.2	8.4	0.2	0.04
3	8.9	8.7	0.2	0.04
4	8.1	8.5	0.4	0.16
5	10.5	10.3	0.2	0.04
6	10.9	10.7	0.2	0.04

Mean = 9.3 $\pm$ 0.2 (SD) $\quad \Sigma D^2 = 0.36$

Coefficient of Variation = 2.2%

c) By separating the components of faecal extracts on columns of opposite polarity (QF-1 and JXR).

d) Thin layer chromatography-gas chromatography: A number of faecal extracts were analysed by TLC. The identity of the main spots was checked by running standards and confirmed by GC after eluting the compounds from the silica gel.

e) Gas chromatography-mass spectrometry: The identity of the main bile acids found in faecal extracts was confirmed by GC-MS made available by the kindness of Queen Charlotte's Hospital, London.

2.13 Comment on a new stationary phase SP 2250

Among the numerous stationary phases tested during the course of this work a material, SP 2250 (Supelco Inc.), was found to offer in some circumstances advantages over both QF-1 and JXR. This material has not been previously used for separating neutral sterols present in faeces or the methyl esters of faecal bile acids.

Figure 2.10A shows the typical tracing obtained for pure neutral sterols analysed on SP 2250 whilst Figure 2.10B shows the tracing obtained for a faecal extract.

The tracings obtained for pure methyl esters of bile acids and for the bile acids found in a faecal extract are shown in Figure 2.10C and 2.10D. The tracings

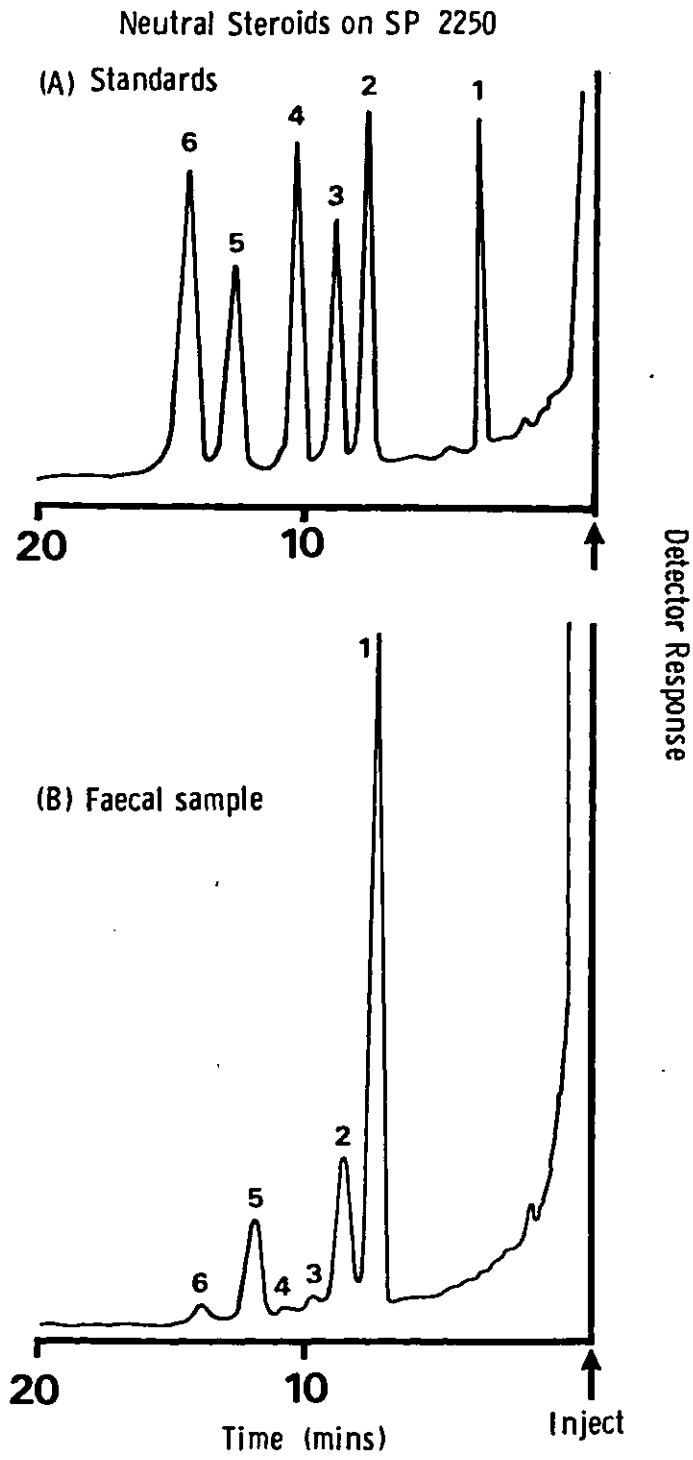


Figure 2.10A & B Tracings obtained for neutral steroids present in a standard mixture (upper trace, A) and neutral sterols found in a faecal sample (lower trace, B). Peak identity, upper trace: 1) 5α-cholestane, 2) coprostanol, 3) cholesterol, 4) coprostanone, 5) stigmasterol, 6) β-sitosterol; lower trace: 1) coprostanol, 2) cholesterol; 3,4,5 & 6) plant sterols. GC conditions for SP2250 as in Appendix F.

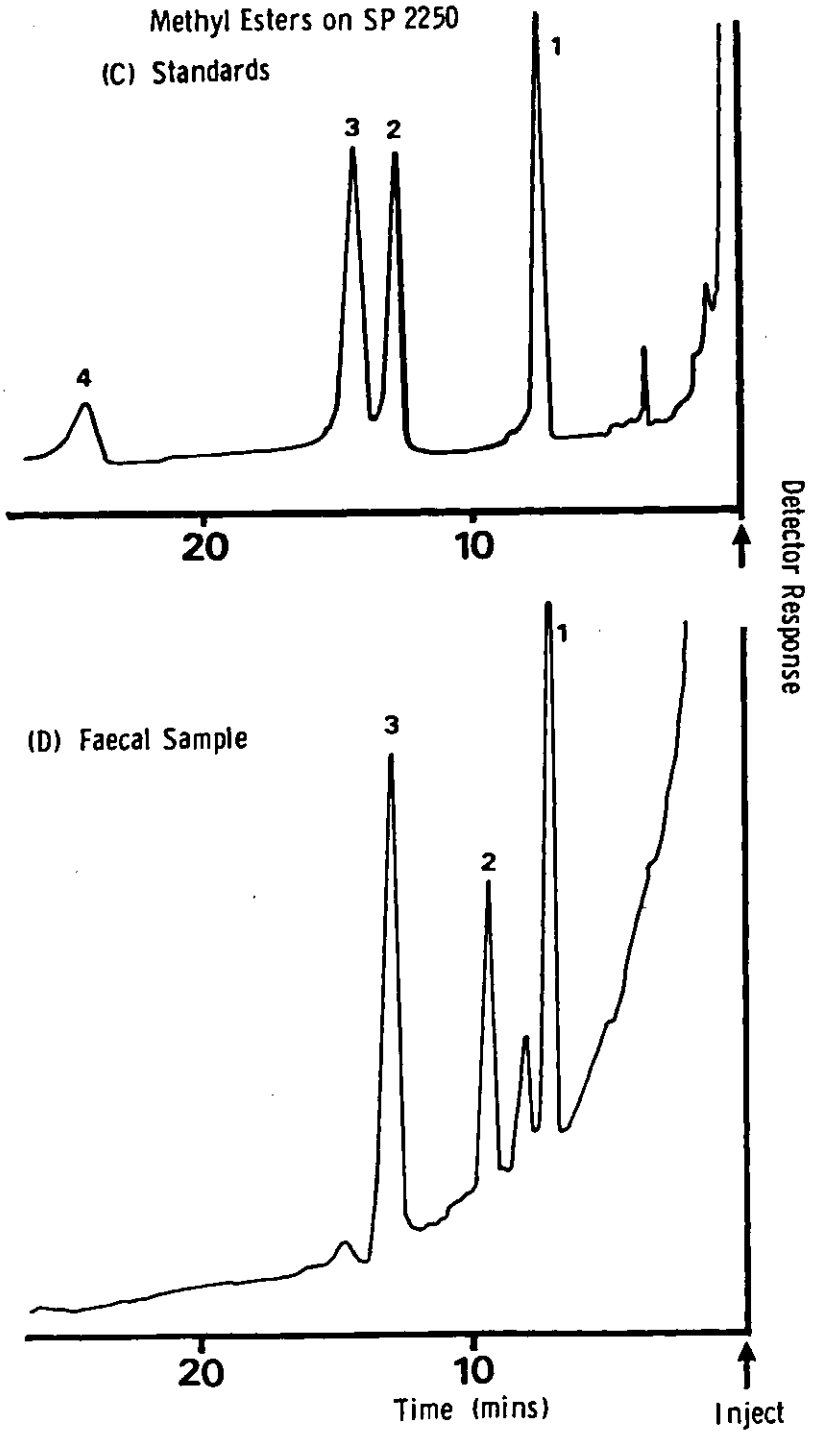


Figure 2.10C & D Tracings obtained for bile acid methyl esters present in a standard mixture (upper trace, C) and bile acids found in a faecal extract (Subject, S.L.; lower trace, D). Peak identity, upper trace: 1) LC, 2) DC, 3) CDC, 4) C; lower trace: 1) LC, 2) 23-Nor-DC, internal standard, 3) DC. Peaks at 8 min and 15 min, unidentified. GC conditions for methyl esters on SP 2250 as in Appendix F.

obtained for the analysis of a complex mixture of methyl esters of bile acids on QF-1 and SP 2250 columns are shown in Figures 2.10E and 2.10F. On the new stationary phase, the analysis time is shorter and 'peak tailing' is reduced; but the peak for UDC (Peak 7 Figure 2.10F) is not resolved. The retention times of a number of methyl esters analysed on SP 2250 are listed in Appendix Table 2.12.

Since the discovery of this material occurred late in the course of this work, all quantitative results reported have been obtained using either QF-1 or JXR columns.

2.14 Discussion

At the present stage of development, GC has several advantages over other methods for the determination of bile acids and neutral sterols in complex mixtures such as human faeces. In this section which follows are discussed various modifications we have introduced into the bile acid methodology in an attempt to overcome some of the problems discussed in the introductory chapter.

One of the first problem was that of sample preparation. A rapid and improved method for freeze-drying faeces has been developed which unlike previous method does not require the use of 'filter aid'; further, drying is completed within 4-6 hours.

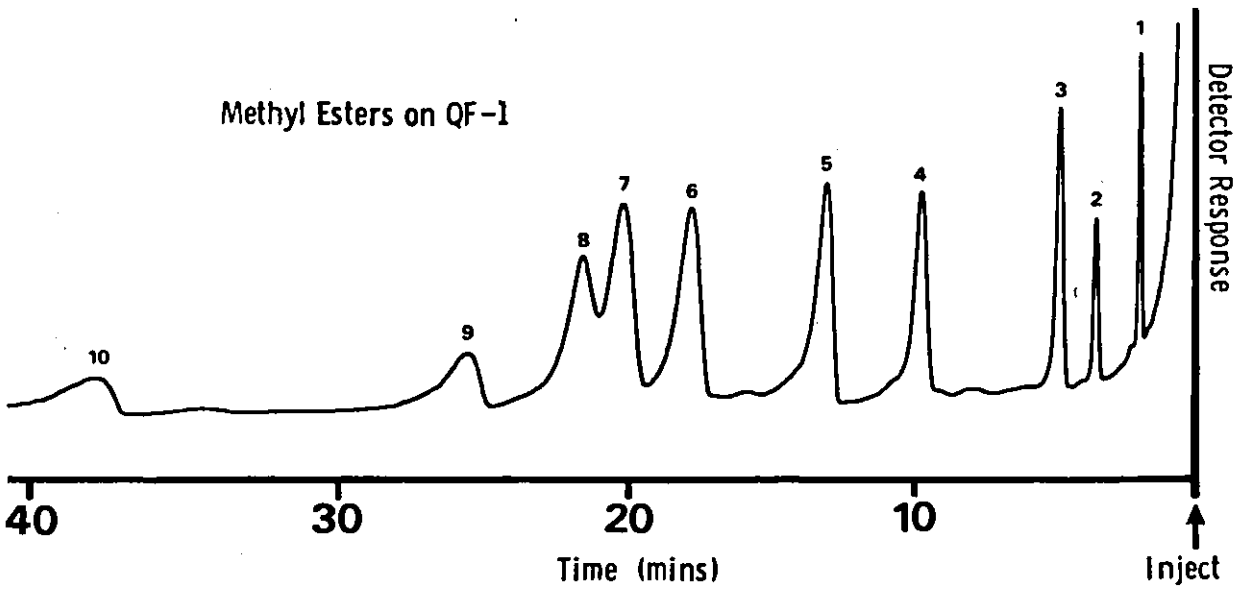


Figure 2.10E Separation of bile acid methyl esters present in a complex mixture of standards. Peak identity: 1) 5α-c 2)cholanic, 3) cholesterol, 4) LC, 5) 23-Nor-DC, 6) DC, 7) CDC, 8) UDC, 9) hyodeoxycholic, 10) C. GC conditions, as for Figure 2.8A.

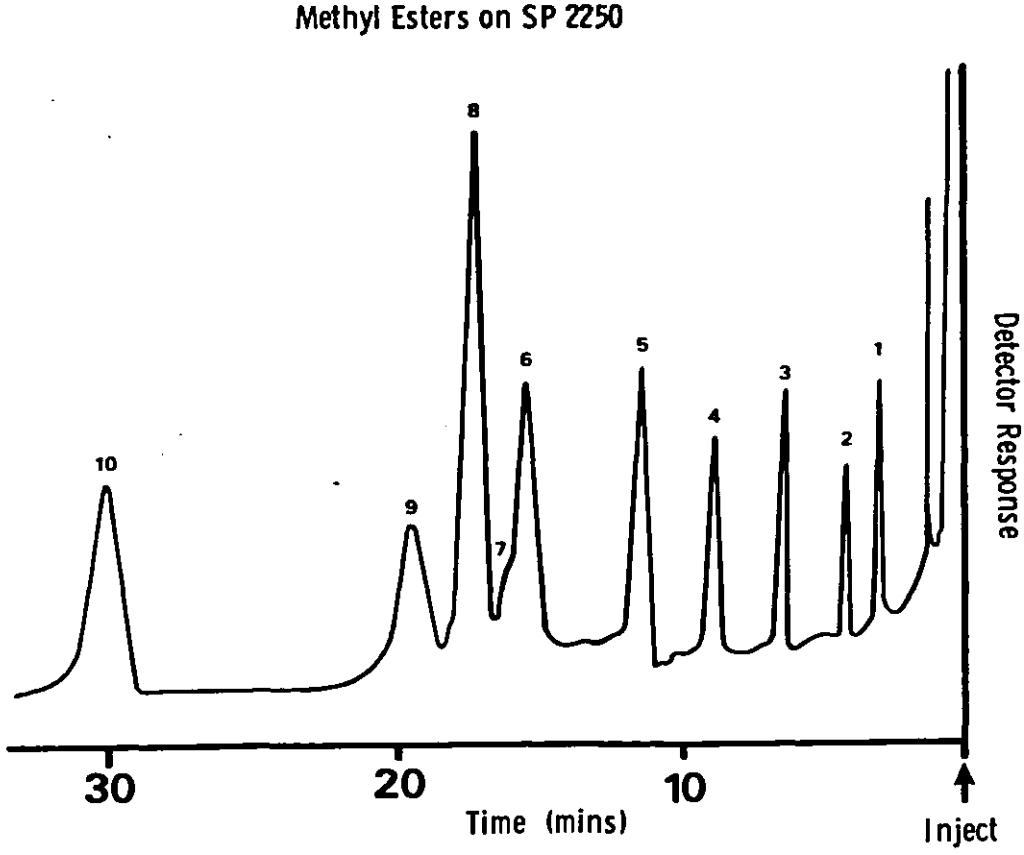


Figure 2.10F Separation of bile acid methyl esters present in a complex mixture of standards analysed on SP 2250 column. Peak identity: 1 to 6) as for Figure 2.10E; 7) UDC, 8) CDC, 9) hyodeoxycholic, 10) C. GC conditions, as for Figure 2.10C.

The extraction of bile acids and neutral sterols from human faeces has been a tedious and lengthy procedure. A simple and rapid method was valuable therefore a semi-automated extraction procedure was developed. This method has been used routinely for over three years without any difficulties. Some of the obvious advantages are as follows: a) speed of analysis; b) reduced operator time; c) increased number of samples extracted simultaneously (24 versus 6 previously) and the possibility of increasing by scaling up the apparatus; d) the method can be used for extracting cholesterol from bile; e) in contrast to any manual method, this method is operator independent, and all samples receive the same treatment thus reducing human error; f) introduction of a 'rest period' before the next 'shake' allows aspiration of ethereal layer before the next extraction.

In further tests, the modified procedures were shown to conform to the criteria of accuracy, precision and repreducibility. Steriod extractions were found to be reliable and quantitative. It is claimed that this procedure is an improvement in the methodology of these compounds and should be useful for routine use.

In all GC work on bile acids, methylation is an essential stage. This is normally carried with diazomethane; but the preparation of this reagent is dangerous because its starting materials and

diazomethane itself are both explosive and carcinogenic. Other methods investigated included the use of dimethyl sulphate, boron trifluoride-methanol complex, methanolic- H_2SO_4, and methanolic-HCl but none of these procedures were found satisfactory for methylating bile acids. Some of the difficulties were, the inability to produce absolutely dry conditions, the long reaction times, identification of spurious peaks in GC chromatograms, the formation of bi-products due to side reactions and the inconsistency of the results obtained using these procedures. The method developed for generating and methylating bile acids, is safer, simple and does not require the distillation of the product which is associated with explosions. The starting material N-methyl-N-nitrosourea used in this study reacts rapidly but it may be possible to use a milder and less toxic reagent N-methyl-N-nitroso-p-toluenesulphonamide (de Boer & Backer, 1963) instead. For methylating bile acids the reaction time was kept to a minimum (15 minutes) to minimise the risk of side reactions. The use of lower temperatures ($-10^{o}C \pm 5^{o}$) ensured that the diazomethane generated was collected immediately into ether. Some of the advantages of this method are: that the preparation of the reagent and methylation is carried out in a fully sealed system which reduces the risks involved. Other advantages are that diazomethane can be condensed into solvents other than ether; and that at the end of the preparation

of the reagent, the material left can be decomposed by introducing acidified water (10-15 ml) with a syringe without opening the system to the atmosphere.

The method introduced has been routinely used for over three years without problems. With the present apparatus, upto 20 samples can be methylated in one session and this number can be increased by scaling up the apparatus.

The Chromatograph (Pye Series 104, Model 64) used in this work was found inadequate when used as purchased. The following modifications were made: Firstly, thermocouples were installed at different parts to give a strict temperature control and a temperature differential between the column and the oven was corrected by modifying the position of the mecury thermometer (Appendix Fig. 2.11). Secondly, the control of gas flows was improved by constructing 'flow meters'. These improved the ease with which the same flow rate could be repeated on another day. Thirdly, 'flow switching' of Nitrogen, Hydrogen and Air allowed the measurement of flow rate of any one of these without turning off the others. Finally, the injection technique was modified, and a 'needle guide' was found useful; the empty space between the injector and the column packing was reduced. Such modifications improved the resolution of steroid separations and the reproducibility of estimations. All GC columns were packed using a 'suction technique'

in order to reduce the damage to the geometry of the coated support material which could occur with a vibrator. The majority of the separations were carried out with glass columns of 1.5 m in length and 4 mm internal diameter. Longer columns of 2-3 m were used for the purpose of GC-MS where increased separation factor was more important; whereas shorter columns of 1 m in length and 3 mm internal diameter were useful for fast separations where only the classes of bile acids were separated (e.g. 'oxo' derivatives on JXR columns). The longer columns demonstrated a 'concertina effect' by pulling the mixture of bile acids apart to give increased resolution of bile acids.

Although stainless steel columns were easier to manipulate and had no temperature limitation, the separation of bile acids were poor compared with those obtained using glass columns. The poor separation may be due to adsorption effects of metal tubing or the inhomogeneity of column packing material which cannot be observed using metal columns. When GC columns were not in use for several months, a deterioration was noticed in column efficiency (N). This problem was solved by re-silanising the column *in-situ* which restored the selectivity and column efficiency. The procedure therefore prolonged column life. We also found that aged columns gave superior separations. It is possible that the active sites on column support are reduced by constant use.

Generally, shorter columns were preferred since bile acid losses are kept to a minimum. Column 'bleed' was also small in these columns but it posed a problem in longer columns. Running two columns using 'dual FID' and using a stationary phase with a higher thermal stability (SP 2250) gave relatively low column bleed compared to QF-1 and JXR columns.

It is believed that the modified extraction and the improved GC procedures are a step towards a fully automated method for estimating these compounds in biological samples. These techniques were used to investigate a problem involving 'fenfluramine' which forms the subject of the next chapter.

CHAPTER 3

APPLICATION OF THE DEVELOPED METHODS TO STUDY BILE ACIDS AND NEUTRAL STEROLS PRESENT IN THE FAECES OF SUBJECTS TREATED WITH FENFLURAMINE

3.1 Introduction

The GC methods previously described (Chapter 2) were used to follow the changes which occur in the faecal bile acid and neutral sterol excretion in subjects taking fenfluramine, an antiobesity agent.

The objective was to investigate:
1) any changes in the excretion of bile acids and neutral sterols occurring on ingestion of fenfluramine; 2) whether fenfluramine caused the excretion of any bile acid not normally found in faeces and 3) any possible relationship between the occurance of diarrhoea, the ingestion of fenfluramine and the kind or the amount of bile acids excreted.

In order to answer these questions two investigations have been carried out. The details of Study I are bound separately in this thesis (Sian & Rains, 1979 a) and only the salient points from the first investigation are mentioned below.

3.2 STUDY I

No. of subjects who completed Study I	= 16
No. who had fenfluramine-induced diarrhoea	= 3
No. of pooled stool collections per subject	= 9
Duplicate estimations per subject (2 x 9)	= 18
Minimum number of bile acid estimations (16 x 18)	= 288
Minimum number of neutral sterol estimations (16 x 18)	= 288
Total number of estimations	= 576

A summary of the results of Study I (Table 3.1)

The results of the first study demonstrated that in a group of 16 subjects the mean bile acid excretion (mg/g dry weight of faeces; mean ± SD) increased from 9.3 ± 3.1 during the control period to 12.1 ± 4.5 during the drug period ($P < 0.02$); and to 11.6 ± 3.4 during the post-drug period ($P < 0.05$).

The mean neutral sterol excretion (mg/g dry weight of faeces; mean ± SD) increased from 16.7 ± 4.9 during the control period to 23.3 ± 8.6 during the drug period ($P < 0.02$). The excretion remained high at 24.1 ± 9.0 during the post-drug period ($P < 0.05$).

The mean excretion of total faecal steroids defined as the sum of bile acids and neutral sterols increased from 26.0 ± 7.0 during the control period to 35.2 ± 10.7 during the drug period ($P < 0.01$); and to 36.7 ± 11.5 during the post drug period ($P < 0.01$).

The mean daily faecal fat excretion (g/day; mean ± SD) measured in six subjects increased from 2.6 ± 0.4 during the pre-drug period to 3.9 ± 1.1 during the drug period ($P < 0.05$). The excretion remained at 3.1 ± 0.5 g/day during the recovery period ($P < 0.01$).

Of the 16 subjects, eight were more than 10% above their ideal body weight (for details see Appendix Table 3.3). During the control period, the mean bile acid excretion (mg/g dry weight of faeces, mean ± SD) for the 'overweight subjects' was 10.8 ± 3.8 ($N = 8$), and for those with

Table 3.1 Quantitative changes in faecal steroid (bile acid and neutral sterol) excretion in subjects taking fenfluramine (Study I)

Faecal Steroid Excretion (mg/g dry weight of faeces) N = 16 subjects	Pre-Drug	Drug	Post-Drug
Bile Acids:			
Mean	9.3	12.1**	11.6*
SD	±3.1	± 4.5	± 3.4
Range	(7-17)	(6-21)	(7-18)
Neutral Sterols:			
Mean	16.7	23.1**	25.1**
SD	± 4.9	± 8.6	± 9.0
Range	(10-28)	(10-36)	(13-38)
Total Steroids (Bile Acids + Neutral Sterols):			
Mean	26.0	35.2***	36.7***
SD	± 7.0	±10.7	±11.5
Range	(16-40)	(19-51)	(19-55)

Significantly different from the excretion during the Pre-Drug period, *$P<0.05$; **$P<0.02$; ***$P<0.01$

'normal weights' 7.7 ± 0.7 (N = 8). The difference in the bile acid excretion was significant ($P < 0.01$).

Qualitatively, the excretion of bile acids before and after giving the drug remained unaltered except in three subjects who had a fenfluramine-induced diarrhoea. These subjects previously excreted trace amounts of CDC and C, eliminated significant amounts of CDC and C after ingesting fenfluramine.

The pattern of neutral sterol excretion in all subjects except one was markedly altered. The excretion of coprostanol was diminished to a trace amount and that of cholesterol was increased.

3.3 Further observations made during Study I

Subjects with fenfluramine-induced diarrhoea

Confirmation of the nature of bile acids found in the stools collected from those with fenfluramine-induced diarrhoea (Subjects: 8, 14 and 16) was obtained using GC-MS made available by the kindness of Queen Charlotte's Hospital, London. The identity of the major bile acids in faeces analysed as their methyl esters was confirmed but a number of small peaks in the tracings could not be identified because of lack of sensitivity.

The mean bile acid excretion (mg/g dry weight of faeces; mean ± SD) in subjects 8, 14 and 16 during the 'control' period was 11.2 ± 5.6. The excretion was increased to

18.3 ± 7.2 during the 'drug period'. This represents a 63% rise in excretion. The excretion returned to 10.9 ± 4.8 during the recovery period.

The excretion of neutral sterols (mg/g dry weight of faeces; mean ± SD) followed a similar pattern to bile acids. This was increased from 12.5 ± 4.8 during the control' period to 23.2 ± 5.4 during the drug period and returned to 15.9 ± 3.9 during the recovery period.

Total faecal steroid excretion (i.e. bile acids + neutral sterols; mg/g dry weight of faeces; mean ± SD) increased from 23.7 ± 6.3 during the 'control' period to 41.5 ± 11.4 during the drug period. The excretion returned to 26.8 ± 8.5 during the recovery period. The results are summarised in Table 3.2.

Table 3.2 Quantitative changes in faecal bile acid and neutral sterol excretion in subjects suffering from fenfluramine-induced diarrhoea

Faecal Steroid Excretion (mg/g dry weight of faeces)			
N = 3 subjects	Pre-Drug	Drug	Post-Drug
Bile Acids:			
Mean	11.2	18.3	10.9
S D	±5.6	±7.2	±4.8
Range	(6-18)	(13-29)	(7-18)
Neutral Sterols:			
Mean	12,5	23.2	15.9
S D	±1.8	±5.4	±2.9
Range	(8-17)	(14-35)	(9-25)
Total Steroids (Bile Acids + Neutral Sterols):			
Mean	23.7	41.5	26.8
S D	±6.3	±11.4	±8.5
Range	(13-33)	(29-64)	(17-44)

3.4 Bile salts in the faeces of subjects without diarrhoea

Both GC and TLC were used to investigate the qualitative changes following treatment with fenfluramine. Of the 13 subjects who did not suffer from a diarrhoea after fenfluramine (i.e. 13 x 9 = 117 extracts) only one subject showed the presence of bile salts (conjugated bile acids). The typical TLC separations for this individual during the three periods of the study are shown in Figure 3.1.

Since the majority of the bile acids found after treatment with the drug were in the unconjugated form, it is suggested that the bacterial activity of the enzyme cholanyl hydrolase (Aries & Hill, 1970) responsible for deconjugating bile salts was not affected. The detection of bile salts in the faeces of just one subject may be a reflection of a rapid intestinal transit of these compounds with less exposure to bacterial enzymes.

3.5 Bile salts in the faeces of subjects suffering from fenfluramine-induced diarrhoea

TLC analyses of stools collected from those suffering from fenfluramine-induced diarrhoea (Subjects, 8, 14 and 16) showed the presence of both bile salts and bile acids. The increased excretion of primary bile acids CDC and C together with smaller amounts of secondary bile acids DC and LC in these subjects was demonstrated by GC. Typical chromatograms obtained for one of these subjects showing

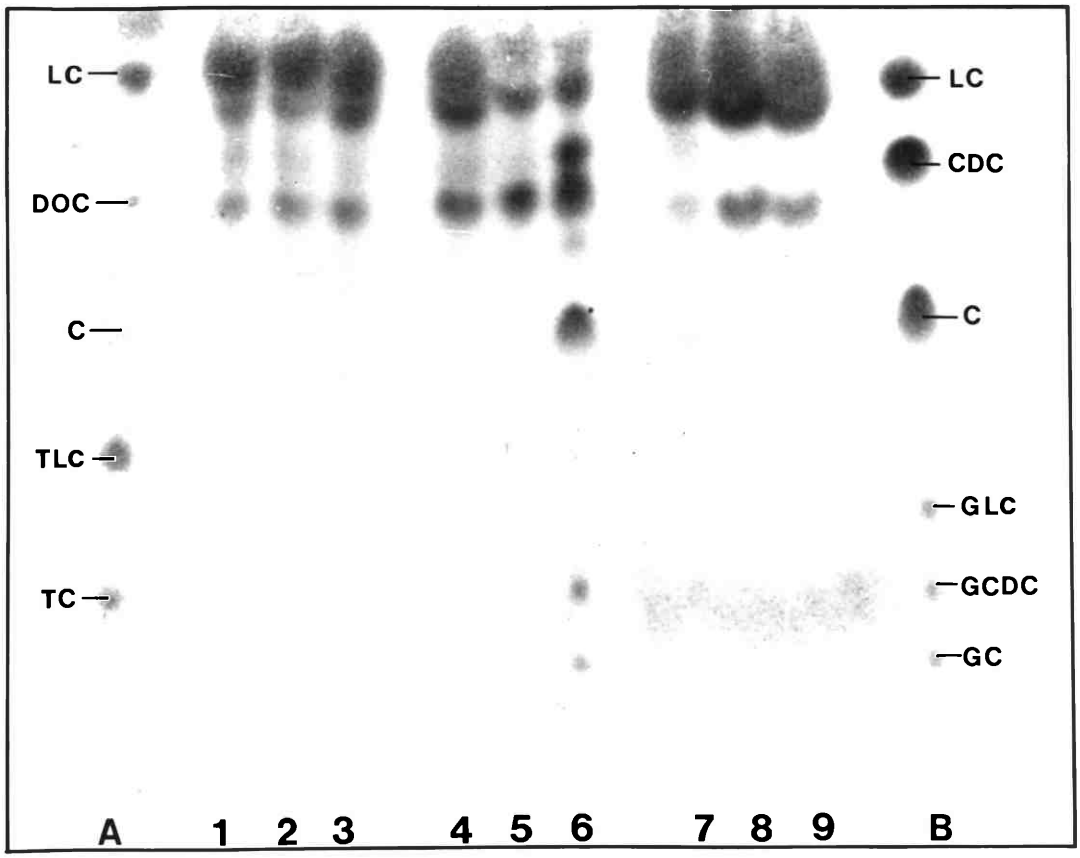


Figure 3.1 TLC chromatogram showing the separation of bile acids and bile salts present in faecal samples collected during fenfluramine study. Samples; 1, 2 & 3, control; 4, 5 & 6, drug; 7, 8 & 9, post drug. A and B are mixtures of known standards. TLC conditions, as in Appendix E.

marked qualitative and quantitative changes in bile acid excretion are shown in Figures 3.2A and 3.2B

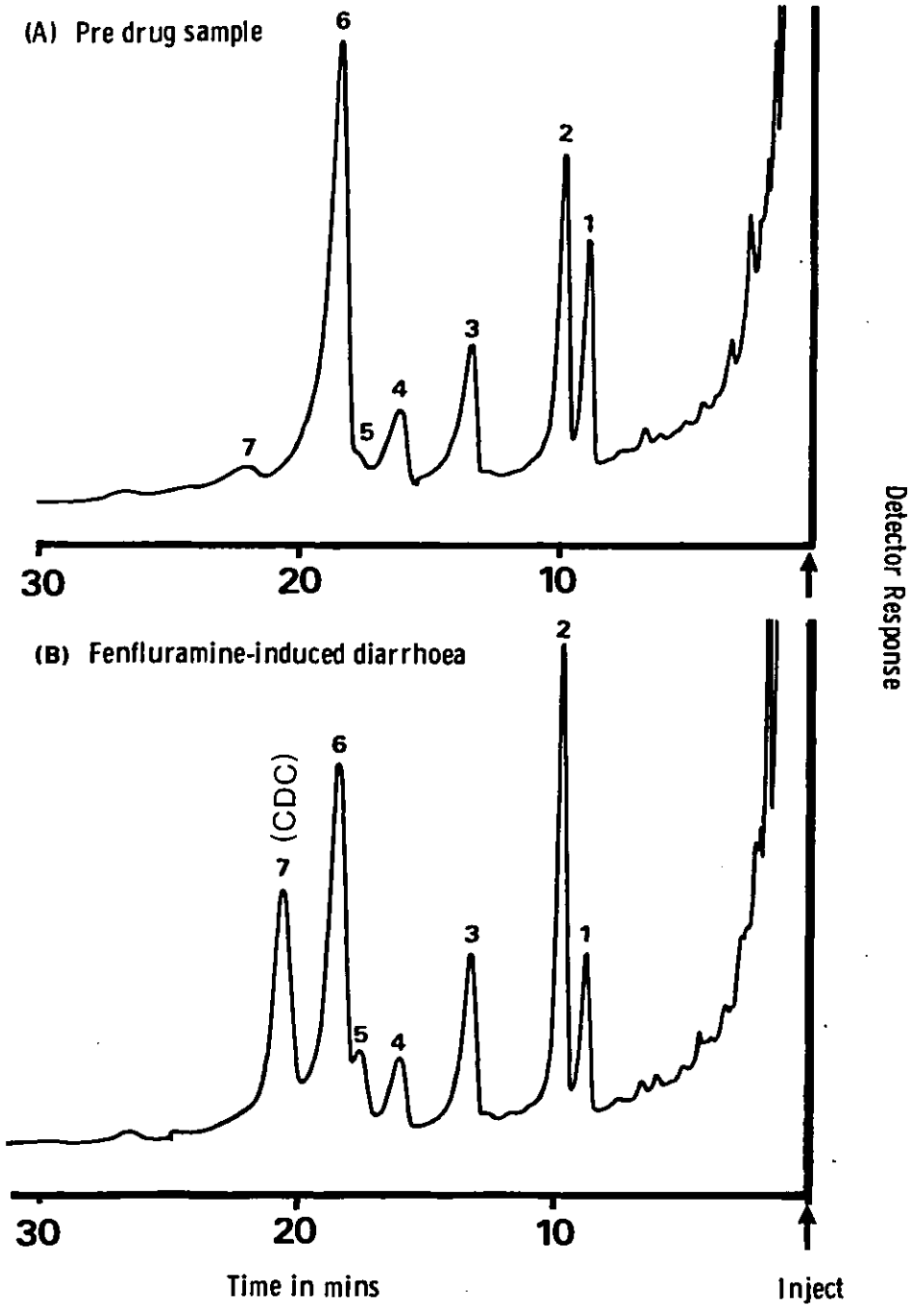


Figure 3.2A & B Tracings obtained for bile acids found in faecal samples (Subject, J.W.) collected before treatment (A) and during fenfluramine-induced diarrhoea (B). Peak identity and GC conditions for methyl esters as for Figure 2.8B.

3.6 STUDY II

Study I (Sian & Rains, 1979a) has been extended as follows: Six subjects have received larger doses of the drug for a longer period of time.

Experimental design: subjects: collection of samples

The design of Study II was similar to that of Study I and consisted of three consecutive periods of, 1) control, 2) drug and 3) recovery. The dosage of the drug was increased from 60-80 mg/day to 80-100 mg/day and the drug period extended from three weeks to eight or ten weeks. Each subject made at least one 3-day stool collection each week. The stools were brought to the laboratory daily where they were pooled. Homogenised faeces were freeze-dried and the faecal steroid estimated as before. The details of the subjects are included in Appendix Table 3.3.

Subjects investigated in Study II Nos. 1 to 6	= 6
Duplicate estimations for 3-week control (2 x 3 x 6)	= 36
Duplicate estimations for 8-week on drug (2 x 8 x 6)	= 96
Duplicate estimations for 3-week recovery (2 x 3 x 6)	= 36
Minimum number of bile acid estimations	= 168
Minimum number of neutral sterol estimations	= 168
Total number of estimations	= 336

3.7 Bile acid and neutral sterol excretion in subjects taking fenfluramine (Study II)

Bile acid excretion in subjects taking fenfluramine (Study II) did not differ from that found in Study I.

Typical GC chromatograms obtained for one of the six subjects (L.S.) showing bile acid methyl esters found in the stools collected before and after treatment with fenfluramine are shown in Figures 3.3A and 3.3B

These tracings show that qualititavely the bile acids remain similar but there are marked quantitative differences.

The excretion of neutral sterols in these subjects did not differ from the excretion found in Study I.

Typical GC tracings obtained for neutral sterols found in the stools collected before, during ad after treatment with fenfluramine (Subject, S.M.) are shown in Figure 3.4.

The mean bile acid excretion (mg/day; mean $\pm$ SD) for six subjects during the 'control' period was 223 $\pm$ 26. The excretion increased to 334 $\pm$ 76 during the drug period ($P < 0.01$); and remained significantly high during the post-drug period (326 $\pm$ 80; $P < 0.02$). The increase in excretion during these periods is equivalent to 50% and 46% respectively.

The mean neutral sterol excretion (mg/day; mean $\pm$ SD) for the six subjects during the pre-drug period was 424 $\pm$ 82. This was increased to 694 $\pm$ 291 after giving the drug ($P < 0.05$). The excretion remained significantly high at 731 $\pm$ 202 during the post-drug period ($P < 0.01$). The increase observed during the drug and the post-drug period is equivalent to 64% and 72% respectively.

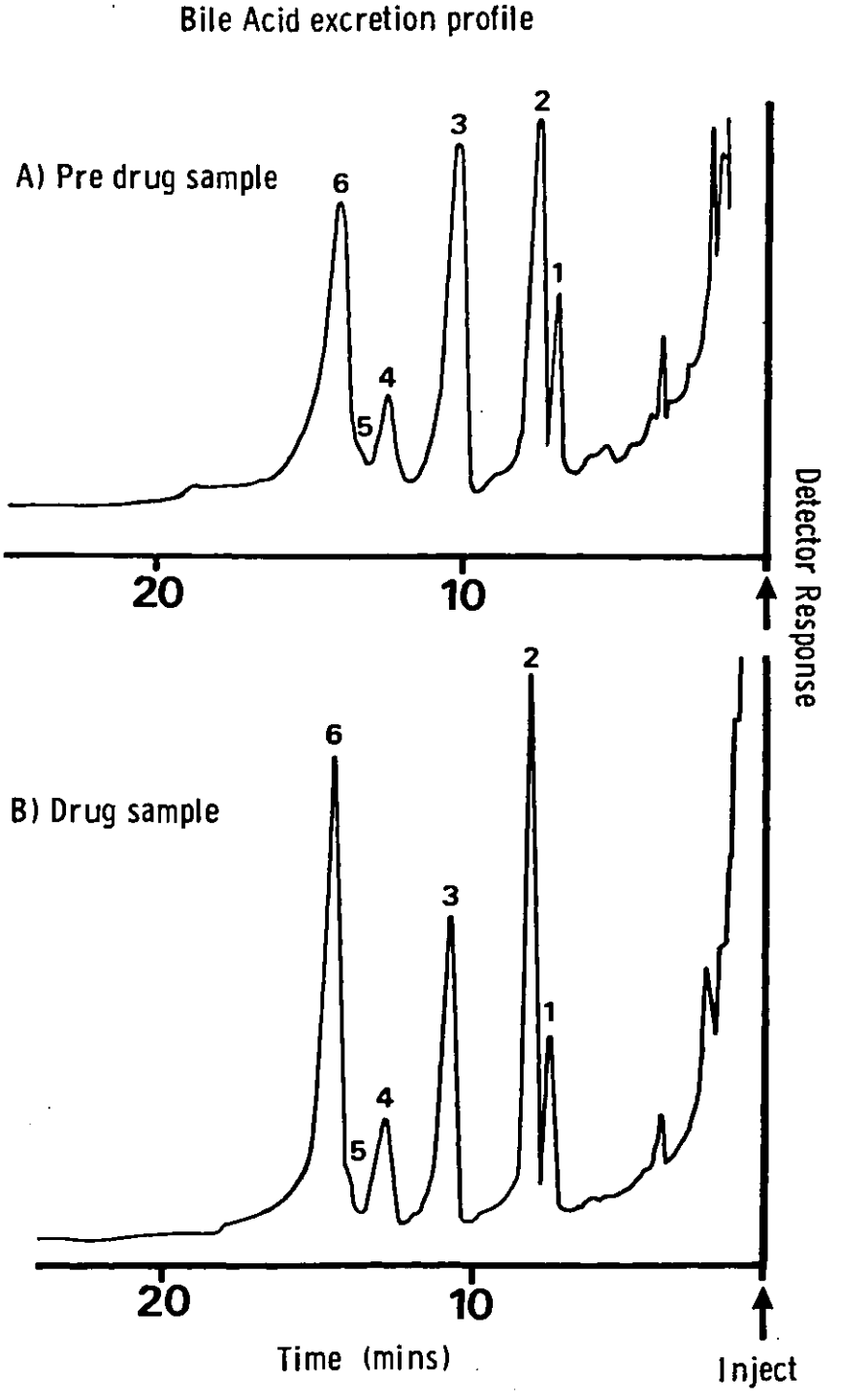


Figure 3.3A & B Tracings obtained for bile acids found in faecal samples (Subject, S.L.) collected before treatment(A) and during treatment with fenfluramine (B). Peak identity and GC conditions for methyl esters as for Figure 2.8B.

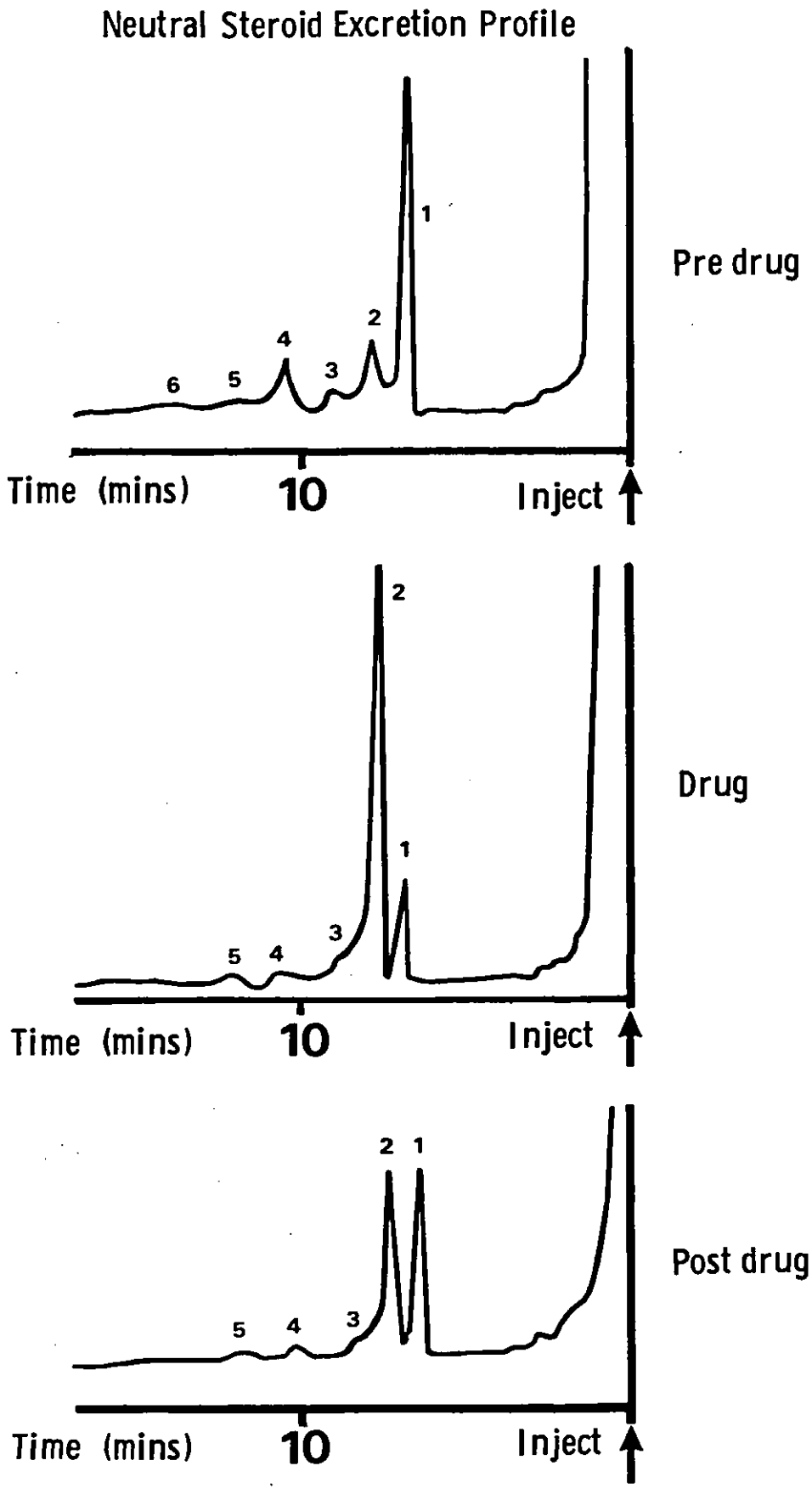


Figure 3.4 Typical chromatograms obtained for neutral sterols found in faecal samples collected during pre-drug, drug and post-drug periods. Peak identity: 1) coprostanol, 2) cholesterol; 3, 4, 5 &6) plant sterols. GC conditions, as for JXR columns (Appendix F).

The mean daily total steroid (i.e. bile acid + neutral sterol) excretion (mg/day; mean ± SD) increased from 647 ± 98 to 1028 ± 331 during treatment ($P < 0.05$) and remained significatnly high at 1057 ± 260 during the post-drug period ($P < 0.01$). The increase during the drug and the post-drug period is equivalent to 53% and 57% respectively. These results are summarised in Table 3.4.

The relative increase in faecal sterols was higher than that observed for bile acids. The ratio of 'neutral sterols to bile acids' was 1.9 during the pre-drug period; 2.1 during drug period and 2.3 during the post-drug period.

The ratio of the amount of coprostanol to cholesterol in the faeces was decreased from a mean value of 8:1 during the 'control' period to 0.2:1 during the drug period. The ratio remained low at 0.7:1 during the post-drug period. The value of 8:1 found in this study, during the 'control' period is similar to the ratio of 7:1 reported for 'normals' by Eneroth, Hellstrom & Ryhage (1964).

Table 3.4. Quantitative changes in faecal steroid (bile acid + neutral sterol) excretion in subjects taking fenfluramine (Study II)

Faecal Steroid Excretion (mg/day)			
N = 6 subjects	Pre-Drug	Drug	Post-Drug
Bile Acids:			
Mean	223	334**	326**
S D	±26	±76	±80
Range	(202-271)	(210-440)	(236-438)
Neutral Sterols:			
Mean	424	694*	731**
S D	±82	±291	±202
Range	(272-504)	(302-1162)	(456-1002)
Total Steroids (Bile Acids + Neutral Sterols):			
Mean	647	1028*	1057**
S D	±98	±331	±260
Range	(477-776)	(512-1527)	(692-1363)

Significantly different from the excretion during the Pre-Drug control period, *P<0.05; **P<0.01

3.8 Comparison of bile acid and neutral sterol excretion in men and women.

During the course of the fenfluramine study an opportunity was taken to compare bile acid and neutral sterol excretion in men and women. The mean daily faecal excretion (mg/day; mean ± SD) during the pre-drug period differed between a group of five men with ideal body weights and a group of five women also with ideal body weight. The value for the male group was 840 ± 68 and that of the female group 638 ± 90. The difference is significant at $P < 0.05$ (Appendix Table 3.5). The observation of increased excretion in men is in accord with that reported by Miettinen (1971). Our value of 4.6 ± 0.4 (N = 5) for bile acid excretion in mg/kg/day for men with ideal body weight compares with the value of 4.2 reported by Connor, Witiak, Stone & Armstrong (1969).

3.9 Relationship between faecal steroid excretion (bile acids + neutral sterols) and body weight

The bile acid and neutral sterol (faecal steroid) excretion (mg/day) found in the 16 subjects before giving the drug was plotted against their 'actual' body weights. The relationship observed for neutral sterols, bile acid and total steroids (bile acids + neutral sterols) is shown in Figure 3.5. These results suggest that the greater the body weight the higher is the faecal steroid excretion. This observation confirms the results reported previously (Miettinen, 1971; Nestel, Schreibman & Ahrens, 1973).

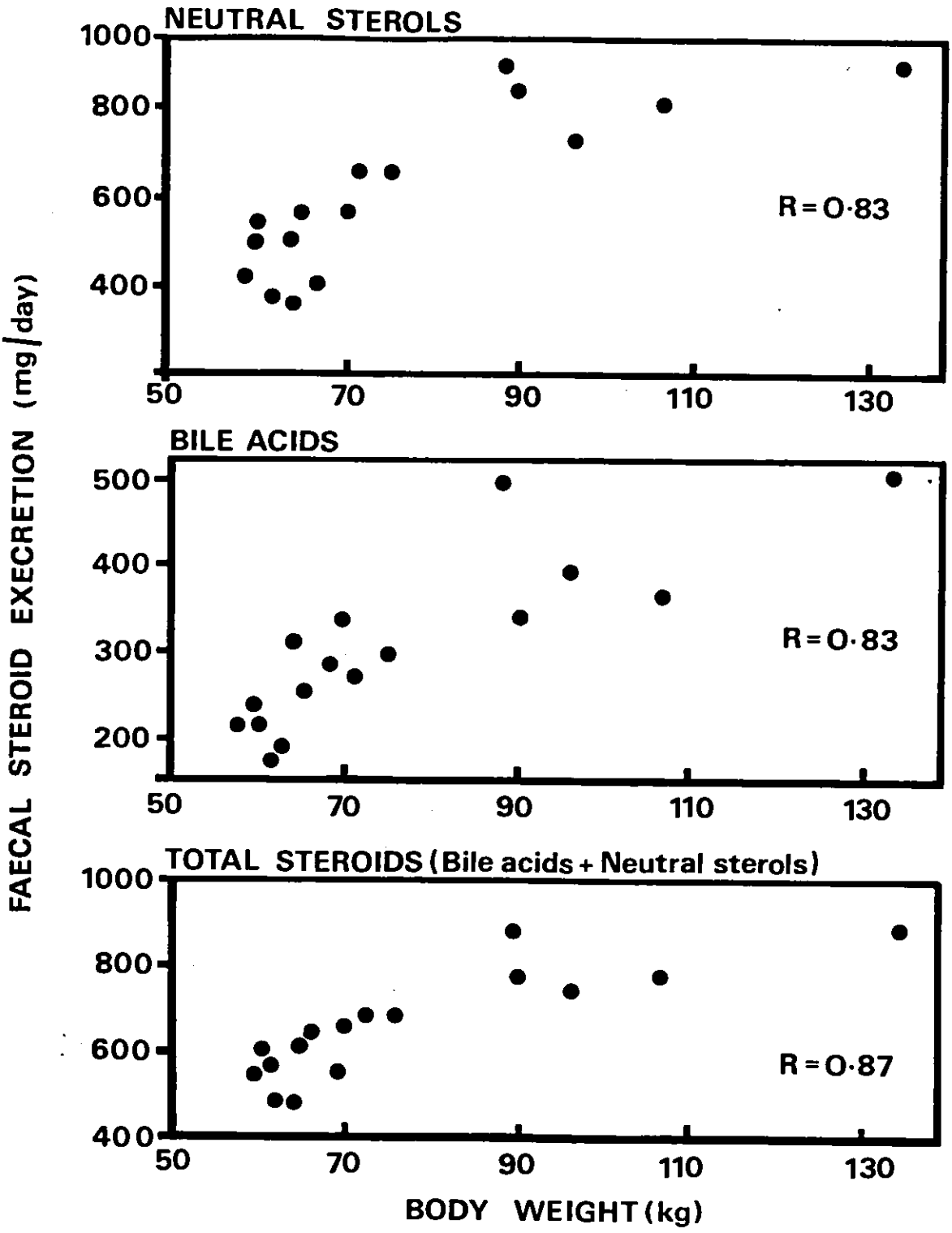


Figure 3.5 Relationship between faecal steroid excretion (bile acid + neutral sterol) and body weight.

3.10 Comparison of bile acid and neutral sterol excretion in 'overweight subjects' and in subjects with 'normal weights'

The daily faecal loss of bile acids and neutral sterols is believed to give an approximate value for the daily synthesis of endogenous cholesterol (Grundy et al, 1965; Dowling et al, 1970). Obesity is said to be associated with an increased synthesis of cholesterol (Nestel, Schreibman & Ahrens, 1973; Nestel & Hunter, 1974).

Bile acids and neutral sterol excretion in 'overweight subjects' was compared with the excretion in subjects with 'normal weights'. Qualitatively, bile acids and neutral sterols found in the faeces collected from the two groups were the same, but there were marked quantitative differences. The mean faecal steroid excretion (mg/day) for a group of 'overweight subjects' during the control period was 1144 $\pm$ 178 (mean $\pm$ SD; N = 8). The value for the subjects with 'normal weights' was 698 $\pm$ 97 (mean $\pm$ SD; N = 8). The difference was highly significant ($P < 0.001$; Appendix Table 3.6 and 3.7). The increased excretion of faecal steroids in the'overweights' may be due to the higher rate of production of endogenous cholesterol in the obese (Nestel & Hunter, 1974; Low-Beer, 1977).

3.11 Discussion

This study shows that the excretion of bile acids and neutral sterols is increased after ingestion of fenfluramine and that the bile acids remain qualitatively unaltered except in those who had a fenfluramine-induced diarrhoea.

It is not known whether the increased excretion of bile acids after fenfluramine is due to increased hepatic synthesis. Although there is no information available regarding the effect of fenfluramine on bile acid synthesis, it may be suggested that the increased faecal loss of bile acids reduces the amount returning to the liver via the enterohepatic circulation and this stimulates the hepatic bile acid synthesis. A second question is whether the increased excretion of bile acids after fenfluramine is due to reduced reabsorption. This would seem to be a likely explanation. Even though we have no information on the reabsorption of these compounds after fenfluramine, there is some evidence of an alteration in gastric and intestinal motility after fenfluramine (Coupar, Hedges, Metcalf & Turner, 1969; Garattini, 1971; Bizzi, Veneroni & Garattini, 1973), a change in gut motility may inhibit the normal reabsorption of bile acids from the ileum. The effect of this would be to increase the concentration of these within the gut. This would favour the argument that excessive amounts of bile salts passing into the colon cause a watery diarrhoea.

Fenfluramine is believed to produce a hypocholesterolaemia (Dannenburg & Chremos, 1971; Tomlinson, Lines & Greenfield 1975). In this laboratory fenfluramine was found to cause a small but significant reduction in the serum cholesterol of patients (Bliss, Kirk & Newal, 1972). A possible explanation for the reduction in serum cholesterol may be by way of increased excretion of bile acids and cholesterol after fenfluramine. Bile acids are necessary for normal absorption of chodesterol from the intestine into the lymphatic system and its inclusion into chylomicrons (Wilson, 1972). Increased faecal loss of bile acids after treatment with fenfluramine may reduce the intraluminal concentration of bile salts and decrease the micellar solubilisation of cholesterol leading to a decrease in the amount of cholesterol absorbed by the intestine.

There is no information available regarding the effect of fenfluramine on the rate of synthesis of cholesterol in the liver or in the intestinal epithelium. However, it could be speculated that a reduction in cholesterol absorption leading to a reduced return to the liver may result in a compensatory increase in cholesterol synthesis (Dietschy & Wilson, 1970).

In this study subjects with fenfluramine-induced diarrhoea excreted primary bile acids rather than secondary bile acids in their faeces. Similarly, coprostanol, the normal faecal sterol was replaced by cholesterol. The excretion of primary bile acids

and cholesterol instead of coprostanol is associated with other forms of diarrhoea: a) diarrhoea with ileal dysfunction (Mitchell & Eastwood, 1972), b) bile and diarrhoea (Hoffman, 1972), c) a mannitol-induced diarrhoea (Meihoff & Kern, 1968), d) fatty acid diarrhoea i.e. steatorrhoea (Hoffman & Poley, 1972), e) non-specific diarrhoea (Heaton, 1972) and f) an antiobiotic diarrhoea (Rubulis, Rubert & Faloon, 1970).

A rapid transit of steroids through the gut following the ingestion of fenfluramine will result in, a) a reduced exposure of bile acids and cholesterol to bacterial enzymes, b) shortened contact time with intestinal mucosa c) reduced absorption of intestinal contents including bile salts and cholesterol from the ileum. The effect of these will be that bacterial transformations would be reduced and increased amounts of unconverted steroids lost in the stools.

Another possible explanation for the changes in bile acid and sterol excretion is a reduction in bacterial activity but we have no evidence for this. An inhibition of normal bacterial activity after treatment with fenfluramine could reduce the conversion of primary bile acids to secondary bile acids and cholesterol not being converted to coprostanol (Rubulis, Rubert & Faloon, 1970). Taking into consideration the effect of fenfluramine on gut motility (Coupar, Hedges, Metcalf & Turner, 1969; Bizzi, Veneroni & Garattini, 1973) and the reduction in lipid

absorption (Garattini, 1971) it may be suggested that the concentration of bile salts within the lumen of the small bowel increases. This may be the 'primary effect' of fenfluramine. The excessive amount of bile salts then enters the colon where bile salts are known to exert 'carthartic effects' such as:a) the stimulation of colonic motility and b) the inhibition of water and electrolyte absorption from the colon or even the net secretion of these elements into the colon (Forth, Rummel & Glasner 1966; Mekhjian, Phillips & Hofmann, 1971). The result of this is a 'watery diarrhoea'. On the basis of these arguments, the changes in intestinal motility and the qualititative alterations in steroid excretion are 'secondary'. This is in accord with the current belief that most of the effects of agents causing diarrhoea, primarily increase the amount of intestinal content by their effects on absorption and that motility changes are secondary (Binder & Donowitz, 1975). The finding of increased amounts of CDC and C in patients with fenfluramine induced diarrhoea is similar to the observations of Mitchell & Eastwood (1972) Kirwan, Smith, Mitchell, Falconer & Eastwood, (1975); who found that CDC was more important cathartic agent than C acid in the diarrhoea of ileal dysfunction.

The increased loss of bile acids and cholesterol after fenfluramine may be beneficial in obesity. A possible disadvantage of such a loss might be increased risk of

gallstone formation. A normal bile acid pool is maintained by an efficient ileal reabsorption of bile salts (Hoffman, 1972) but under the influence of fenfluramine, excessive loss of bile salts may reduce the size of the bile acid pool(Dowling, Mack & Small, 1970; Marks, Bonorris & Shoenfield, 1977). A 'lithogenic bile' may be produced. A high incidence of cholesterol gallstones in patients with increased faecal bile acid excretion or in patients with ileal dysfunction is well documented (Woodbury & Kern, 1971; Baker et al. 1974). It is now generally agreed that gallstone formation results from the secretion by the liver of bile with excess cholesterol or a relative lack of bile salts (Admirand & Small, 1968; Rains, 1976; Bouchier, 1979). However, no information is available regarding the incidence of gallstones following treatment with fenfluramine.

CHAPTER 4

THE DEVELOPMENT OF METHODS USING 'HIGH PRESSURE LIQUID CHROMATOGRAPHY' FOR THE ANALYSIS OF BILE SALTS PRESENT IN HUMAN BILE

4.1 Introduction

It has been known for some time that there are changes in the proportions of bile salts present in human bile of patients with hepato-biliary disease (Sjovall, 1960) but few detailed studies have been reported (Dam, Kruse, Kallehauge, Hartkopp & Jensen, 1966; Nakayama, 1967). Further, the differences which have been demonstrated between samples of bile obtained from subjects with and without cholesterol gallstones have not shown a consistent pattern (Admirand & Small, 1968). This is mainly due to the lack of suitable methods for the analysis of bile salts as they exist in bile (Nakayama, 1969; Shioda, Wood & Kinsell, 1969).

The limitations of the methods previously used for the analysis of bile salts in human bile have been reviewed in the INTRODUCTION.

In order to overcome some of these difficulties, an improved method using 'high pressure liquid chromatography' (HPLC) has been developed. The details of the chromatographic procedures used are bound separately in this thesis (Sian & Rains, 1979b).

Since the above publication, a number of modifications have been introduced and the method compared with TLC and GC. This section describes the modifications, the results and details not included in the 1979b publication.

4.2 Improved method used for estimating bile salts present in gall bladder bile

i) Extraction

0.5 ml of gall bladder bile was added slowly with agitation to 5 ml ethanol (AR) in a 25 ml ethanol (AR) in a 25 ml conical flask. The mixture was heated briefly on a hot plate to precipitate the proteins, cooled under a tap and transferred quantitatively with washings to a centrifuge tube. The deproteinised mixture was centrifuged at 2 000 rpm for 5 mins to separate the proteins. The supernatent was removed and the precipitate washed with ethanol (2 x 2 ml); each time the supernatant was pooled with the original. The pooled supernatant was diluted with 4 ml of 20% NaCl (w/v) and extracted three times with 5 ml of petroleum ether (b.p. 40-60^{o}C) to remove biliary cholesterol. The aqueous alcohol layer was reduced to a small volume using a rotary evaporator, and finally taken to dryness using a water bath (45^{o}C). The residue was dissolved in 0.5 ml of appropriate mobile phase and the solution filtered through a micron filter (Sartorius) before injecting 1-2 µl to a HPLC column.

ii) Chromatographic conditions

All analysis were carried out using columns of Partisil-10 ODS using methanol/water (HPLC system II) or a µ Bondapak FAA using isopropanol/8.8 mM potassium phosphate (HPLC System III); as described in the 1979b publication.

iii) Detection

The separations were monitored using a Cecil Model 2012 variable wavelength UV spectrophotometer equipped with a 8 µl flow cell of 10 mm path length.

iv) Qualitative Analysis

For tentative identification of the major bile salts present in gall bladder bile, a reference mixture of standard bile salts containing 1 mg/ml each of: TC, TCDC, TDC, GYLC, GCDC and GDC was prepared and chromatographed just before or immediately after the sample run. The retention times (R_t) of bile salts were calculated relative to the retention time of TDC (i.e. $R_{t\ (TDC)} = 1.0$).

v) Quantitative Analysis

All quantitative measurements were made using Waters µ Bondapak FAA columns. The peak area counts were recorded using a Varian electronic integrator, Model CDS 111A. Calibration curves covering a range from 0.1 to 5.0 µg were prepared daily for the main bile salts present in bile. The general layout of the set up is shown in Figure 4.1.

4.3 Comments on Liquid Chromatograph and Modifications

The performance of the Du Pont Model 840 Liquid Chromatograph was improved by making the following modifications;

a) The tubing used to transfer the mobile phase from its reservoir to the pump was replaced

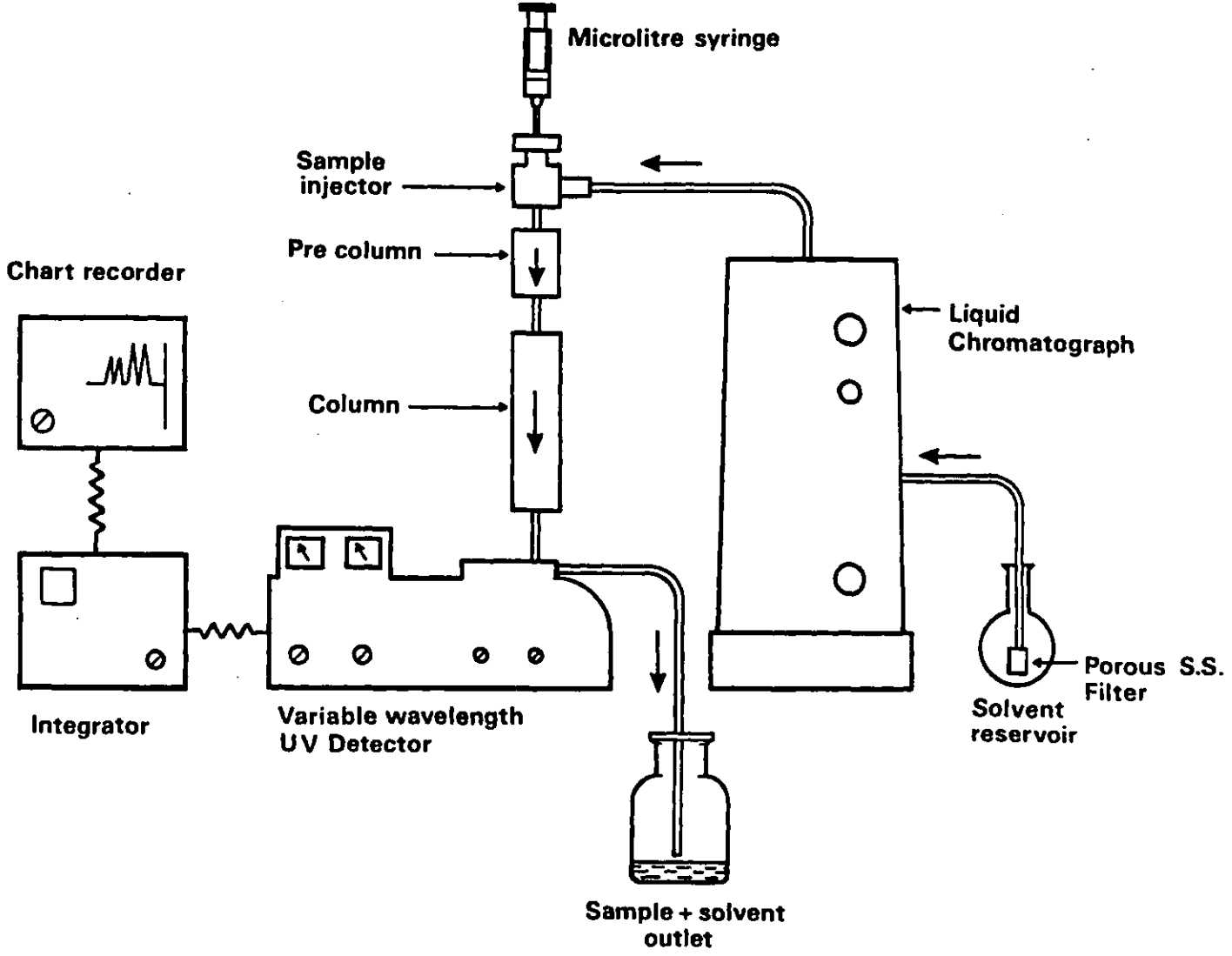


Figure 4.1 Schematic of 'high pressure liquid chromatography' system connected to a variable wavelength UV detector suitable for the analysis of bile salts present in human bile. Further details of the apparatus and the supplier are included in the text.

with a shorter and narrow tubing. This reduced the equilibration time.

b) The life of HPLC columns was prolonged by introducing a stainless steel porous filter of 2 micron porosity (Chrompak, U.K. Ltd.) between the pump and the solvent reservoir.

c) A needle guide constructed from a piece of Nylon tubing (3mm o.d. 1mm i.d. and 2.5 cm in length) was used for all injections. This increased the septum life and also improved the reproducibility of the injections.

d) A device for measuring the flow rate was contructed by modifying an old burette. The effluent was allowed to flow into the burette and the stopcock left 'on' to drain. When the flow rate was to be measured, the burette was turned 'off', allowed to fill to the 'zero' mark and the time taken to fill a given volume measured with a stop watch. The side arm of the burette serves as an overflow for the effluent if the burette is left in the 'off' position by mistake.

e) A device to pack our own pre-columns was constructed and shown to work effectively. This reduced the time spent on sample preparation and also prolonged column life (Figure 4.2 and Appendix Figure 4.3).

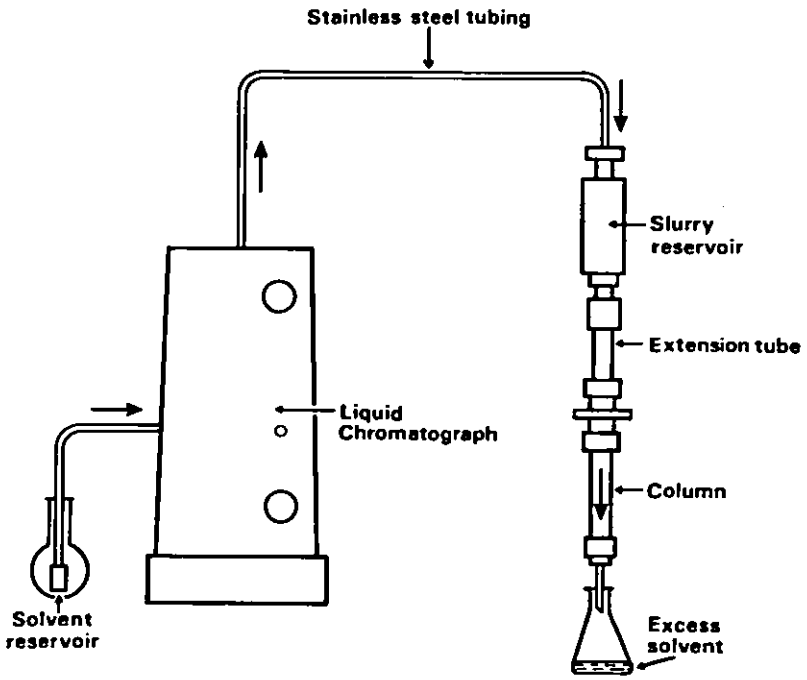


Figure 4.2 Design of the apparatus used for slurry-packing 'guard' columns for 'high pressure liquid chromatography'

4.4 Examples of separation of bile salts

PARTISIL-10 ODS COLUMNS

Figure 4.4A shows the typical tracing obtained using pure bile salts commonly found in human bile, whilst Figure 4.4B shows a similar trace obtained for bile salts found in a sample of gall bladder bile.

μ BONDAPAK FAA COLUMNS

Figures 4.5A and 4.5B show similar tracings obtained using μ Bondapak FAA columns.

For the complete analysis of bile salts present in gall bladder bile obtained from patients with cholesterol gallstones, the use of μ Bondapak FAA was preferred since this stationary phase separated all the major bile salts present in human bile. The retention times of bile salts analysed on Partisil-10 ODS and μ Bondapak FAA are listed in Appendix Table 4.1.

4.5 Assessment of Methods

i) Accuracy

a) Comparison with other methods:

The amounts of bile salts found in gall bladder bile using this method and those reported by others are shown in Table 4.2.

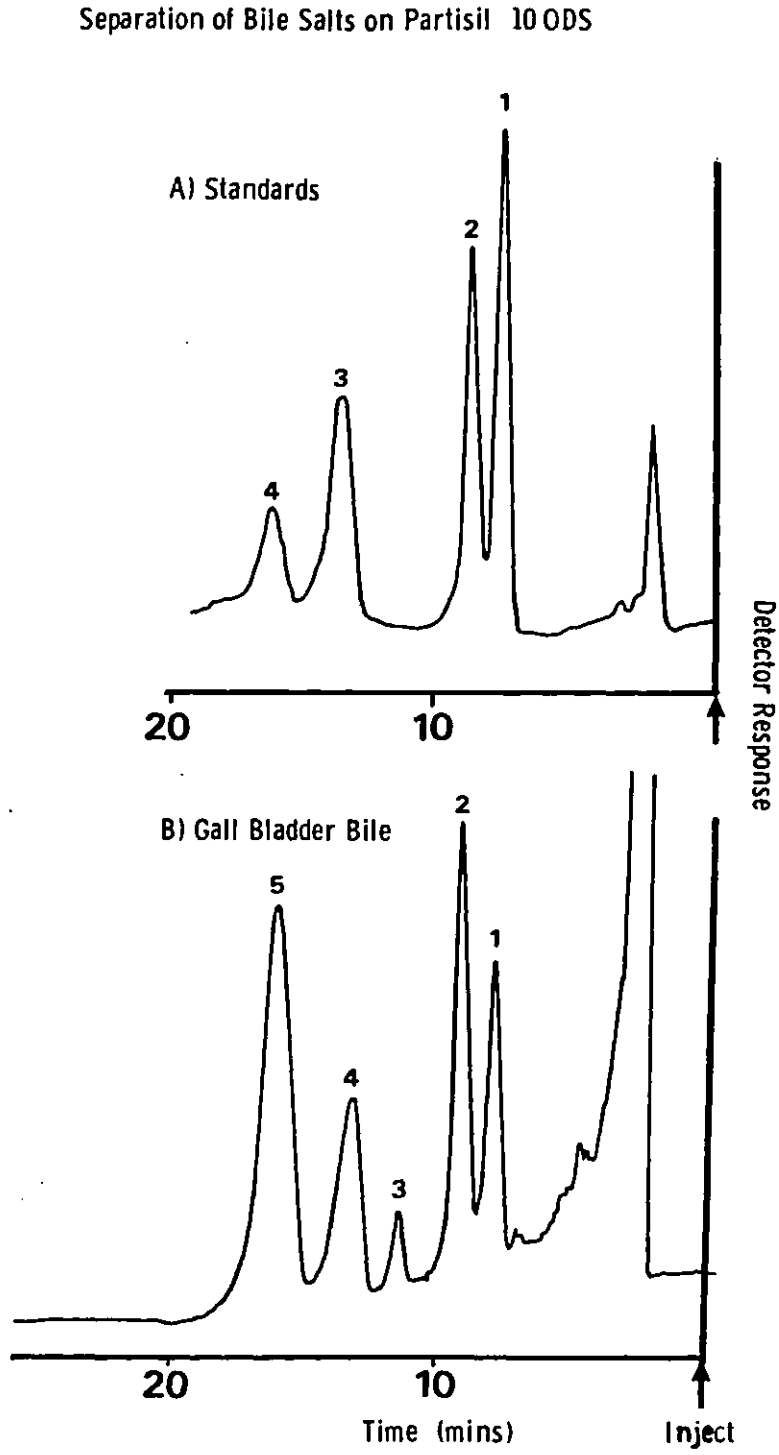


Figure 4.4A & B Tracings obtained for bile salts present in a standard mixture (upper trace, A) and bile salts found in an extract of gall bladder bile (lower trace, B). Peak identity, upper trace: 1) TC, 2) GLYC, 3) TCDC, 4) GCDC; lower trace: 1) TC, 2) GLYC, 3) non-bile salt peak, 4) TCDC + TDC, 5) GCDC + GDC. HPLC conditions as in text (4.2).

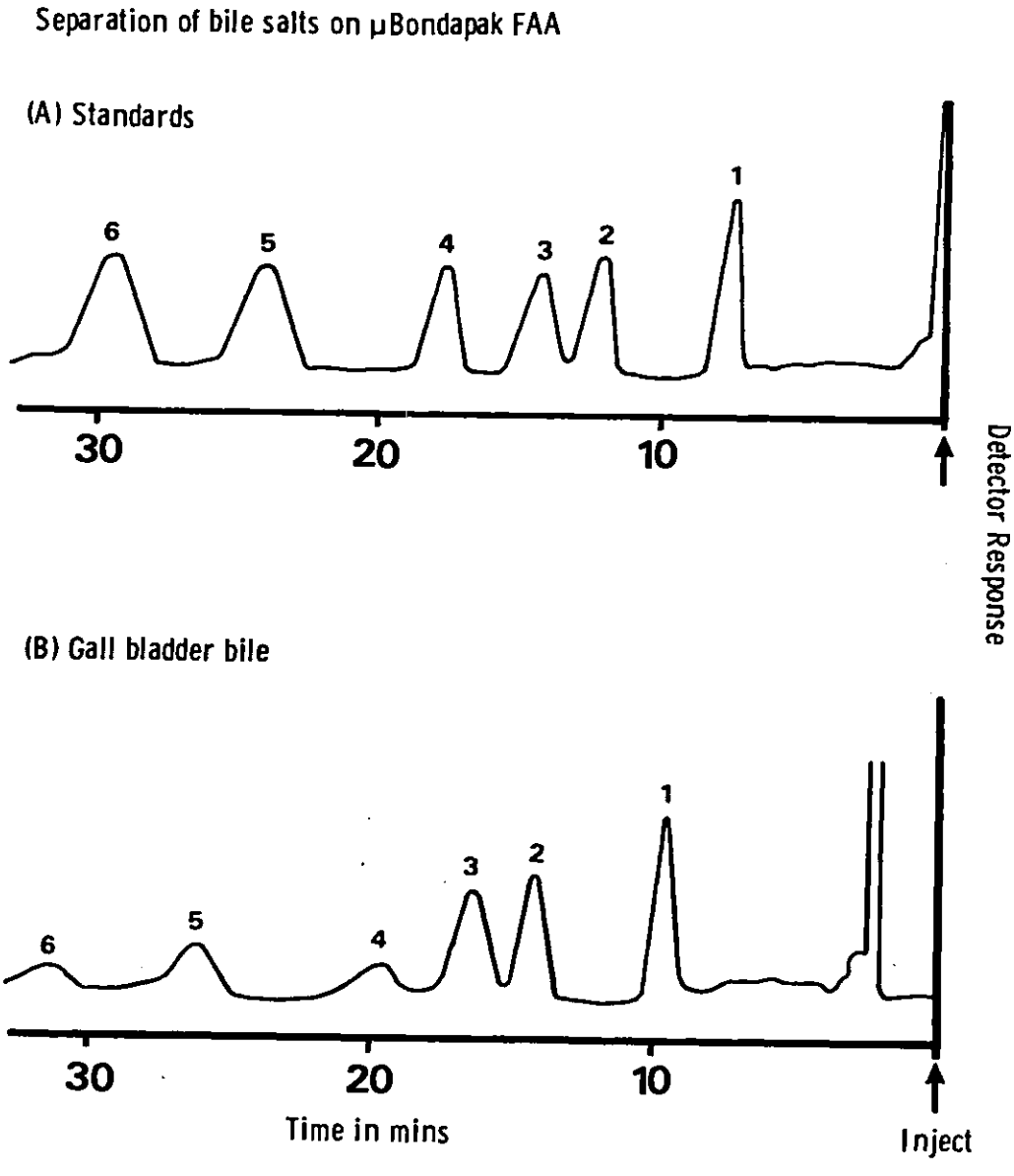


Figure 4.5A & B Tracings obtained for bile salts present in a standard mixture (upper trace, A) and bile salts found in an extract of gall bladder bile (lower trace, B). Peak identity, upper and lower trace: 1) TC, 2) GLYC, 3) TCDC, 4) TDC, 5) GCDC, 6) GDC. HPLC conditions as in text (4.2)

Table 4.2 The amounts of bile salts found in gall bladder bile when different methods of estimation are used.

Author	Method	Gall bladder Bile	Bile Salts (mg/ml) Mean ± SD
Sjovall (1960)	Paper Chromatography	Non-gallstone (4)	47.0 ± 31
Shioda et al (1969)	TLC-Spectrophotometry	Non-gallstone Duodenal or T-tube bile (4)	44.0 ± 0.3
Nakayama (1969)	GC	Non-gallstone (10)	37.9 ± 20.6
This study	HPLC	Non-gallstone (16)	46.6 ± 7.3
		Gallstone (16)	28.2 ± 8.3

b) Use of two different techniques:

As a check of the accuracy of the final quantitation, the bile salt content of gall bladder bile was estimated by the HPLC method descibed and compared with the results obtained when the same bile samples were estimated by gas chromatography.

Method: 0.5 ml of gall bladder bile was extracted with 5 ml of ethanol (AR) and treated as described in 4.2. The supernatent obtained after separating the proteins was transferred quantitatively with washings to a Pyrex test tube (17 x 160 mm) and reduced to a small volume. The extract was finally taken to dryness using a rotary evaporator and a water bath (45^{o}C). The residue obtained was dissolved in 2ml of 20% KOH in ethylene glycol (w/v) and the mixture refluxed as before (2.2b). Cholesterol present in bile was removed with petroleum ether and the bile acids taken into di-ethyl ether after acidification. The bile acid content of gall bladder bile was estimated as methyl esters by gas chromatography using QF-1 columns (2.2d & 2.2e).

Table 4.3 shows that the amount of bile salts found in gall bladder bile estimated by HPLC, agrees with the amount of bile acids found by the longer and more complicated procedure of GC. There are small insignificant differences between the results but most of the differences are in the same direction suggesting that HPLC results are slightly higher than those obtained by GC.

Table 4.3 Comparison of the results obtained by HPLC and GC

Subject	Bile Salt content of gall bladder bile (mg/ml) HPLC (A)	GC (B)	Difference (A-B)	Difference (%)
1	22.4	21.2	- 1.2	- 5.4
2	43.5	44.2	+ 0.7	+ 1.6
3	23.5	22.4	- 1.1	- 4.7
4	40.1	38.4	- 1.7	- 4.2
5	19.4	18.5	- 0.9	- 4.6
6	36.5	35.4	- 1.1	- 3.0
Mean =	30.9	30.0		$\frac{A-B}{A} \times 100$
S D = $\pm$	10.3	$\pm$ 10.7		

HPLC results were obtained using μ Bondapak FAA column and GC estimates were made using methyl esters on QF-1 columns.

ii) Reproducibility of HPLC method

The reproducibility of HPLC method for estimating bile salts present in gall bladder bile was examined in a similar manner to that described by gas chromatography (2.12).

a) The results of nine replicate analyses made during the course of one day on samples taken from a single bladder bile collection are shown in Table 4.4. The standard deviation was small (± 1.1 mg/ml) and the percentage differences from the mean less than 6.4%. The interassay coefficient of variation was 4.2% indicating good reproducibility.

b) When the variability of bile salt estimations was assessed more realistically by the method suggested by Snedecor (1952) the results obtained on six different days using six different samples are shown in table 4.5.

By both methods the standard deviation and the coefficient of variation appeared to be satisfactory.

iii) Sensitivity

The sensitivity of the estimates was calculated as for GC methods and was found to be approximately 30 µg/ml of gall bladder bile.

Table 4.4 Reproducibility of HPLC method for estimating bile salts present in gall bladder bile

Replicate	Bile Salt Content of Bile (mg/ml)	Difference From Mean	Difference From Mean %
1	25.2	- 0.2	- 0.8
2	23.8	- 1.6	- 6.3
3	25.8	+ 0.4	+ 1.6
4	27.0	+ 1.6	+ 6.3
5	25.4	0.0	0.0
6	26.6	+ 1.2	+ 4.7
7	24.2	- 1.2	- 4.7
8	26.2	+ 0.8	+ 3.1
9	24.6	- 0.8	- 3.1

Mean = 25.4 $\pm$ 1.1 (SD)

Coeffiecient of variation = 4.2%

Table 4.5 Reproducibility of HPLC method using duplicate estimates (bile salt content, mg/ml).

Subject	Duplicate Estimates A	B	Difference (A-B)=D	Difference $(D)^2$
1	25.4	24.6	0.8	0.64
2	31.6	30.4	1.2	0.49
3	23.5	24.2	0.7	0.49
4	42.5	40.9	1.6	2.56
5	22.4	22.9	0.5	0.25
6	19.2	20.6	1.4	1.96

Mean = 27.4 $\pm$ 0.8 (SD) $\quad \Sigma D^2 = 7.34$

Coefficient of Variation = 2.9%

iv) Specificity

This implies that the areas under the peaks correspond to the named bile salts. The identity of the main peaks in the tracings obtained for the analysis of gall bladder bile was checked by:

a) Retention times:

The retention times (R_t) of bile salts found in gall bladder bile agreed with those recorded using pure compounds. The order of elution and the elution patterns found using μ Bondapak FAA columns were similar to those reported by Shaw, Smith & Elliott (1978).

b) Analysis before and after adding pure bile salts to bile samples: This was repeated for all the six major bile salts present in bile. The peaks obtained for the bile salts were found to overlap.

c) Analysis on two different columns:

Bile samples were analysed on Partisil-10 ODS columns and the separations compared with those recorded using μ Bondapak FAA columns.

d) Thin layer chromatography - gas chromatography:

The dried ethanolic extract of gall bladder bile prepared for HPLC (4.2a) was dissolved in 0.5 ml of methanol and 20-40 μl of this solution used for TLC (Kieselgel 60 F254; 10 x 20 cm and 0.25 mm thick, Merck). A solution of bile salt standards was applied in parallel with the sample and the plate developed using a solvent mixture comprising,

butanol/acetic acid/water (10:1:1 v/v).

The bile salts were detected by spraying the plate with 10% phosphomolybdic acid in ethanol (w/v) and heating the plate for 2-3 mins at 110°C.

The identity of spots was checked by comparing the mobility (R_f = distance moved by spot ÷ distance moved by solvent front) of pure standards.

Figure 4.6A shows the typical TLC chromatogram obtained for bile salt standards and the bile salts found in a sample of gall bladder bile.

Additional evidence was obtained by GC after eluting the material from the silica gel (Figure 4.6B).

4.6 Comments on another HPLC method (HPLC System I)

During the course of this work another HPLC method suitable for the analysis of 'free' bile acids obtained after hydrolysis of conjugated bile salts present in gall bladder bile has also been developed even though 'free' bile acids are not normally found in bile. This procedure has not been reported previously and has a number of advantages when compared with TLC or GC. As distinct from GC no methylation is required and the difficulties of quantitative measurements met with in TLC are avoided.

Estimation of 'free' bile acids after hydrolysis of bile salts present in gall bladder bile:

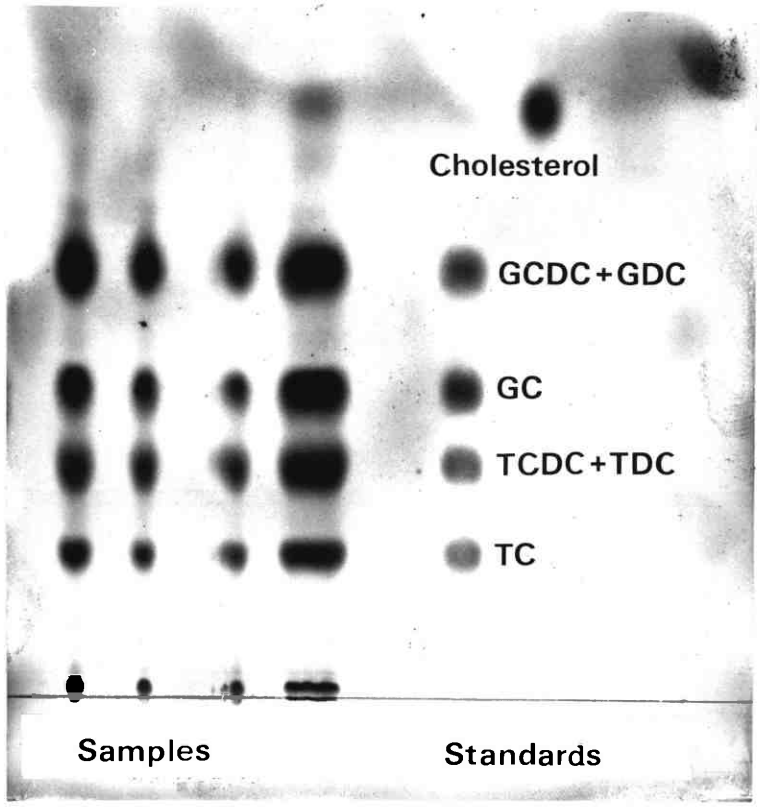


Figure 4.6A TLC separation of bile salts found in an extract of gall bladder bile (Patient, H.T.). TLC conditions, as in Appendix E.

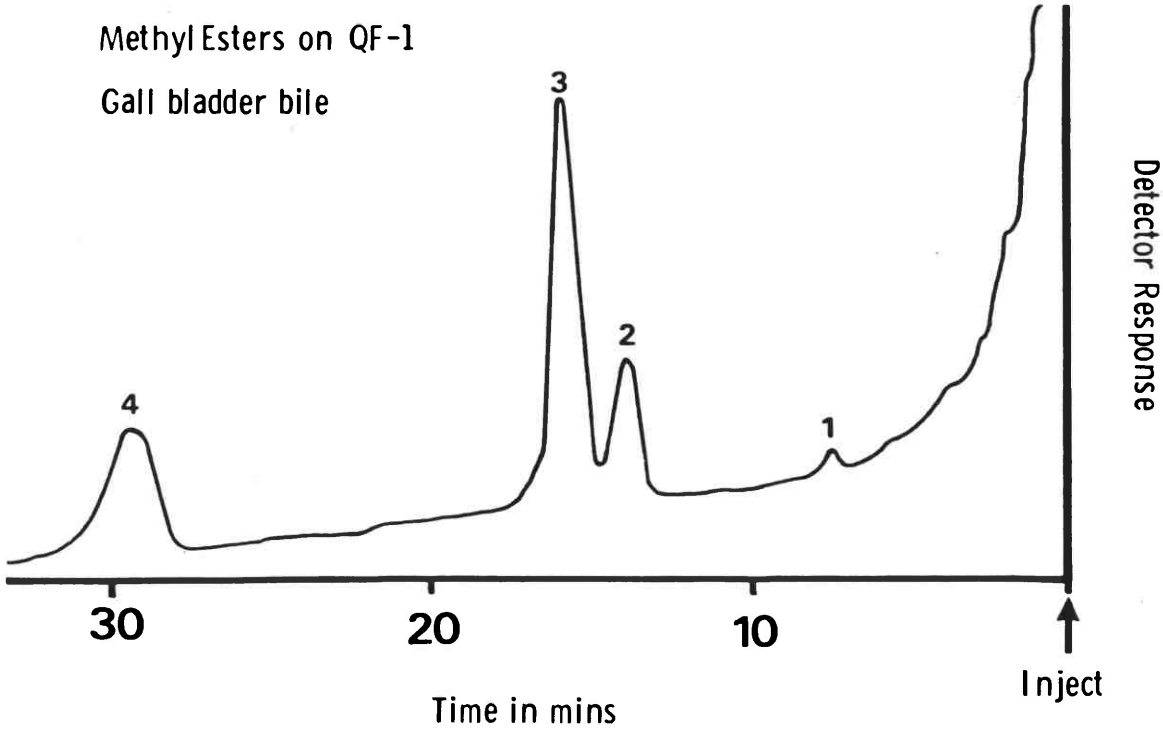


Figure 4.6B Typical tracing obtained for bile acids found in the material collected after TLC separation of bile salts present in the same sample as that used in Figure 4.6A (Patient, H.T.) and analysed as methyl esters using gas chromatography. Peak identity: 1) LC, 2) DC, 3) CDC, 4) C. GC conditions, as for QF-1 column (Appendix F).

i) Extraction: An ethanolic extract of gall bladder bile was prepared as for GC (4.5b). The residue obtained after evaporation of di-ethyl ether was dissolved in 0.5 ml of the mobile phase and 2-3 µl of this solution used for HPLC.

ii) Liquid Chromatograph: The basic Du Pont Model 840 Liquid Chromatograph modified to provide improved performance was used.

iii) Columns: Of the various columns tested, best separations were recorded on Micro Pak Si-10 columns (25 cm in length, 2 mm i.d. 6.35 mm o.d.; Varian Associates) factory packed with microparticulate material of 10 micron particle size. The column ends were modified to suit Du Pont system and a Whatman LIB septum injector. For analysis, two identical columns were connected in parallel: one for the analysis of sample the 'analytical column' and the other, 'reference column' for the flow of mobile phase alone. The general layout is shown in Figure 4.7.

iv) Mobile phase: a suitable mobile phase was found to be isooctane/ethyl acetate/acetic acid, (55:40:5, v/v). The optimal flow rate was 1.5 ml/min.

v) Detection: The separations were monitored using a Du Pont Model 845 Refractive Index (RI) detector fitted with two, flow cells of 3 µl cell volume. This monitors the refractive index by difference of the effluent from the two columns.

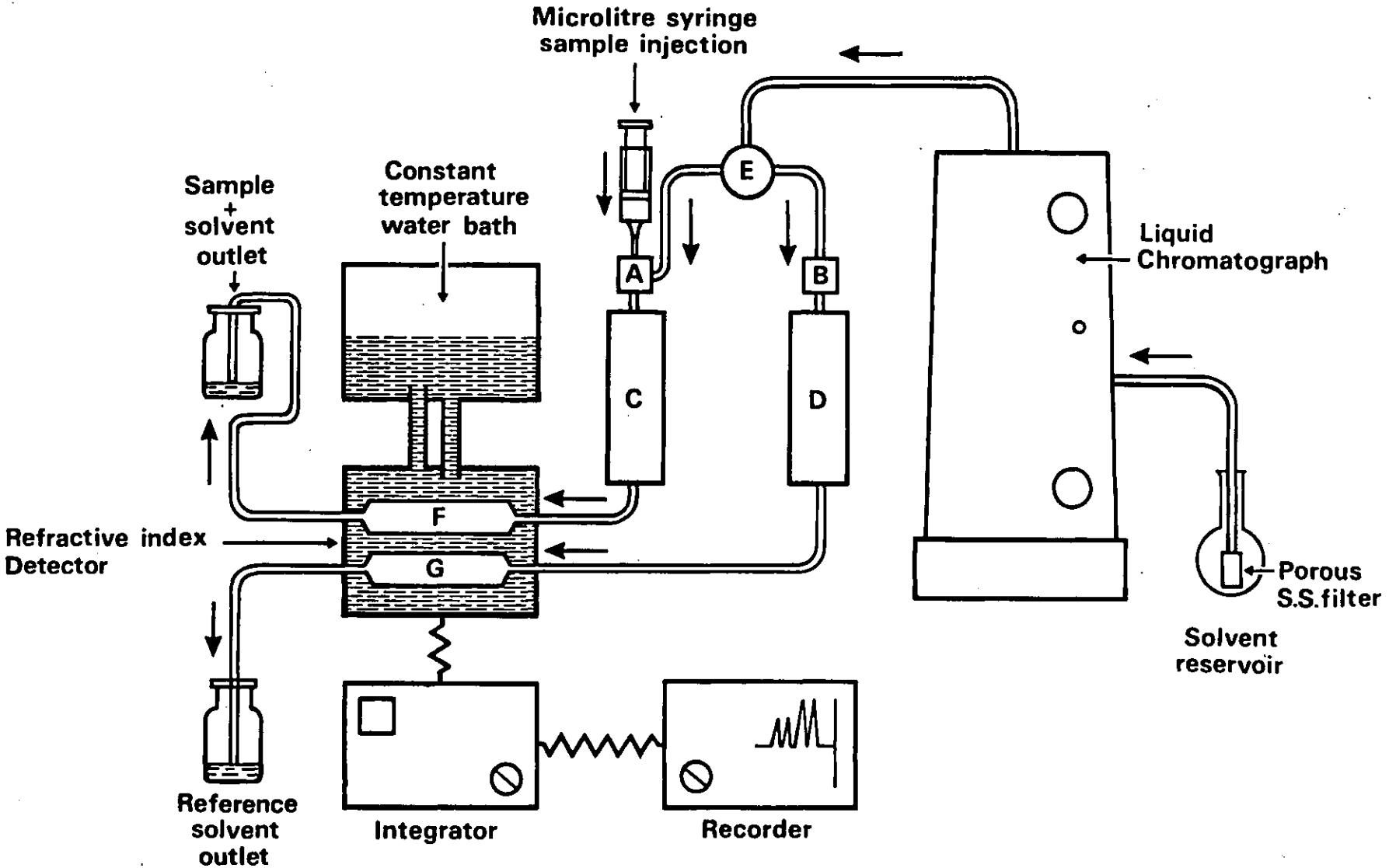


Figure 4.7 Schematic of HPLC system connnected to a RI detector suitable for the analysis of 'free' bile acids obtained after hydrolysis of bile salts present in bile: A) sample injector, B) micro needle valve, C) analytical column, D) reference column, E) 3-way 'T', F) sample cell, G) reference cell.

The detector response measured as peak height was highly reproducible (Appendix Table 4.6).

vi) Qualitative analysis: For tentative identification of the main bile acids present in bile, a standard mixture containing, LC, DC, CDC, and C (1 mg/ml of each) was analysed just before or immediately after the sample run. The standard deviation of retention time (R_t) of DC calculated from six replicate injections was very small ($\pm$ 0.03 min) and the coefficient of variation less than 2% (Appendix Table 4.7).

vii) Quantitative analysis: Curves relating the peak height response of RI detector to known amounts of bile acids were prepared. For quantities between 1 to 40 µg injected, the detector response was linear. The slope of each line was different, highest for LC and lowest for C. The minimum detectability at an attenuation of 1×10^{-10} RI units was, 2.8, 4.8, 6 and 8 µg respectively.

4.7 Examples of separations of 'free' bile acids

Figure 4.8 shows the typical tracing obtained by this technique. In studies to optimise separations an increase in the proportion of ethyl acetate favoured a rapid elution of bile acids, but the separation of DC and CDC was improved when the proportion of isoctane was increased. Flow rates lower than 1.5 ml/min gave distorted peaks and faster flow rates decreased the column efficiency (Appendix Tables 4.8 and 4.9).

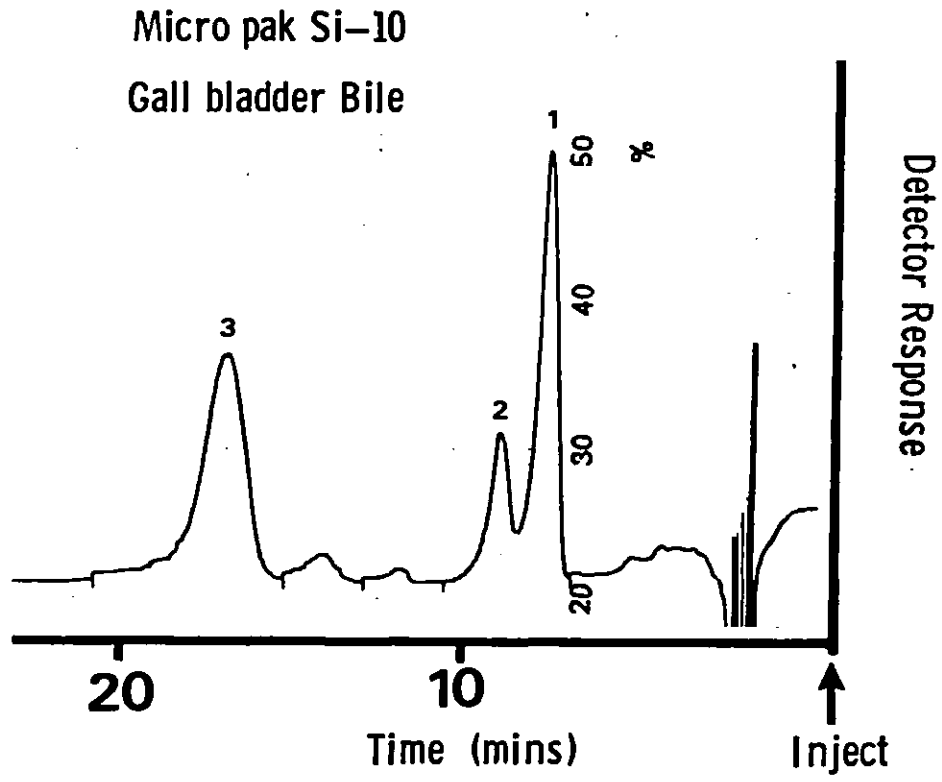


Figure 4.8 Typical separation obtained for bile acids analysed after hydrolysis of bile salts present in an extract of gall bladder bile. Peak identity: 1) DC, 2) CDC, 3) C; HPLC conditions, as in 4.6.

4.8 Discussion

Initially it was hoped to separate bile salts present in gall bladder bile on silica gel columns. These attempts proved unsuccessful and 'reverse-phase' (R-P) partition chromatography thought to offer possibilities. The method described (4.2) has proved useful in estimating the amount of individual bile salts and the ratio of 'glycine to taurine' (G:T) conjugates present in human gall bladder bile without previous hydrolysis or methylation. The G:T ratio of bile salts is regarded by some to be important in the formation of cholesterol gallstones (Earnest & Admirand, 1971; Thistle & Hofmann, 1973; McDougal, Walker & Thurston, 1976).

The manufacture of ultraviolet (UV) absorbing detectors for use in HPLC and capable of working at 210 nm (Carr, 1974) made it possible to estimate the concentration of bile salts present in bile.

The introduction of chemically-bonded stationary phases for HPLC allowed the manufacture of columns as efficient as those associated with gas chromatography.

It is interesting to note that for each individual bile salts, the more soluble taurine conjugate eluted ahead of the corresponding glycine compound. Though, failing to give as detailed a separation of the individual bile salts as the method described by Shaw, Smith & Elliott, (1978), the more rapid procedure

described (HPLC System II) allows for the estimation of total bile salts and G:T ratios in bile.

Methods suitable for estimating bile acids and bile salts using HPLC were not available when the present work was started. During the course of the work Shaw & Elliott (1976) used reverse-phase (R-P) HPLC but failed to separate GDC and GCDC because of tailing. Okuyama, Uemura & Hirata, (1976) using R-P were unable to separate TDC and TCDC. Similarly, Shimada, Hasegawa, Goto & Nambara, (1978) and Goto, Hasegawa, Kato & Nambara, (1978), also used R-P but TUDC and TC were not separated without a double elution. Shaw, Smith & Elliott, (1978) modified a recently reported R-P system (Waters Bulletins No. 3484 and No. 3485) on which our simplified HPLC Method III is based making it suitable for routine analyses of bile salts in gall bladder bile. The introduction of a pre-column reduced the time spent on sample preparation and prolonged column life.

With regards to the 'free' bile acids, the HPLC procedure described (HPLC System I) is as far as is known the only one of its kind capable of separating and estimating LC, DC, CDC, and C acids found in gall bladder bile.

Jefferson & Chang (1976) used HPLC to separate a number of bile acid methyl esters; and Shaikh, Pontzer, Molina & Kelsey (1978) reported another HPLC method but they only dealt with LC and ILC; furthermore, these bile acids were analysed after forming the UV-absorbing

p-nitro benzyl (PNB) derivatives.

The method described for the analysis of bile salts present in gall bladder bile offers a big saving in analysis time. To analyse six bile samples in duplicate using gas chromatography takes approximately two days as compared with the method described the same number of samples take about four hours.

CHAPTER 5

APPLICATION OF THE DEVELOPED METHODS TO STUDY BILE SALTS PRESENT IN BILE TAKEN FROM GALL BLADDERS WHICH DID OR DID CONTAIN CHOLESTEROL GALLSTONES

5.1 Introduction

Few qualitative and quantitative studies on bile salts present in human bile have been reported (Sjovall, 1960; Burnett, 1965). The limited amount of data available indicate large interlaboratory differences, and consistent qualitative changes in bile salt composition of bile obtained from patients with cholesterol gallstones have been difficult to demonstrate (Mirvish, 1964; Dam, Kruse, Kallehague, Hartkopp & Jensen, 1966; Nakayama, 1967; Admirand & Small, 1968).

The HPLC methods described in Chapter 4 have been used to investigate bile salt composition of gall bladder bile taken from patients suffering from cholesterol gallstones and in gall bladder bile obtained at post mortem of individuals without cholesterol gallstones.

The objective was to investigate the kind and quantity of bile salts present in gall bladder bile of patients suffering from gallstones and to examine any differences between the bile salts present in these subjects as compared with those present in 'fresh' post mortem bile without cholesterol gallstones.

5.2 Design of Study

Ethical consideration prevented bile from subjects not suffering from biliary disease being obtained at operation. Bile obtained within 48 hours of death has been regarded as "normal". Only samples which showed no

growth on bacterial examination were used, and these were obtained from subjects without biliary or hepatic disease.

"Fresh" gall bladder bile from patients with cholelithiasis was obtained at operation and only samples uncontaminated with blood were used. All samples were stored at -20°C until analysed.

5.3 Bile salt patterns in bile obtained from gall bladders which did or did not contain cholesterol gallstones

A typical HPLC chromatogram showing the pattern of bile salts in gall bladder bile taken from a patient suffering from gallstones is shown in Figure 5.1A. A typical pattern of bile salts found in 'fresh' post mortem gall bladder bile without gallstones is shown in Figure 5.1B. Comparison of individual bile salts show marked differences. The amount of TCDC is higher in post mortem bile while that of GDC is lower than in gallstone bile. All the samples tested showed the presence of six main bile salts: TC, TCDC, TDC, GLYC, GCDC and GDC but a number of samples showed a number of small peaks between the solvent peak and the first bile salt peak. The retention time of these peaks did not correspond to the retention time of any bile salt available to us. The identity of the six bile salts was established using methods described previously.

Further examples of the patterns recorded for bile salts present in bile taken from gall bladders containing

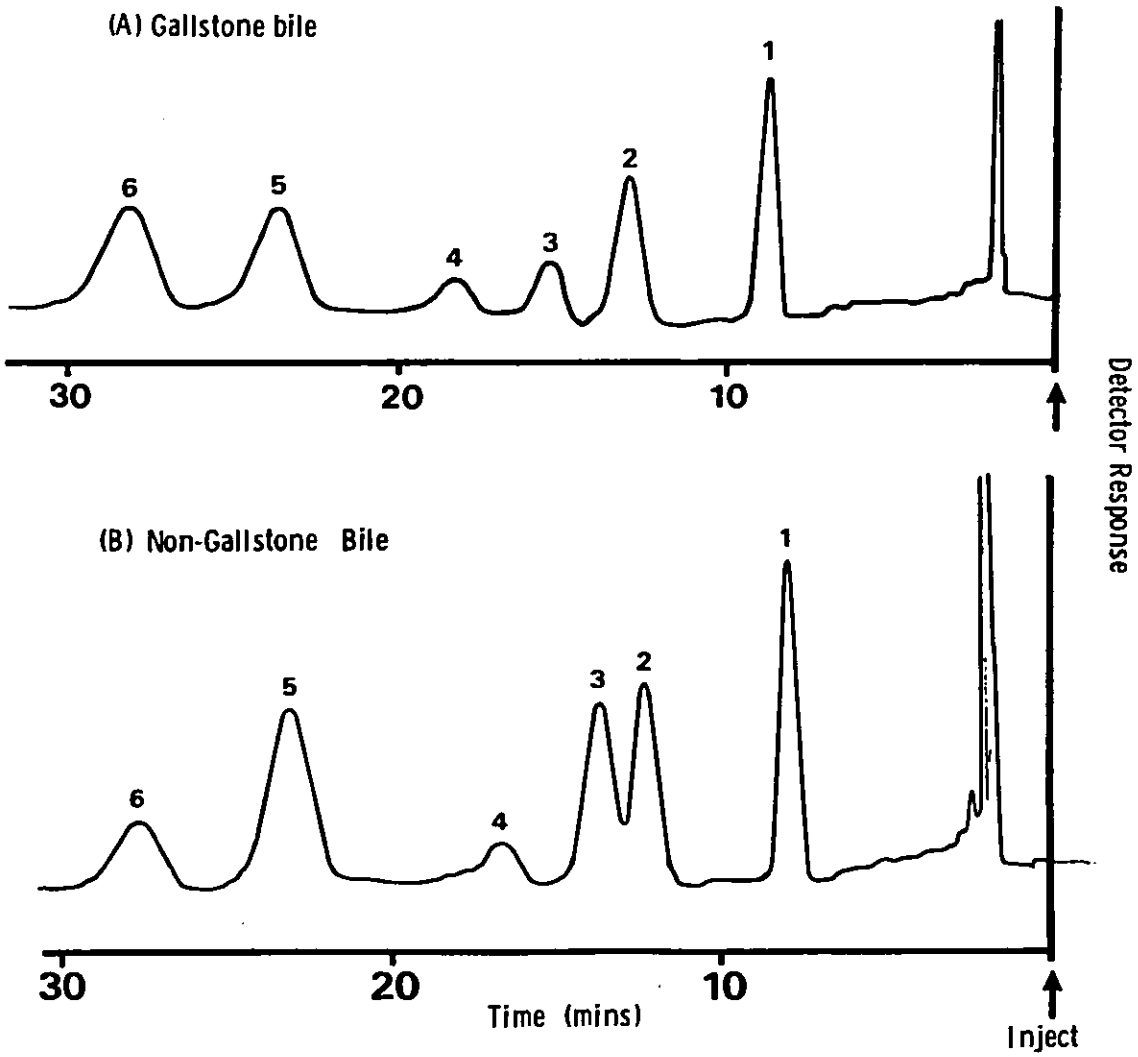


Figure 5.1A & B Tracings obtained for bile salts present in 'gallstone bile' (Patient, L.J., upper trace, A) and those found in 'non-gallstone bile' (Sample 1B, lower trace, B). Peak identity, upper and lower trace: as for Figure 4.5A.

gallstones (i.e.'gallstone bile') and bile obtained from gall bladders without gallstones (i,e. 'non-gallstone bile') are shown in Figures 5.1C-H.

5.4 Differences in the bile salt composition of gall bladder bile

The percentage composition with respect to bile salts of gall bladder bile obtained from patients suffering from gallstones differed from that found in gall bladder bile obtained from post mortem.

In gall bladder bile from those suffering from gallstones, the mean percentages of the glycine conjugates, GLYC, GCDC and GDC were: 27, 20 and 17%; and in post mortem bile from gall bladders without gallstones the percentages were: 24, 19 and 8% respectively.

The mean percentages of the taurine conjugates, TC, and TCDC and TDC in those with gallstones were: 18, 11 and 7% compared with 21, 21 and 7% in post mortem bile without gallstones. There was no difference between the percentages of GLYC and GCDC in the two groups but the percentage of GDC was significantly higher in gallstone patients than in gall bladder bile without gallstones (17% in gallstone patients, N = 16; compared with 8% in post mortem bile without gallstones, N = 16 $P<0.001$). The mean percentage of TC in gallstone bile was 18% compared with 21% in bile without gallstones but the difference was of low significance. Similarly, there was no signfficant difference between the percentages of TDC in the two groups but the percentage of TCDC was

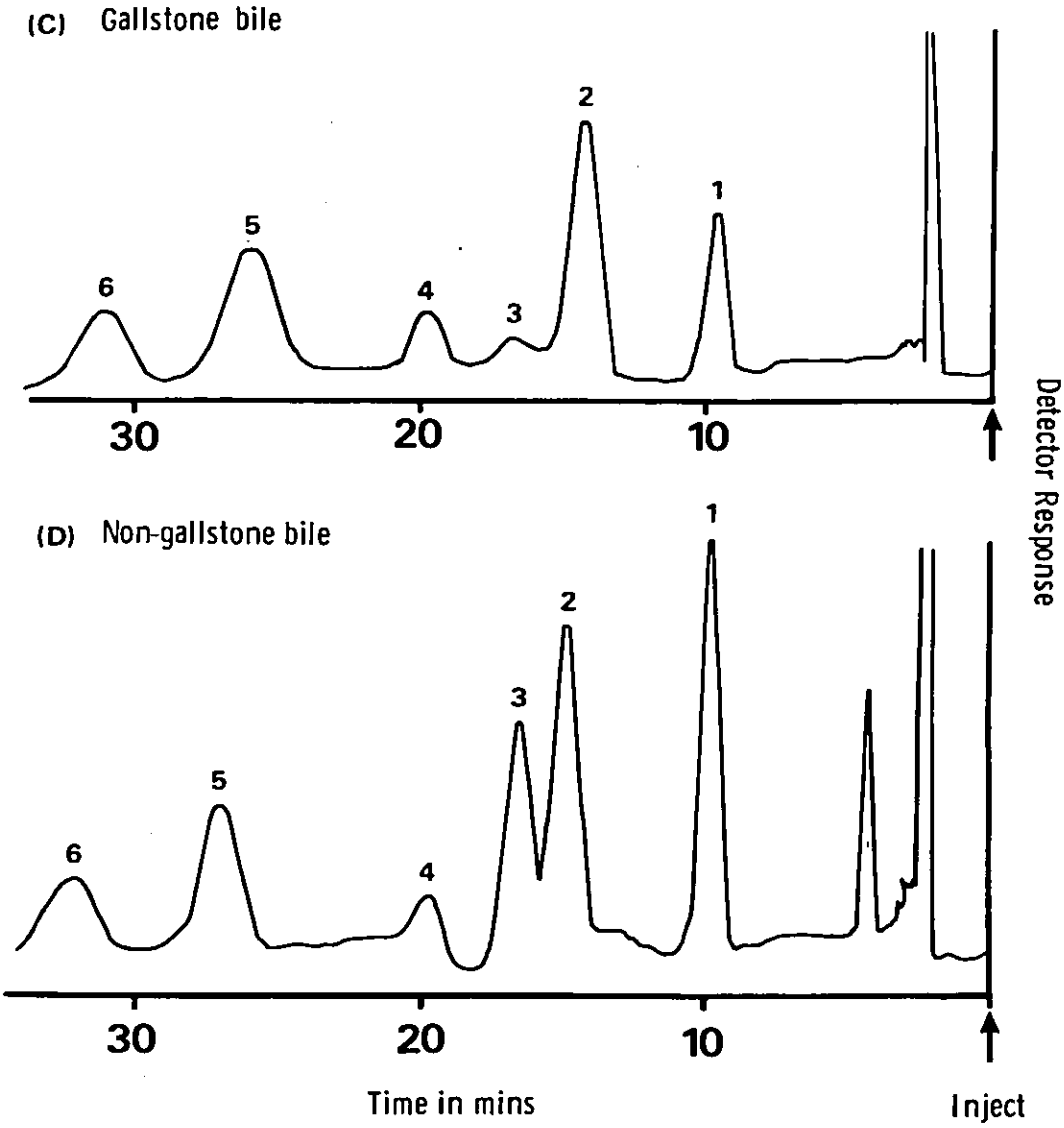


Figure 5.1C & D Tracings obtained for bile salts present in 'gallstone bile' (Patient S.W., upper trace, C) and those found in 'non-gallstone bile' (Sample 1D, lower trace, D). Peak identity, upper and lower trace: as for Figure 4.5A.

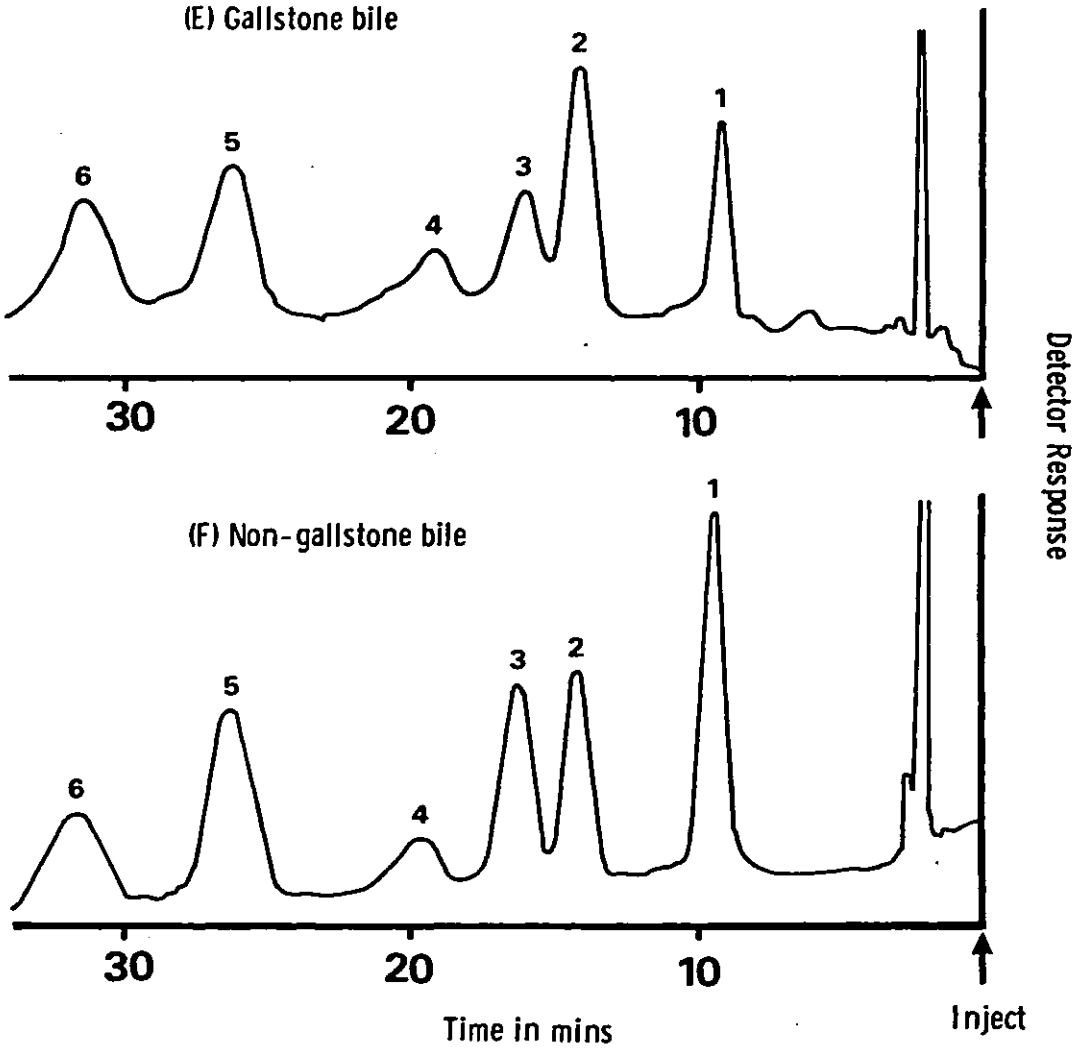


Figure 5.1E & F Tracings obtained for bile salts present in 'gallstone bile' (Patient, P.B., upper trace, E) and those found in 'non-gallstone bile' (Sample 1F, lower trace, F). Peak identity, upper and lower trace: as for Figure 4.5A.

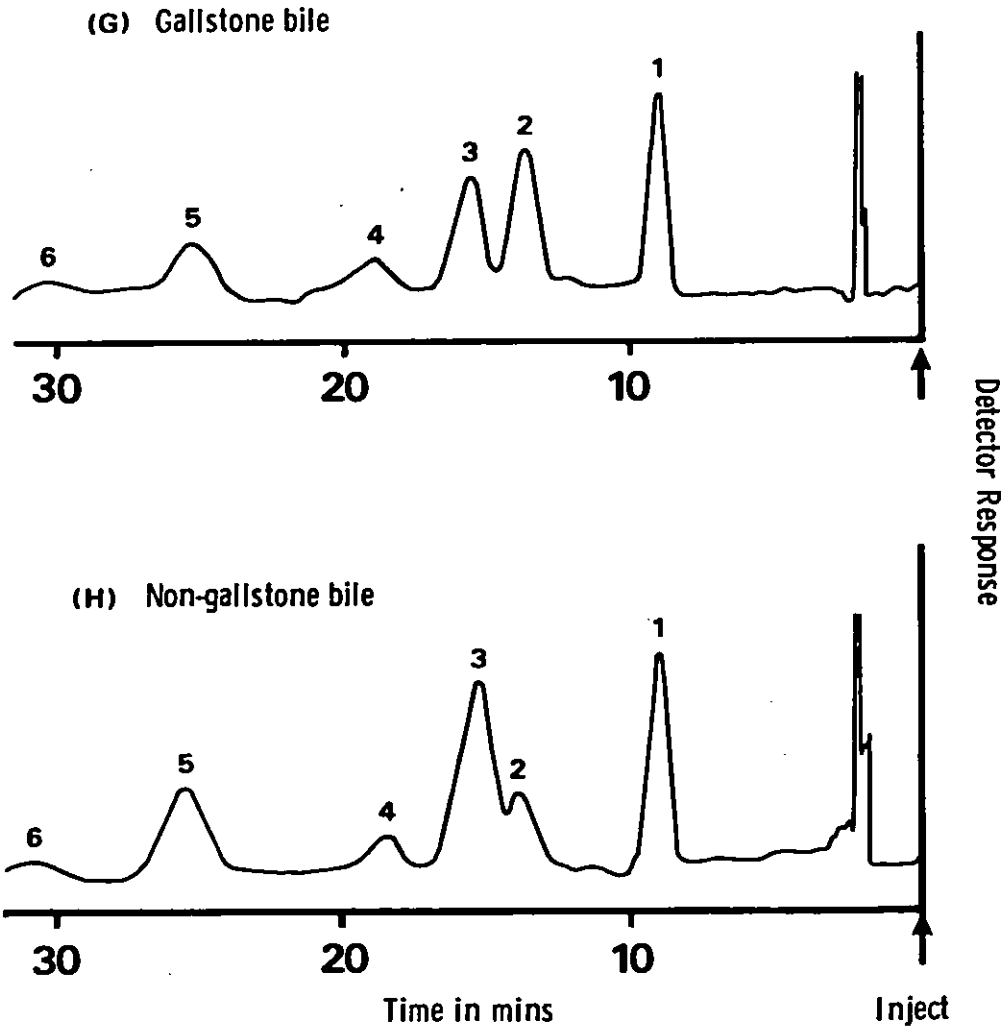


Figure 5.1G & H Tracings obtained for bile salts present in 'gallstone bile' (Patient, H.T., upper trace, G) and those found in 'non-gallstone bile', (Sample 1H, lower trace, H). Peak identity, upper and lower trace: as for Figure 4.5A.

lower in gallstone bile (11% in gallstone bile compared with 21% in non-gallstone bile). The difference was highly significant ($t = 7.3$, $P<0.001$). These results are summarised in Table 5.1, (Figures 5.2 and 5.3).

Table 5.1 Differences in the proportions of glycine and taurine bile salts found in patients suffering from gallstones and in post mortem bile without gallstones.

	% Composition of gall bladder bile							
Bile Salt	GLYC	GCDC	GDC	Total G	TC	TCDC	TDC	Total T
Gallstone Group N=16	27	20	17	64%	18	11	7	36%
Non-gallstone Group N=16	24	19	8 *	51% *	21	21 *	7	49% *

*P < 0.001; G = glycine conjugates; T = taurine conjugates

The observation of increased proportion of GDC and decreased TCDC when gallstones are present is consistent with the current concept that changes of this kind increase the 'lithogenic potential' of bile and favour gallstone formation (Thistle & Hofmann, 1973; McDougal, Walker & Thurston, 1976). The observation of a higher proportion of glycine conjugates in 'gallstone bile' (64%) compared with 51% in 'non-gallstone bile' is difficult to explain since glycine conjuages have been reported to solubilise more cholesterol than taurine conjugates (Earnest & Admirand, 1971). The concentration

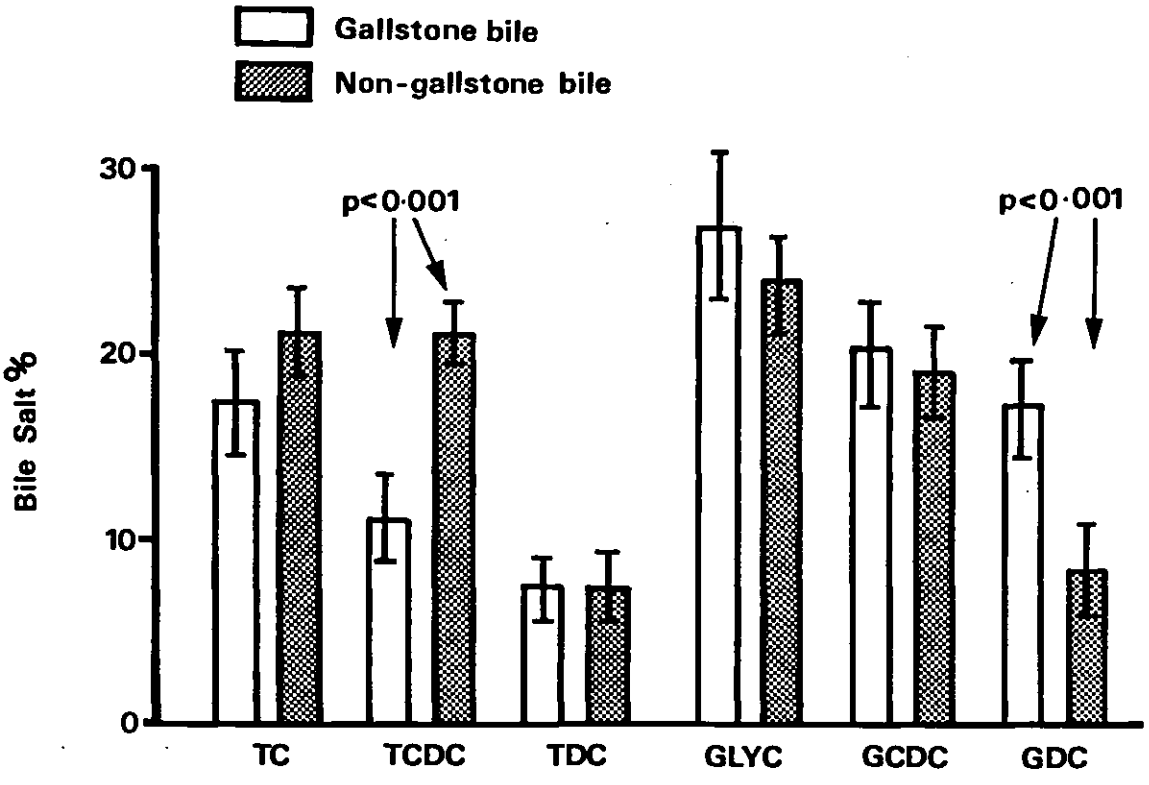


Figure 5.2 Percentage composition of 'gallstone bile' and 'non-gallstone bile' expressed as the percentage of total bile salts present in bile. (Mean ± SD)

of total bile salts (i,e. the sum of the concentration of six main bile salts) found in patients with gallstones was different from that in post mortem bile without gallstones: 28.2 ± 8.3 mg/ml in patients with gallstones, N = 16 compared with 46.6 ± 7.3 mg/ml in post mortem bile N = 16 (mean ± SD; P<0.001; Table 5.2). Further details of these results are included in Appendix Table 5.3 A & B.

Table 5.2 Differences in the concentration of bile salts found in patients with gallstones and in post mortem bile without gallstones.

	Bile Salts (mg/ml)			
Bile Salts	'Non-gallstone' Post mortem Bile	'Gallstone Bile'	Difference	%
GLYC	11.2	7.6	-3.6	-32
GCDC	8.7	5.8	-2.9	-33
GDC	3.8	4.8	+1.0	+26
Total G				
Mean ± SD	23.7 ± 4.9	18.2 ± 5.9	-5.5	-23
TC	10.0	5.0	-5.0	-50
TCDC	9.8	3.0	-6.8	-69
TDC	3.1	2.0	-1.1	-35
Total T				
Mean ± SD	22.9 ± 4.4	10.1 ± 3.1	-12.8	-56
Total (T+G):	46.6 ± 7.3 (N=16)	28.2 ± 8.3 (N=16)		
Range:	(34-60)	(13-44)		
Level of Significance:		P<0.001		

G = glycine conjugates; T = taurine conjugates

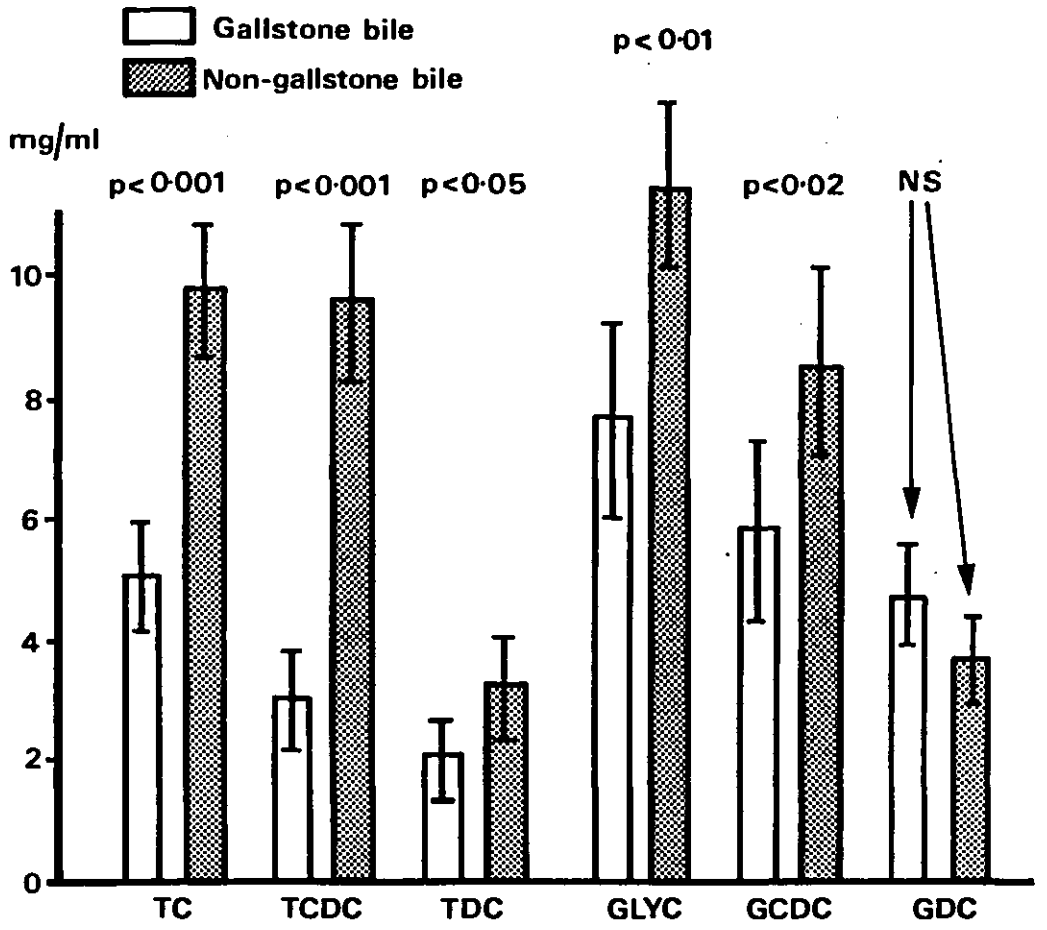


Figure 5.3 Concentration of individual bile salts (mg/ml) present in 'gallstone bile' and in 'non-gallstone bile' (Mean ± SD)

In gall bladder bile from patients with gallstones, 64% of bile salts were conjugated with glycine compared with 51% in post mortem bile without gallstones. The difference is significant ($P<0.001$). Similarly, in 'gallstone bile' 36% of bile salts were conjugated with taurine compared with 49% found in 'non-gallstone bile'; $P<0.001$; Table 5.1).

The mean and standard deviation of glycine to taurine (G:T) ratio in gallstone patients was 1.85 ± 0.53 ($N = 16$) and in post mortem bile it was 1.07 ± 0.34 ($N = 16$; $P<0.001$ Appendix Table 5.4).

Table 5.5 "Glycine to taurine" (G:T) ratio found in this study and those reported by others in patients with gallstones and in those without.

Author	N	Gallstone Group	N	Non-gallstone Group	Difference
Sjovall (1960)	8	3.4	4	3.2	-0.2
Dam et al (1966)	14	3.14	14	3.34	+0.2
Burnett (1965)	11	1.8	10	2.2	+0.4
Kuroda et al (1974)	4	4.1	10	3.14	-0.96
Correia et al (1977)		---	10	1.69	
This Study	16	1.85	16	1.07	-0.78

N = number of subjects studied

The results of present study suggest that in gallstone disease there is a distinct change in the conjugation of glycine and taurine bile salts. This is in line with previous studies which show that the G:T ratio was variable and influenced by diet (Haslewood, 1965).

5.5 Differences in 'Tri- hydroxy to Di-hydroxy' (T:D) bile salt ratios

There was no difference in the tri-hydroxy (T:D) bile salt ratio in bile obtained from gall bladders with and without gallstones (0.86 ± 0.39 N=16 in 'gallstone bile' compared with 0.88 ± 18, N=16 in 'non-gallstone bile' Mean ± SD; Appendix Table 5.6).

The ratios found in this study and those reported by others are shown in Table 5.7.

Table 5.7 Tri-hydroxy (T:D) bile salt ratio found in this study and those reported by others in patients with and without gallstones.

Author	N	Non-gallstone Group	N	Gallstone Group	Difference
Sjovall (1960)	4	0.66	8	0.56	-0.10
Burnett (1965)	10	1.04	11	0.79	-0.25
Dam et al (1966)	14	0.70	14	0.64	-0.06
Vlahcevic et al (1970)	2	0.69	14	0.62	-0.07
Nakayama et al (1970)	20	1.16	20	0.66	-0.50
Kuroda et al (1974)	10	0.56	4	0.53	-0.03
This Study	16	0.88	16	0.86	-0.02

N = number of patients studied

These results show the variation in the T:D ratios reported from different laboratories. It is noteworthy that the differences in the ratios of the two groups are in the same direction including the results of this study.

A lower value of T:D ratio than 0.86 ± 0.39 was expected in those with cholelithiasis because of the relative increase in the proportion of DC. Together with this alteration there was a decrease in the proportion of CDC in 'gallstone bile'. These changes favour the production of bile supersaturated with cholesterol relative to the concentration of bile salts and lecithin (Van der Linden, 1971; Heaton, 1972; Coyne, Marks & Schoenfield, 1977).

5.6 The 'primary bile salt' (C:CDC) ratio in gall bladder bile with and without gallstones

The ratio of 'primary bile salts' (C:CDC) in gall bladder bile obtained from patients suffering from gallstones was 1.80 ± 1.6 (N=16) and that found in post mortem bile without gallstones was 1.20 ± 0.32 (N=16; Mean ± SD) but the difference was of low significance (Appendix Table 5.8). The ratios found are compared with those reported from other laboratories (Table 5.9). These results show wide variations in the values reported by different authors. The higher value of C:CDC ratio found in patients suffering from gallstones (1.80 compared with 1.20) than in post mortem bile without gallstones is most likely due to a reduction in the synthesis of CDC in gallstone patients (Thistle & Schoenfield) 1971; Danielsson & Sjovall, 1975).

Table 5.9 Comparison of the 'primary bile salt' (C:CDC) ratio in patients with gallstones and in those without gallstones.

Author	N	'Gallstone Bile'	N	'Non-gallstone Bile'	Difference
Sjovall (1960)	8	1.00	4	1.10	+0.10
Nakayama (1967)			10	1.3	
Thistle et al (1971)	33	1.00	9	0.56	-0.44
Kuroda et al (1974)	4	0.90	10	1.09	+0.19
This study	16	1.80	16	1.20	-0.60

N = number of patients studied

5.7 The proportions of C: CDC:DC in gall bladder bile

The major bile salts present in human bile are the glycine and taurine conjugates of C, CDC and DC. The relative proportions of these bile acids were different in gall bladder bile obtained from patients with gallstones and in post mortem bile without gallstones. (45: 31: 24:in patients with gallstones, N=16 compared with 46: 40: 14 in post mortem bile, N=16). The proportion of C was unchanged but the proportion of CDC was lower in those with cholelithiasis (31% in 'gallstone group' vs. 40% in 'non-gallstone' group $P < 0.001$). The proportion of DC was higher in those with gallstones (24% in 'gallstone group' vs. 14% in post mortem bile'non-gallstone group', $P < 0.001$; Appendix Table 5.10). The relative proportions of

C: CDC: DC found in this study are compared with those reported by others (Table 5.11).

The decrease in CDC and the relative increase in DC found in patients suffering from gallstones is undesirable since this would increase the 'lithogenic potential' of bile by lowering its capacity to solubilise cholesterol (Danielsson & Sjovall, 1975; McDougal, Walker & Thurston, 1976).

Table 5.11 Comparison of C: CDC: DC ratios in patients with gallstones and in those without gallstones

Author	'Gallstone bile' N	C: CDC: DC	'Non-Gallstone Bile N	C: CDC: DC
Wooton et al (1953)		-- -- --	1	42:40:18
Sjovall (1960)	8	36:36:29	4	41:37:22
Bloomstrand (1961)	4	45:29:26	1	35:39:27
Nakayama (1967)		-- -- --	10	44:33:19
Kuroda et al (1974)	4	35:39:26	10	36:33:31
Thistle et al (1971)	33	37:37:26	9	30:54:16
This study	16	45:31:24	16	46:40:14

N = number of patient studied

5.8 Discussion

The results of this study show marked quantitative differences in the bile salts present in bile taken from gall bladders which did or did not contain cholesterol gallstones. Only the major six bile salts normally found in human bile (i.e. glycine and taurine conjugates of C, CDC and DC) have been

considered. Of these, C and CDC are synthesised *de nova* in the liver from cholesterol (Danielsson & Sjovall, 1975) and conjugated with glycine and taurine (Haslewood, 1967) before being secreted into bile and finally passed into the duodenum. The other bile acid DC, is formed from C by the action of intestinal bacteria which remove the hydroxyl (-OH) group in 7α position (Hill & Drasar, 1968). Approximately 96% or more bile salts secreted into the gut are reabsorbed and returned to the liver via the 'enterohepatic circulation' (Dowling, Mack & Small, 1970) such that a 'bile acid pool' (BAP) is maintained. A small proportion (less than 5%) of this pool escapes reabsorption and is excreted daily in the faeces (Small, Dowling & Redinger, 1972). In order to keep the BAP constant the liver only synthesises enough bile salts to replace the daily faecal loss (Grundy, Ahrens & Miettinen, 1965; Bouchier, 1979). Having this as a background, the results of present study show that 'glycine and taurine'conjugation of bile salts is altered in gallstone disease. The finding of a higher G:T ratio in 'gallstone bile' (1.85 in 'gallstone bile' compared with 1.07 in 'non-gallstone bile'; $P < 0.001$) is in accord with the results reported by a number of other authors (Table 5.5) The increase in G:T ratio found in 'gallstone bile' is due to both an increase in the proportion of glycine conjugates and a relative decrease in taurine conjugates. Recent work (Greim, 1977) suggests

that glycine conjugates predominate in 'normal' bile and appear to be affected more than taurine conjugates. The observation of a relative decrease in the proportion of taurine conjugates in gallstone bile is difficult to explain if taurine conjugates are considered to solubilise less cholesterol than glycine conjugates (Earnest & Admirand, 1971). The effect of this would decrease the 'lithogenic potential' of bile and reduce the tendency to form gallstones.

The 'tri-hydroxy to di-hydroxy' (T:D) bile salt ratio in gall bladder bile from the two groups did not alter significantly. This was mainly because (a) the proportion of C acid has remained unchanged and (b) the reduction in the proportion of CDC in 'gallstone bile' is accompanied by an increase in the proportion of DC such that the proportion of di-hydroxy bile salts has remained unaltered (55% in 'gallstone bile' compared with 54% in 'non-gallstone bile').

Comparison of the relative proportions of C, CDC, and DC in 'gallstone bile' and in 'non-gallstone bile' demonstrated marked differences. These differences suggest changes in the hepatic synthesis of primary bile acids (Danielsson & Sjovall, 1975) and in the formation of secondary bile acid in the colon. The amount of bile acids returning to the liver via the enterohepatic circulation (EHC) exert a 'negative feed back' control on the hepatic synthesis of bile

acids (Mosbach, 1972) and also influence the formation of secondary bile acids in the intestinal tract (Danzinger, Hofmann, Thistle & Schoenfield, 1973). In this way the concentration of CDC in EHC controls the synthesis of C in the liver; and inversely, an increased concentration of C inhibits the synthesis of CDC. In the light of current concepts of the regulation of bile acid synthesis (Wilson, 1972; Hanson & Pries, 1977) the reduction in the relative proportion of CDC in 'gallstone bile' may be due to an abnormality in the hepatic synthesis of this bile acid. The finding of a reduced amount of CDC in gallstone patients supports previous studies (Thistle & Schoenfield, 1971).

Since the hepatic synthesis of C is influenced by the concentration of CDC in bile returning to the liver (Einarsson, Hellstrom & Kallner, 1973) a decreased concentration of CDC will stimulate the synthesis of C. This is evident from our results of 'gallstone bile' where the proportion of C has remained as high as that in 'non-gallstone bile'.(C = 45% in the gallstone group vs. 46% in non-gallstone' group; Table 5.10). This would be the normal response of the liver to compensate for reduced bile acid pool (BAP) found in gallstone patients (Coyne, Marks & Schoenfield, 1977). Evidence suggesting a reduced BAP in gallstone patients comes from our quantitative results which show that the concentration of bile salts in 'gallstone bile' was significantly lower than in 'non-gallstone bile' (28.2 $\pm$ 8.3 in 'gallstone bile' vs. 46.6 $\pm$ 7.3 in 'non-

gallstone bile'; $P < 0.001$; Table 5.2).

The finding of an increased amount of DC in gallstone patients leads one to consider the EHC to explain this observation . The EHC is a physiological conserving mechanism which ensures that the bile salts secreted in bile are subsequently reabsorbed from the intestine and returned to the liver. During their circulation the primary bile acids C and CDC are transformed by bacterial enzymes in the colon to secondary bile acids DC and LC respectively. Most of the DC formed is reabsorbed and returned to the liver. In this way, the observation of increased proportion of DC in gallstone patients may have a number of explanations such as, a) an impairment in the storage capacity of gall bladder (McDougal, Walker & Thurston, 1976). This would increase the number of EHC cycles so that there is an increased bacterial transformation of C acid hence the concentration of DC in bile increases; b) a diseased gall bladder: a diseased gall bladder has been implicated as a less efficient storage chamber (Danielsson & Sjovall, 1975; Mackay, 1975), allowing increased contact between the gut and the primary bile acids; hence the formation of secondary bile acids will increase; c) lack of dietary fibre: it is claimed (Heaton, 1972) that a lack of dietary fibre increases the exposure of bile acids to bacterial enzymes, therefore more secondary products are formed. In this respect, diets with a high fibre content are likely to reduce the risk of developing gallstones; d) decreased synthesis of CDC: it is suggested that there is a strong inverse relationship between CDC

and DC in bile (Low-Beer, 1977); the level of DC in EHC selectively inhibits the synthesis of CDC. Another consideration is the cholesterol solubility in bile. DC in bile is known to be more important determinant of biliary cholesterol saturation (Mackay, Low-Beer, 1975; 1977) and therefore of the tendency to form gallstones.

As regards the primary bile salt (C:CDC) ratio, although the difference is not significant (1.80 $\pm$ 1.60; N=16 compared with 1.20 $\pm$ 0.32; N=16; Mean $\pm$ SD; t=1.49) the results indicate a tendency for the primary bile salt ratio to be high in gallstone patients. The measurement of this ratio is considered to be a good index of bile acid synthesis and has a diagnositic value since it yields the information of the condition of hepto-cytes (Carey, 1973). The higher value found for the ratio in 'gallstone bile' may be due to impaired bile acid synthesis of CDC in gallstone patients. A relative decrease in the concentration of CDC in gall bladder bile has been shown to reduce BAP (Thistle & Schoenfield, 1971) and to decrease the micellar solubilisation of biliary cholesterol (Danzinger et al 1973). This type of bile favours the development of gallstones.

Evidence available suggests that the bile in patients suffering from gallstone disease becomes supersaturated with cholesterol which may precipitate to form crystals leading to the development of cholesterol gallstones (Small, 1968; Rains, 1968; Coyne, Marks & Schoenfield, 1977). It is also generally agreed that supersaturation of bile occurs from either a relative excess of cholesterol or a relative lack of bile salts (Heaton, 1972; Bouchier, 1979). As for

excessive cholesterol in bile it is believed to be due to increased hepatic synthesis in patients suffering from cholesterol gallstones (Miettinen, 1971; Nestel et al. 1973). To support this there is evidence in gallstone patients of increased activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase the rate-limiting enzyme for cholesterol synthesis in the liver (Salen, Nicolau, Shafer & Masbach, 1975).

In this study, the aspect of bile salts in patients suffering from gallstones has been examined. Gall bladder bile from these patients has been shown to contain significantly lower amounts of bile salts than in post mortem bile without gallstones. The deficiency of bile salts in gallstone disease may be due to, a) impaired hepatic synthesis of bile salts (Grundy & Metzger, 1972) associated with a decrease in the activity of 'cholesterol 7α-hydroxylase; b) reduced bile acid pool (BAP) which is unable to solubilise biliary cholesterol (Heaton & Read, 1969; Vlahcevic, Bell, Buhac, Farrar & Swell, 1970); c) abnormal return of bile salts via enterohepatic circulation (EHC) leading to an increased recycling frequency (Mackay, 1975; Low-Beer & Pomare, 1973); d) excessive faecal loss of bile salts in patients suffering from gallstones may reduce the size of BAP (Greim, 1977, Mok, von Bergmann & Grundy, 1977).

The results of this study show marked differences in the bile salt patterns and various bile salt ratios found in gall bladder bile of patients suffering from gallstones when these were compared with the patterns and ratios found

in post mortem gall bladder bile without gallstones. Our finding of increased DC and a relative decrease in the proportion of CDC in gallstone patients provides further evidence to suggest that this kind of bile favours gallstone formation.

One of the main problems of this study has been the difficulty of obtaining "normal" gall bladder bile from healthy subjects. It is realised that the two groups of samples used do not provide "absolute comparisons". Ideally, gall bladder bile obtained from healthy subjects would be preferable but this was not posible for ethical reasons.

It may be useful to estimate bile salts present in gall bladder bile taken at post mortem from individuals with gallstones. As regards 'normal' bile, it would be worth considering to aspirate bile from the gall bladder after 'brain death' of patients not suffering from cholelithiasis or hepato-biliary disease and who have authorised the removal of their organs e.g. kidney donors.

CHAPTER 6

GENERAL DISCUSSION, CONCLUSION AND DIRECTION FOR FUTURE RESEARCH

In this thesis two analytical problems have been considered. The analysis of bile acids and neutral sterols present in human faeces, and the analysis of bile salts present in gall bladder bile. Both kind of analyses have application in two clinical situations, fenfluramine-induced diarrhoea and the problem of cholesterol gallstones.

When the present work was started, both TLC and GC were well established analytical techniques which had been used for the analysis of bile acids in biological samples, but HPLC was a fairly new procedure and there were no HPLC methods available for the estimation of bile acids or bile salts. During the course of this work, methods using GC for the routine estimation of bile acids and neutral sterols present in faeces were developed. The improved procedures were then used to investigate the problem of fenfluramine-induced diarrhoea. Studies described in Chapter 3 demonstrated that in subjects taking fenfluramine, the excretion of bile acids and neutral sterols in the faeces was significantly increased but the bile acids remain qualititatively the same except in those suffering from fenfluramine-induced diarrhoea. The subjects with diarrhoea excreted increased amounts of CDC and C instead of the usual DC and LC.

Even though there is no information available on the incidence of gallstones in subjects taking fenfluramine, a disadvantage of increased faecal loss of bile acids is a tendency to produce 'lithogenic bile' and gallstone formation (Greim, 1977; Mok et al. 1977). Therefore methods using HPLC were developed for estimating bile salts present in gall bladder bile. Unfortunately,

bile from 'normal' healthy subjects taking fenfluramine could not be obtained for ethical considerations, therefore gall bladder bile taken at cholecystectomy for gallstones was used in a comparitive study to investigate the differences in bile salts when compared with post mortem bile without gallstones.

The study described in Chapter 5 demonstrated marked qualitative and quantitative differences in bile salts present in gall bladder bile obtained from those suffering from cholesterol gallstones and in post mortem gall bladder bile without gallstones. The concentration of bile salts was significantly lower in gallstone patients than in post mortem bile. This is consistent with observation of a reduced bile acid pool in patients with cholelithiasis (Vlahcevic et al. 1970). Concomitant with the quantitative changes, the relative proportion of DC in gall bladder bile obtained from patients suffering from gallstones was higher but the proportion of CDC was lower than in post mortem bile without gallstones. This kind of bile has a reduced capacity to solubilise cholesterol (Thistle et al. 1973; McDougal, Walker & Thurston, 1976).

The major conclusion to emerge from this work is that the excretion of bile acids and neutral sterols was significantly increased in subjects treated with fenfluramine. Over a short period this may be beneficial in obesity and lower the serum cholesterol level but a long term effect may be an excessive loss of bile salts with a tendency to form 'lithogenic bile' and to cholelithiasis (Greim, 1977; Mok et al. 1977).

Using the techniques developed in this thesis it has been shown

that the pattern of bile salts present in gall bladder bile obtained from patients suffering from cholesterol gallstones are different from those found in post mortem gall bladder bile without gallstones. The concentration of the total amount of bile salts found in 'gallstone bile' was significantly lower than in post mortem gall bladder bile. The occurance of cholesterol gallstones is believed to be favoured either by impaired hepatic bile acid synthesis leading to a lower concentration of bile salts or by the relative increase in cholesterol production (Coyne et al. 1976).

Direction for Future Research

Gas chromatography in the past has provided the best method for the analysis of bile acids, but it suffers from a number of limitations and is unsuitable for the estimation of bile salts. Work described in this thesis shows that HPLC offers a rapid and reliable alternative. The new procedure should be the method of choice for bile salt analyses.and increase the possibility of finding new, yet undiscovered bile acids. HPLC allows the estimation of bile salts without hydrolysis or the preparation of derivatives, using mild conditions.

A detailed knowledge of the individual bile salts in bile should closely reflect the changes in liver function and increase the present understanding of gallstone disease. Another area where HPLC has a special application is in studies of CDC and UDC therapy' for the treatment of gallstones. In this respect it should be a 'new diagnostic technique' for monitoring the progress of 'lithogenic to litholytic bile'. At the present stage of development of HPLC, the sensitivity provided by UV-detection

is adequate for estimating bile salts in bile but not for estimating serum bile satls. Work is therefore required in this area to improve the sensitivity of HPLC in anticipation of the application to the analysis of serum bile salts. Blood samples are easier to obtain than bile.

If GC is preferred, an increased sensitivity of detection, a liquid phase with separating power better than QF-1 and a thermal stability similar to that of SP 2250 would be useful. One possibility would be to use stationary phases of mixed composition in which the tendency for column bleed of one component was more than compensated for by the presence of another. However, data does not appear to be available concerning the use of a mixture of QF-1 and SP 2250. In faecal bile acid analysis, increased resolution and higher column efficiency was achieved by increasing the column length and by decreasing the internal diameter but these modifications increased the analysis time. In this respect it would be useful to investigate the use of 'capillary columns' for bile acid analysis.

A P P E N D I C E S

APPENDIX A

BILE ACIDS	SYSTEMATIC NAME & SUPPLIER
Cholanic	*5β-cholan-24-oic acid*
Lithocholic	*3α-Hydroxy-5β-cholan-24-oic-acid*
Deoxycholic	*3α, 12α-Dihydroxy-5β-cholan-24-oic-acid*
Chenodeoxycholic	*3α, 7α-Dihydroxy-5β-cholan-24-oic acid*
Ursodeoxycholic	*3α, 7β-Dihydroxy-5β-cholan-24-oic acid*
Hyodeoxycholic	*3α, 6α-Dihydroxy-5β-cholan-24-oic acid*
Dehydrodeoxycholic	*3, 12-Diketo-5β-cholan-24-oic acid*
Cholic	*3α, 7α, 12α-Trihydroxy-5β-cholan-24-oic*
Hyocholic	*3α, 6α, 12α-Trihydroxy-5β-cholan-24-oic*
7-keto-lithocholic	*3α-Hydroxy-7-keto-5β-cholan-24-oic acid*
7, 12-diketo-lithocholic	*3α-Hydroxy-7, 12-diketo-5β-cholan-24-oic*

- Serva (Uniscience Limited)

NEUTRAL STEROLS/STEROIDS

Cholesterol	*5-Cholesten-3β-ol*
Cholestanol	*5α-Cholestan-3β-ol*
Coprostanol	*5β-Cholestan-3β-ol*
Epicoprostanol	*5β-Cholestan-3α-ol*
Lanosterol	*8, 24-Lanostadien-3-ol*
β-sitosterol	*Stigmast-6-en-3β-ol*
Stigmasterol	*Stigmasta-5, 22-dien-3β-ol*
Campersterol	*(24R)-Ergost-5-en-3β-ol*
Cholestane	*5α-Cholestane*
Cholestanone	*5α-Cholestan-3-one*
Coprostanone	*5β-Cholestan -3-one*

- Serva (Uniscience Limited)

BILE SALTS (glycine & taurine Na salts)

Taurocholic, Taurochenodeoxycholic, Tauradeoxycholic,
Glycocholic, Glycochenodeoxycholic, Glycodeoxycholic

- Serva (Uniscience Limited)

Taurolithocholic, Tauroursodeoxycholic
Glycholithocholic, Glycoursodeoxycholic

- International Enzymes Limited

APPENDIX B

Materials used in the construction of apparatus used for freezing homogenised faeces (Figure 2.3).

	Supplier
Citenco variable speed stirrer motor (Cat. No. H82/5)	- Jencons
Adjustable Jacob's Chuck (Cat. No. H82/8)	- Jencons
Stand with Wooden Base (constructed)	- Laboratory Workshop
Flat Botton Glass Tank, Pyrex (Cat. No. H18/3)	- Jencons
1-l Round Bottom Flask (Neck, 34/35)	- Quickfit
Solid CO_2	- Distillers
Acetone (GP)	- Fisons

Materials used in the construction of apparatus designed for extracting bile acids from faeces (Figure 2.1)

Flask Shaker (Model SD 110)	- Gallenkamp
Extension with clamps for test tubes (constructed)	- Hospital Workshop
Test tubes (Pyrex, MF 24/1/6)	- Quickfit
Oil Bath (Type SS30)	- Grant Instruments
Timer Unit (constructed)	- Laboratory Workshop

Materials used for the modification of the operation of Gallenkamp Shaker (Figure 2.4)

Timers (Omron STP YMH: 1, 72 min; 2 & 3, 12 min)	- IMO Precision Controls
Plug-in Relay Coil (Type 6012.06)	- IMO Precision Controls
Connector Socket for Relay Coil (ZB46)	- IMO Precision Controls

APPENDIX C

Preparation of 23-Nor-Deoxycholic Acid (Internal Standard)

23-Nor-Deoxycholic Acid (23-Nor-DC) was prepared by Barbier-Wieland degradation of deoxycholic acid (Riegel, Moffett & McIntosh, 1955). The purified material melted at 213-215°C; and its purity was checked by gas chromatography of the methyl ester derivative on QF-1 column.

Materials for Stage I (Preparation of 3, 12-Diacetoxy-bisnor-cholanyl-diphenylethylene, Riegel, Moffett & McIntosh, 1955)

Grignard Reagent: synthesised (Riegel, Moffett & McIntosh, 1955)

Magnesium turnings (97.2 g); Bromobenzene (450 ml, 4.3 mol); Diethyl ether, AR (b.p. 34-35°C, 1250 ml)	- BDH*
Deoxycholic acid (research grade, 100g)	- Serva (Uniscience Limited)
Benzene, AR (700 ml); NaOH, AR (pellets); conc. HCl (700 ml); dil. HCl (1.1); Acetic acid, glacial, AR (1.1); Acetic anhydride AR (500 ml)	- BDH

Materials for Stage II (Preparation of 23-Nor-DC)

Chromium trioxide (37 g); Chloroform, AR (60 ml); Acetic acid, glacial, AR (500 ml); KOH, AR (pellets); Methanol, AR (1.1); dil. HCl (750 ml); Acetyl chloride, AR (50 ml); Diethyl ether, AR (b.p. 34-35°C, 400 ml); Acetone, AR (600 ml)	- BDH

*British Drug House

APPENDIX D

Preparation of Ethereal Diazomethane

Stage I (Preparation of N-methyl-N-nitrosourea)

N-methyl-N-nitrosourea, the starting material for the preparation of ethereal diazomethane was prepared by the method of Arndt (1950). The product was dried to constant weight and stored at -20°C.

CAUTION Diazomethane and its starting material are potentially dangerous chemicals; precautions similar to those recommended for carcinogens must be taken during the preparation.

Materials of Stage I	Supplier
Methylamine solution (25-30%, w/v, 100 g); conc. HCl (78 ml); Urea, AR (150 g); Sodium Nitrite, AR (55 g); conc. H_2SO_4, AR (50 g); Crushed ice (300 g)	- BDH

Materials for Stage II (Preparation of Ethereal Diazomethane)

N-methyl-N-nitrosourea (0.6 g) synthesised, Arndt (1950); Diethyl ether, AR (b.p. 34-35°C) freshly distilled; Methanol, AR (freshly redistilled); KOH, AR (pellets); Salt/crushed ice mixture	- BDH

Materials for the appratus designed for generating diazomethane (Figure 2.2)

	Supplier
3-Neck flask (1.L capacity; middle next, 30 mm i.d.; side necks, 20 mm i.d.). Reaction tube with bored cap, 'Teflon' lined septum (20 mm O.D., 200 mm in length), Sovirel. The 'oval' window was cut using a circular glass cutter; and oxy-acetylene flame used to smooth the edges.	- V.A. Howe
Reaction tube for methylating bile acids (20 mm o.d. 100 mm in length, fitted with bored cap & 'Teflon' lined septum, Sovirel); Chemically inert 3-way micro valve, (Hamilton); gas tight syringe (20 ml, 'Teflon' plunger, luer lock, Hamilton)	- V.A. Howe
Thermometer (-40°C to +40°C)	- Griffen & George
PTFE tubing (1 m in length)	- A.E.I. Plastics

Procedure:

The apparatus designed for generating ethereal diazomethane is shown in Figure 2.2. Freshly distilled diethyl ether (25 ml) was placed in the 3-neck flask and 0,6 g of N-methyl-N-nitrosourea transferred into the reaction tube with an 'oval' window. The apparatus was assembled using sealing rings between the joints to prevent leakage. The lower end of the reaction tube was allowed to dip into the ether layer to dissipate the heat of reaction and the air tight generating flask partially immersed in a salt/crushed ice mixture. With the 3-way micro valve open to the Hamilton syringe, 1 ml of 50% KOH (w/v) was injected slowly into the reaction tube through the septum. Diazomethane liberated by the action of

KOH on N-methyl-N-nitrosourea was allowed to collect in the cold ether which turns deep yellow as the reaction proceeds (15-20 min).

For methylating, the bile acids were dissolved in diethyl ether: methanol (9:1 v/v) and freshly prepared ethereal solution of diazomethane (3-4 ml) added using the 3-way micro valve to avoid contact with diazomethane. After 15 min at room temperature, the excess reagent and solvents were evaporated at the water pump.

The Hamilton syringe prevents any excess pressure during the preparation.

APPENDIX E

THIN LAYER CHROMATOGRAPHY

Materials	Supplier
Chromatography tank (9 x 230 x 230cm)	- Griffen & George
Lining paper (Whatman No. 1)	- Whatman Lab. Sales
TLC plates (pre-coated, Kieselgel 60 F_{254} 10 x 20 cm, 0.25mm thick)	- Merck
Butanol*/Acetic Acid*/Water (10:1:1, System I)	
Chloroform*/Methanol*/Water/Acetic Acid (75:25:3:2, System II)	- *BDH
Phosphomolybdic Acid*, Ethanol (AR)	

Procedure: Bile Salts in Bile

0.5 ml of gall bladder bile was deproteinised with ethanol (5ml) and the cholesterol present in bile removed as described in Chapter 4.2. The ethanolic extract containing bile salts was applied to pre-coated TLC plate either as a streak or as a spot and the bile salts separated using solvent System I. When the solvent front was about 2 cm from the top edge of the plate, the plate was removed, briefly dried and sprayed with 10% phosphomolybdic acid in ethanol (w/v). The bile salts were detected by heating the plate at 110°C for 2-3 min.

Bile Acids in Faeces

The faecal extract obtained after extracting freeze-dried faeces with toluene/acetic acid (Chapter 2.2) was concentrated and applied to pre-coated TLC plates. The bile acids present in faeces were separated using solvent System II and detected as described above.

APPENDIX F

GAS CHROMATOGRAPHY

1) Neutral Sterols on QF-1 Columns

Gas Chromatograph: Pye Series 104, Model 64 fitted with dual columns and FID. Columns: glass coil, 1.5 m in length, 4 mm i.d. filled with 3% (w/w) (fluoroalkyl polysiloxane, Dow Corning Corp.) on 100-120 mesh Gas Chrom. Q. Nitrogen flow rate: 60 ml/min. Temperatures: Column, 220°C; Injector, 225°C; Detector, 250°C. Hitachi-Perkin Elmer 1 mV Recorder; Chart speed, 0.5 cm/min, Attenuation: 5×10^{-12} amps. R_t (cholesterol) = 8.8 min (isothermal).

2) Bile Acid Methyl Esters on QF-1 Columns

Gas Chromatograph & Columns: as for neutral sterols on QF-1 columns. Nitrogen flow rate: 50 ml/min. Temperatures: Columns, 235°C; Injector 240°C; Detector, 250°C. Recorder: as in above. Attenuation: 5×10^{-12} amps. $R_{t\ (DC)}$ = 14.9 min (isothermal).

3) Neutral Sterols on JXR Columns

Gas Chromatograph: as in above. Columns: glass coil, 1.5 m in length, 4 mm i.d. filled with 1% (w/w) JXR (dimethyl polysiloxane, Applied Science) on 100-120 mesh Gas Chrom. Q. Nitrogen flow rate: 50 ml/min. Temperatures: Column, 220°C; Injector, 225°C; Detector, 250°C. Recorder and Attenuation: as in above. $R_{t(cholesterol)}$ = 10.8 min (isothermal).

4) Bile Acid Methyl Ester Ketors ('oxo' derivatives) on JXR Columns

Gas Chromatograph & Columns: as in above. Nitrogen flow rate: 46 ml/min. Temperatures: Column, 240°C, Injector, 245°C; Detector, 250°C. Recorder and Attenuation: as in above. $R_{t(DC)}$ = 9.2 min (isothermal).

5) Neutral Sterols on SP 2250 Columns

Gas Chromatograph: as for QF-1 columns. Columns: 1.5 m in length, 4 mm i.d. filled with 3% (w/w) SP 2250 (methyl phenyl polysiloxane, Supelco Inc.) on 100-120 mesh Supelcon AW-DMCS. Nitrogen flow rate: 50 ml/min. Temperatures: Column, 270°C; Injector, 275°C; Detector 280°C. Recorder and Attenuation: as for QF-1 columns. $R_{t\ (cholesterol)}$ = 8.9 min (isothermal).

6) Bile Acid Methyl Esters on SP 2250 Columns

Gas Chromatograph & Columns: as in above. Nitrogen flow rate: 60 ml/min. Temperatures: Column, 280°C, Injector, 285°C, Detector, 290°C. Recorder and Attenuation: as for QF-1 columns. $R_{t(DC)}$ = 12.6 min (isothermal).

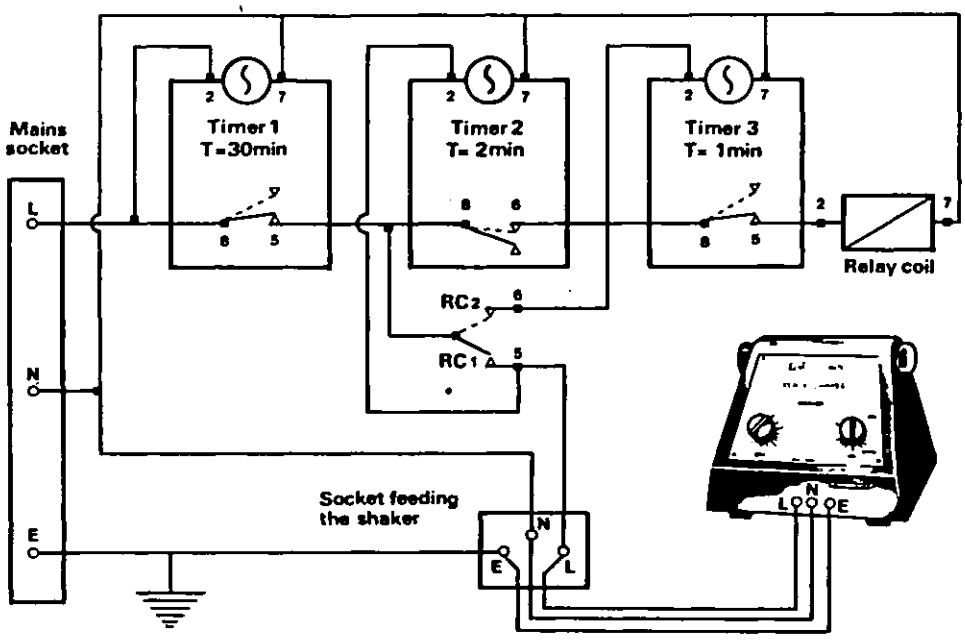
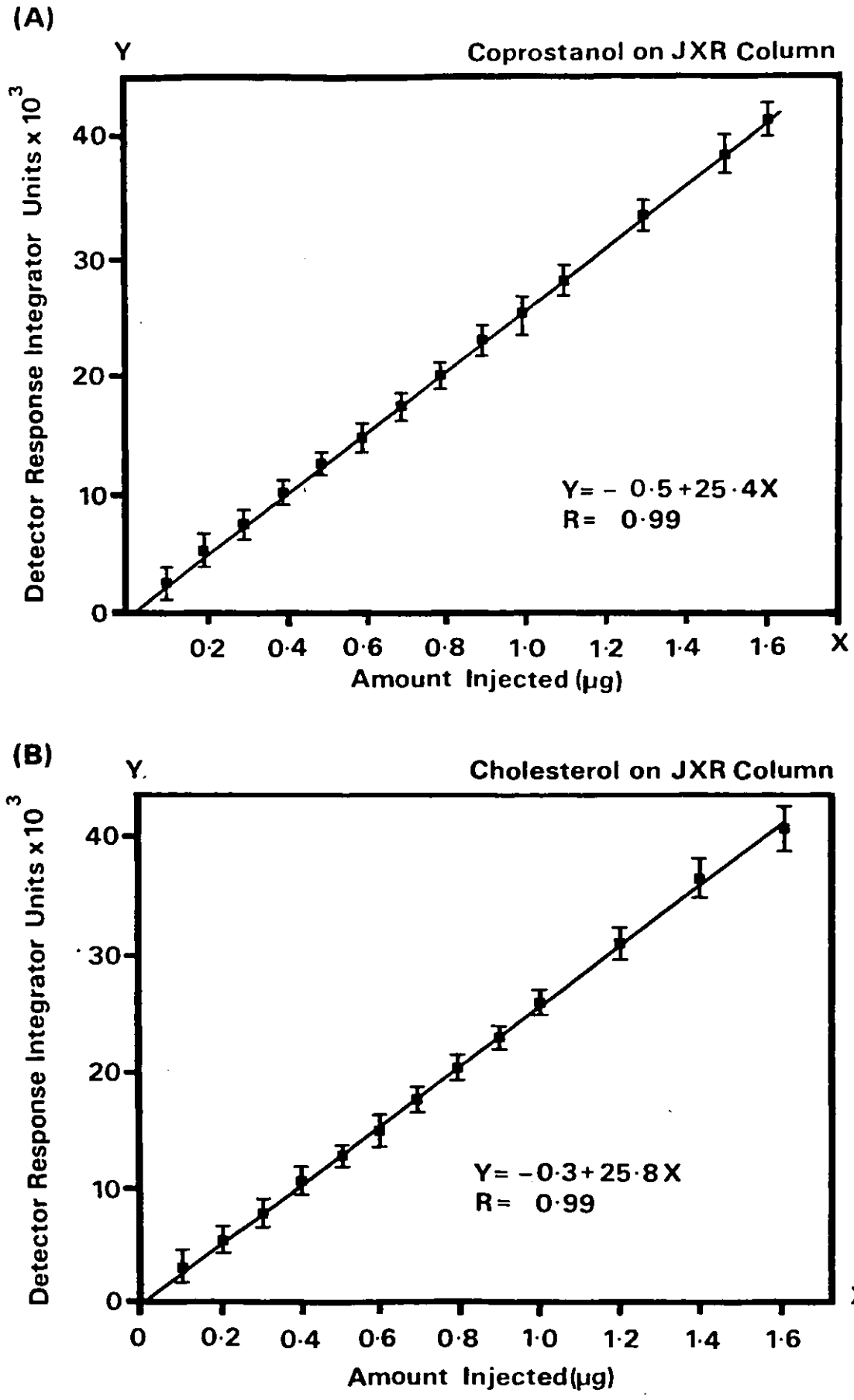


Figure 2.4 Circuit diagram for modified operation of Gallenkamp shaker. The desired mode of operation was achieved by wiring 3 timers with an interposing relay coil. The relay contacts (RC 1 and RC 2) allow Timer 2 and Timer 3 to be switched on and off after the pre set time. The details of the materials and the supplier are included in Appendix B.



Figures 2.5A and 2.5B Calibration curves relating the amount of neutral sterols injected on JXR columns and the detector response: A, coprostanol and B, cholesterol. GC conditions, as in Appendix F. Bars represent 95% confidence limits to the points on the calibration curve.

(C)

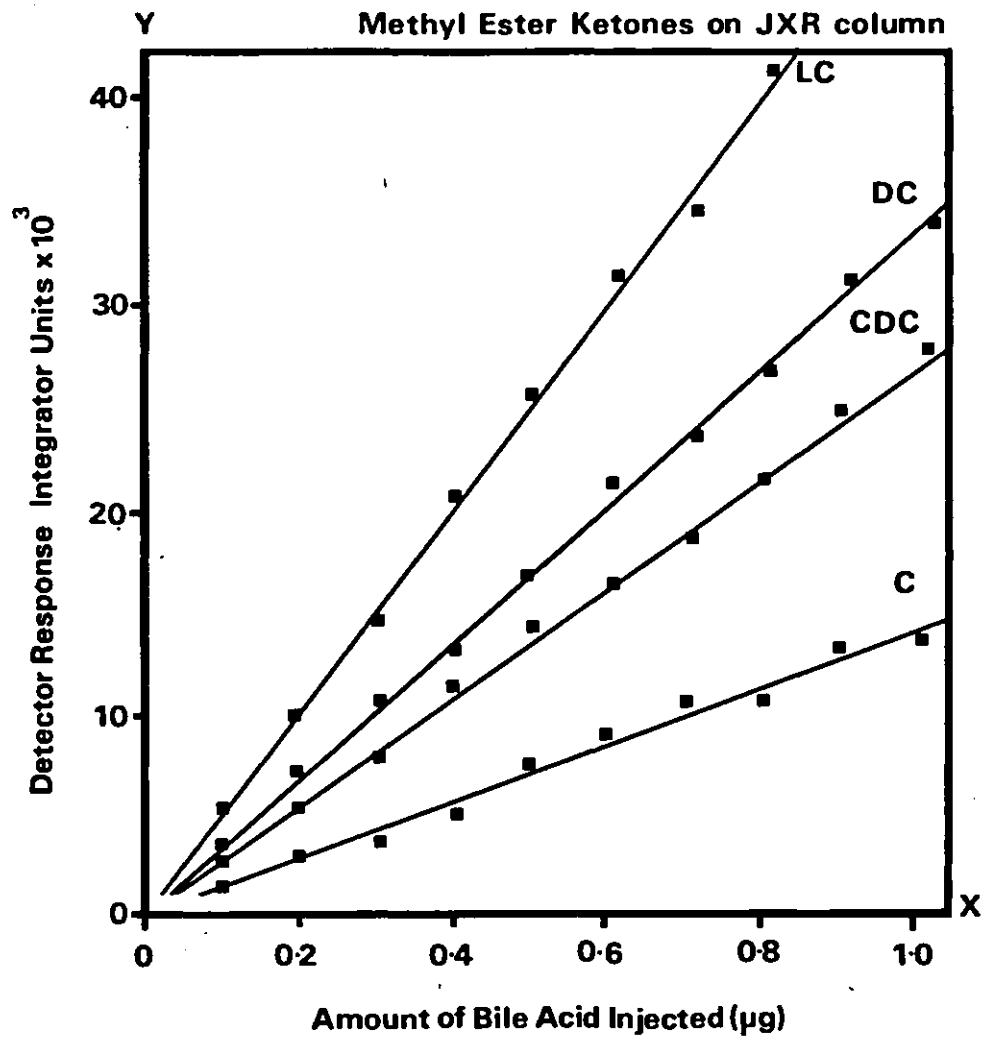
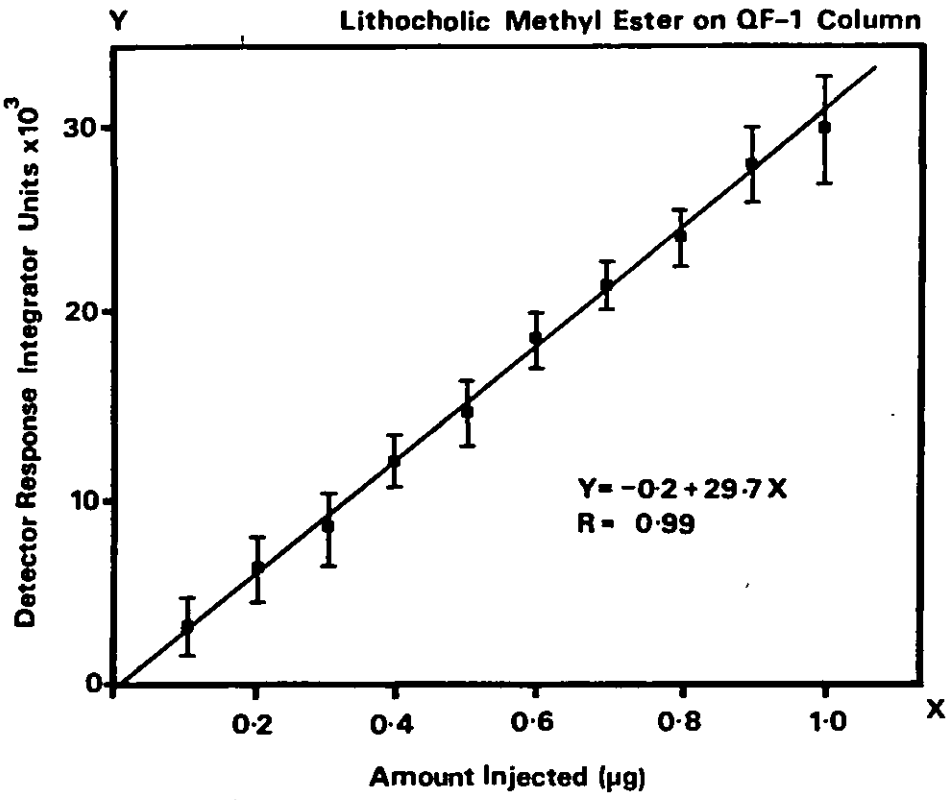


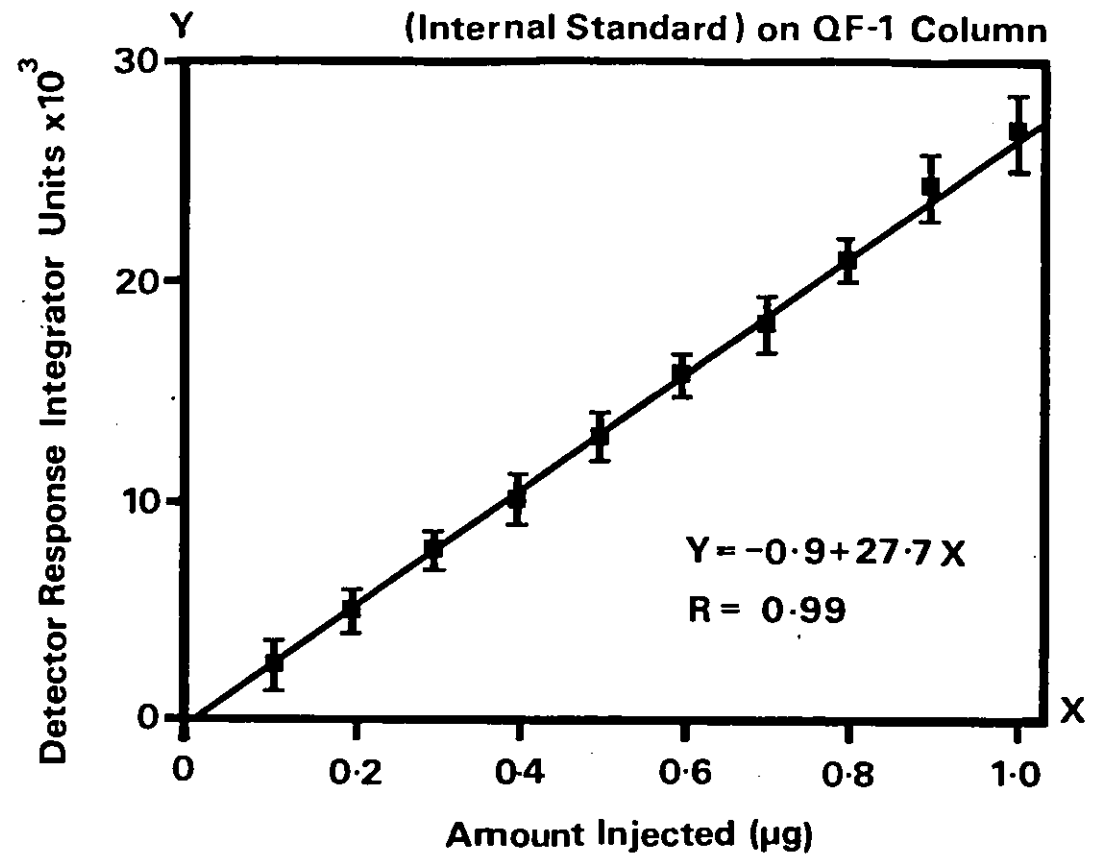
Figure 2.5C Calibration curves relating the amount of bile acid methyl ester ketones injected on JXR columns and the detector response. GC conditions, as in Appendix F.

(D)

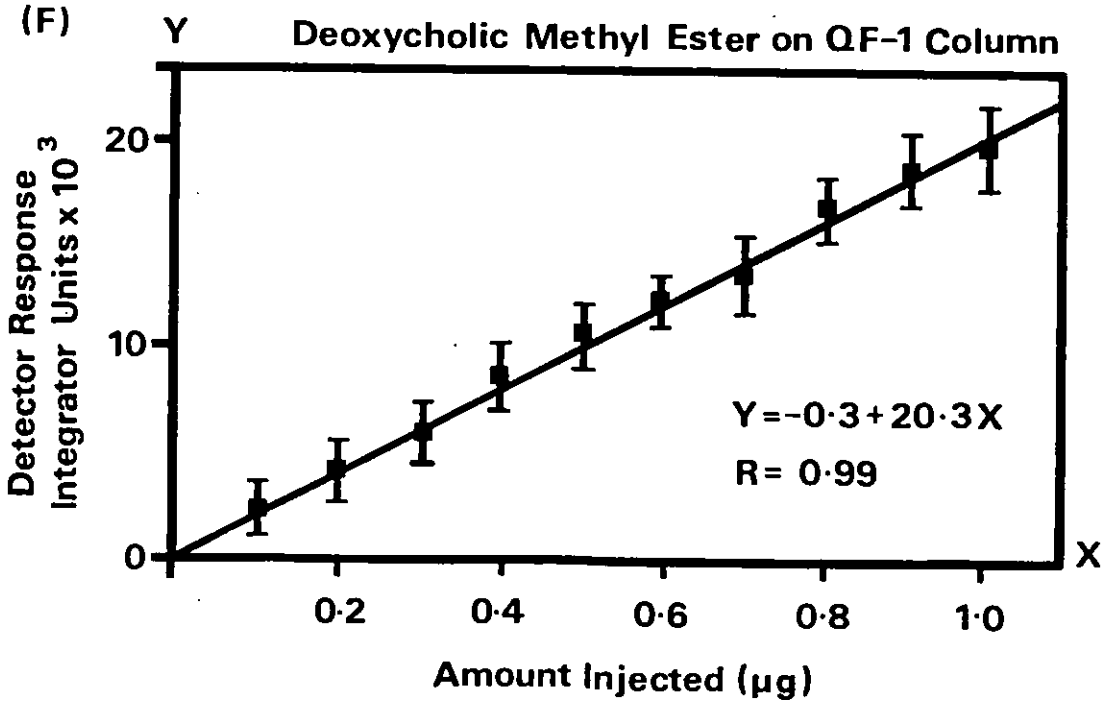


Figures 2.5D - 2.5H Calibration curves relating the amount of bile acid methyl esters injected on QF-1 columns and the detector response: D) LC; E) 23-Nor-DC; F) DC; G) CDC; H) C. GC conditions, as in Appendix F.

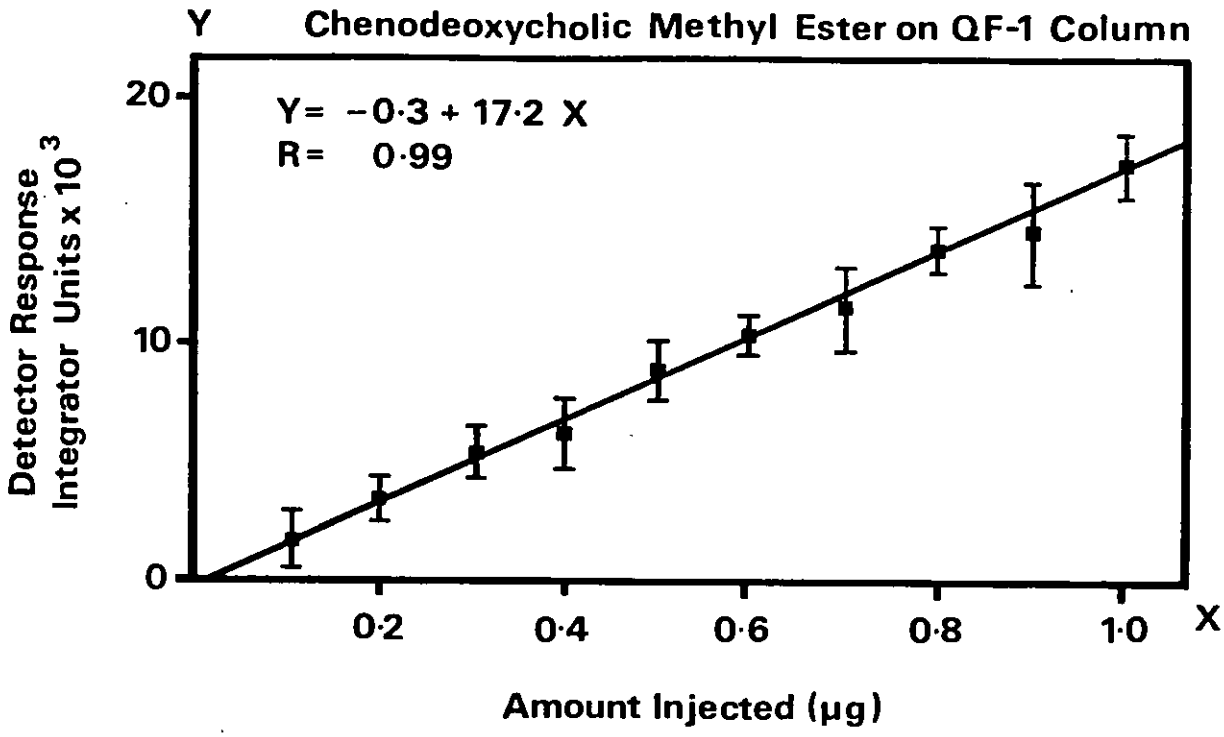
(E)


23-Nor-Deoxycholic Methyl Ester
(Internal Standard) on QF-1 Column
Y
X
Detector Response Integrator Units x10³
30
20
10
0
0
0·2
0·4
0·6
0·8
1·0
Y = -0·9+27·7 X
R = 0·99
Amount Injected (µg)

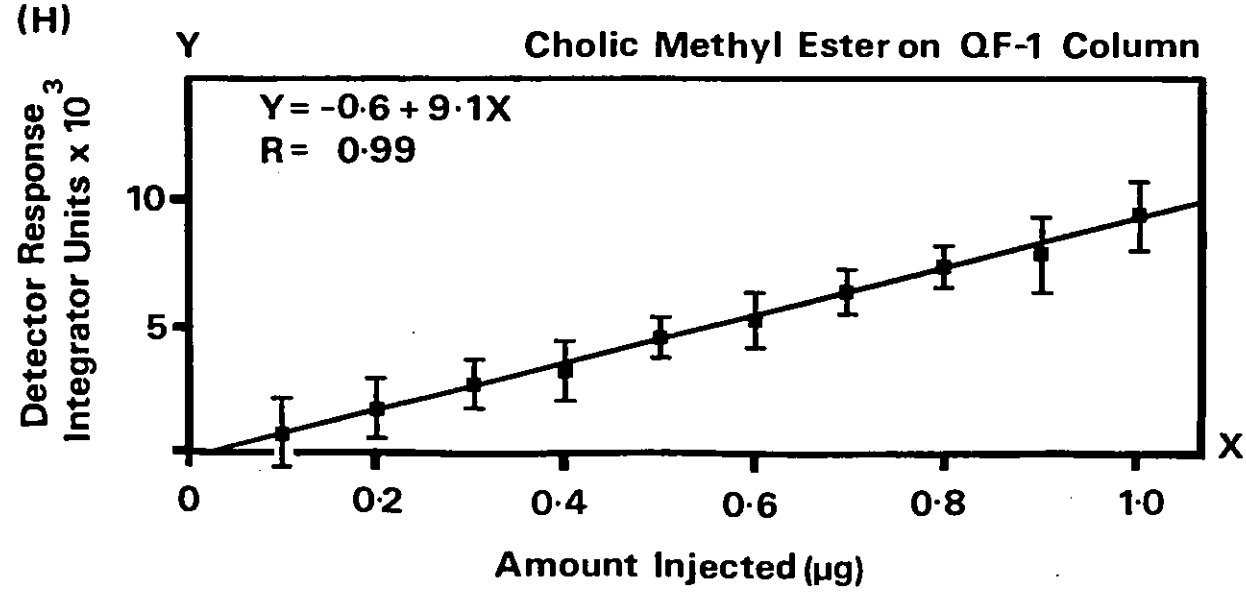
(F)


Deoxycholic Methyl Ester on QF-1 Column
Y
X
Detector Response Integrator Units x 10³
20
10
0
0·2
0·4
0·6
0·8
1·0
Y=-0·3+20·3X
R= 0·99
Amount Injected (µg)

(G)


Y
Chenodeoxycholic Methyl Ester on QF-1 Column
Y= −0·3 + 17·2 X
R= 0·99
Detector Response
Integrator Units x 10³
20
10
0
0·2
0·4
0·6
0·8
1·0
X
Amount Injected (µg)

(H)


Y
Cholic Methyl Ester on QF-1 Column
Y= -0·6 + 9·1X
R= 0·99
Detector Response
Integrator Units x 10³
10
5
0
0·2
0·4
0·6
0·8
1·0
X
Amount Injected (µg)

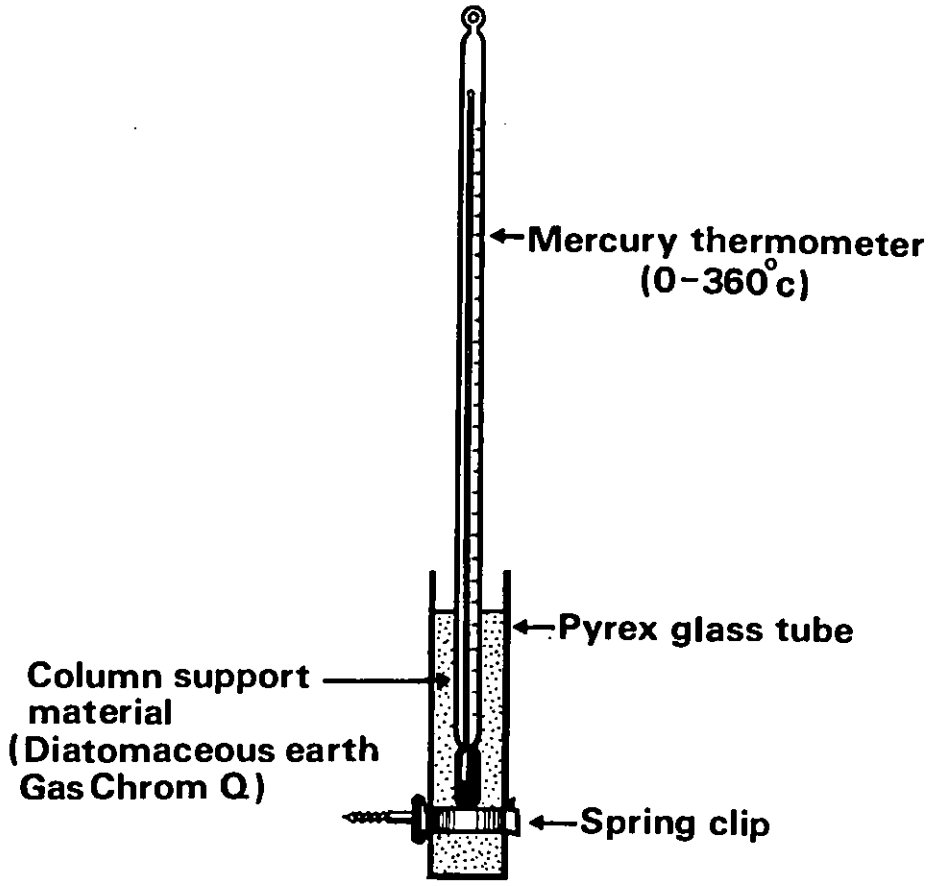


Figure 2.11 Device for correcting the temperature differential between the GC column and the column oven. This modification was suggested by Dr. G.S. King of Queen Charlotte Hospital.

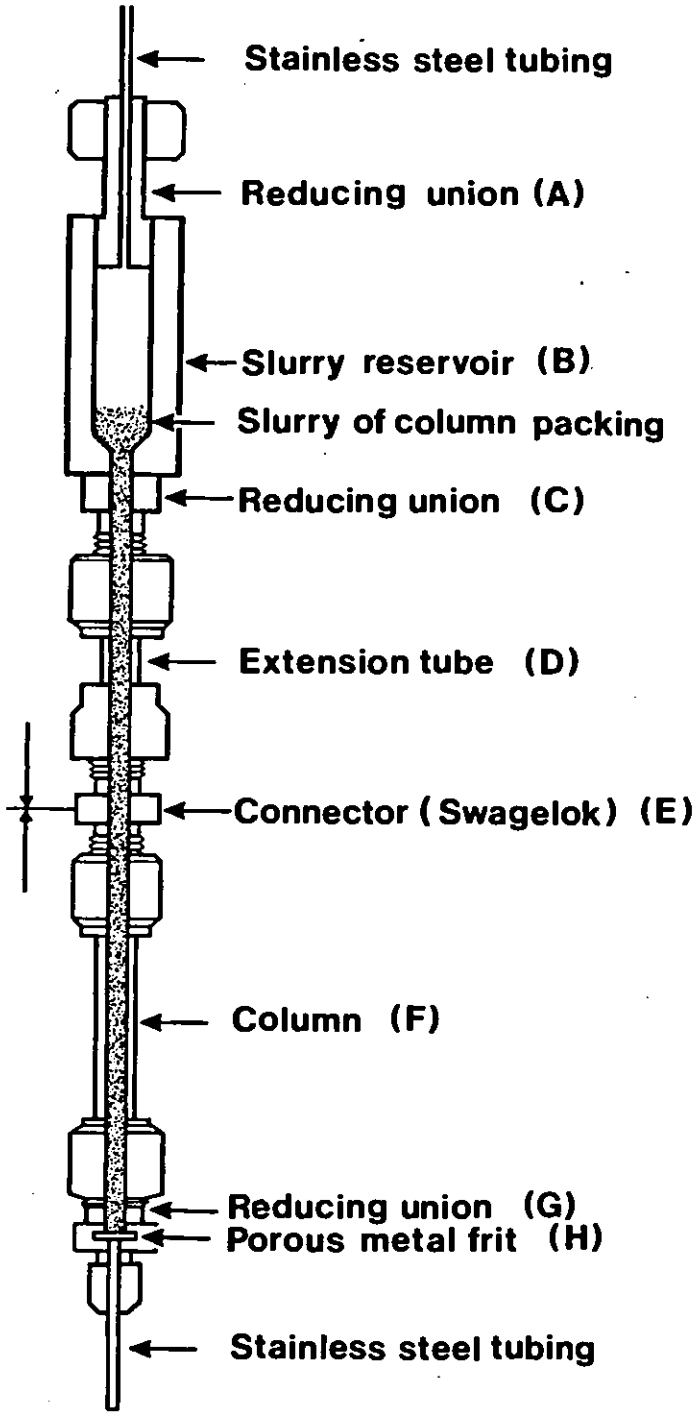


Figure 4.3 Schematic of the apparatus constructed for slurry-packing 'guard' columns: A) reducer, 6.35 mm to 1.5 mm; B) slurry-reservoir, 40 mm x 15 mm; C) reducer, 6.35 mm to 3.2 mm; D) tubing, stainless steel, 40 mm in length, 6.35 mm o.d., 3 mm i.d.; E) connector, 6.35 mm to 6.35 mm; F) column, 50 mm in length, 6.35 mm o.d., 3.0 mm i.d.; G) 'zero dead volume' reducer; H) metal frit, 6.35 mm o.d., mesh, 2 micron.

Table 2.1. Reproducibility of bile acid analysis of faecal samples freeze-dried and prepared by the present method

Sample	Apparent Bile Acid Content of Faeces (mg/g dry weight) Duplicate Estimates A	B	Difference (A-B) =D	Difference $(D)^2$
1	10.4	10.9	0.5	0.25
2	11.6	11.0	0.6	0.36
3	10.1	9.3	0.8	0.64
4	11.0	11.7	0.7	0.49
5	9.5	10.5	1.0	1.00
6	10.7	10.0	0.7	0.49

Mean $\pm$ SD = 10.6 $\pm$ 0.5 $\quad \Sigma D^2 = 3.23$

Coefficient of Variation = 5%

Bile acid content of 3-day stool (Subject, H.P.) was estimated as 'oxo' derivatives on JXR columns. Standard deviation was calculated from the difference between duplicate estimates (Snedecor, 1952).

Table 2.2 The effect of sample storage on bile acid content of faeces

Apparent Bile Acid Content of Faeces (mg/g dry weight)				
Replicate Sample	Fresh Sample (A)	9 weeks at -20°C (B)	1 year at -20°C (C)	3 years at +4 °C after freeze drying (D)
1	9.8	8.7	9.3	9.0
2	9.1	9.2	9.0	8.7
3	8.6	9.8	8.3	9.8
4	9.3	8.9	9.2	9.3
5	8.9	9.7	9.5	9.6
6	9.0	9.6	8.8	8.9
Mean ± SD =	9.1 ± 0.4	9.3 ± 0.4	9.0 ± 0.4	9.2 ± 0.4
Coefficient of Variation =	4.4%	4.3%	4.5%	4.4%

A 3-day stool collection was treated in four different ways and the apparent bile acid content estimated as for Table 2.1.

Table 2.5 Recovery of 23-Nor-Deoxycholic acid (internal standard) added to freeze-dried faeces

Faecal Sample	Amount of Internal Standard Added (mg)	Duplicate Estimates A	B	Recovered (mg) Mean	% Recovery
1	0.1	0.07	0.09	0.08	80
2	0.2	0.18	0.14	0.16	85
3	0.3	0.24	0.26	0.25	83
4	0.4	0.33	0.31	0.32	80
5	0.5	0.41	0.43	0.42	84
6	0.6	0.47	0.46	0.55	92
7	0.7	0.59	0.55	0.57	82
8	0.8	0.70	0.66	0.68	85
9	0.9	0.86	0.90	0.88	98
10	1.0	0.80	0.90	0.85	85
N = 20 duplicate estimates				Mean recovery = 85% $\pm$ 6 (SD)	

Known amounts of the internal standard were added to faecal samples, extracted, hydrolysed, methylated and estimated on QF-1 column as for bile acids. For controls, similar amounts of the internal standard were estimated directly after methylation.

2.6 Comparison of modified extraction procedure (Method A) with that of Evrard & Janssen's procedure (Method B)

Bile Acid Content of Faeces (mg/g dry weight)		
Faecal Sample	Method A	Method B
1	8.8	8.2
2	8.1	8.0
3	7.9	8.9
4	8.1	7.5
5	6.8	5.2
6	9.8	9.5
7	8.5	7.8
8	8.0	8.6
9	9.2	8.5
Mean $\pm$ SD = 8.4 $\pm$ 1.0		8.0 $\pm$ 1.0
Coefficient of Variation = 11.9%		12.5%

3-day stools were collected from a subject S.K. and the faecal extract prepared by 30 min semi-automated Method A and by 60 min (Evrard & Janssen, 1968) Method B.

Table 2.7 Reproducibility of bile acid estimations using the semi-automated procedure

Replicate Sample	Bile Acid Content of Faeces (mg/g dry weight)	Difference From Mean	%Difference From Mean
1	9.1	-0.3	-3.2
2	8.9	-0.4	-4.3
3	9.4	+0.1	+1.0
4	9.7	+0.4	+4.3
5	8.9	-0.4	-4.3
6	9.3	0.0	0.0
7	9.6	+0.3	+3.2
8	9.8	+0.5	+5.3

Mean $\pm$ SD = 9.3 $\pm$ 0.4

Coefficient of Variation = 4.3%

Replicate samples were taken from a 3-day stool collection and the bile acid content estimated as for Table 2.3

Table 2.8 Recovery of bile acids and bile salts after hydrolysis as estimated by GC of methyl esters

Bile acid/ Bile salt	No. of Estimates	% Recovery	Range (%)
TC	24	84	80 - 90
GLYC	"	81	80 - 88
LC	"	95	87 - 97
23-Nor-DC	"	93	85 - 96
DC	"	92	87 - 96
CDC	"	91	86 - 96
C	"	88	84 - 94

Mean Recovery after hydrolysis = 89% $\pm$5 (SD)

A stock solution was prepared for each compound (concn. 1 mg/ml) in AR methanol, and 0.1, 0.2, 0.3, 0.4, 0.5, to 1.2 mg was taken for duplicate estimations (12 x 2 = 24) Recoveries of bile acids were estimated by GC of methyl esters on QF-1 column.

Table 2.9 Same day variation in detector response to known quantities of coprostanol injected to a JXR column

Amount Injected (ug)	Detector Response ($\times 10^3$ Integrator Units) Mean (AM) A	Mean (PM) B	Difference (A-B)	% Difference $\frac{A-B}{A} \times 100$
0.1	20	21	+ 1	+ 5.0
0.2	47	50	+ 3	+ 6.3
0.3	72	70	- 2	- 2.8
0.4	102	104	+ 2	+ 2.0
0.5	104	108	+ 4	+ 3.8
0.6	140	138	- 2	- 1.4
0.7	167	163	- 4	- 2.4
0.8	190	196	+ 6	+ 3.2
0.9	219	224	+ 5	+ 2.3
1.0	243	239	- 4	- 1.6
1.1	263	267	+ 4	+ 1.5
1.3	322	317	- 5	- 1.6
1.5	379	373	- 6	- 1.6
1.6	404	400	- 4	- 1.0
m =	254	250.0		
b =	- 9.0	- 6.0	$Y = m X + b$ *	
R =	0.99	.0.99		

* m = slope of regression line, b = Y-intercept, R = correlation coefficient.

Table 2.10 Retention times of neutral steroids analysed on three different stationary phases

Steroid	3% QF-1		1% JXR		3% SP2250	
	R_t (min)	RR_t	R_t (min)	RR_t	R_t (min)	RRt
5 α-cholestane	3.29	0.37	5.43	0.52	4.04	0.45
coprostanol	7.73	0.88	9.51	0.91	7.81	0.8
epicoprostanol	8.50	0.97	9.45	0.90	7.76	0.87
cholestanol	9.41	1.07	10.70	1.02	8.84	0.94
cholesterol	8.81	1.00	10.49	1.00	8.88	1.00
stigmasterol	11.74	1.33	15.24	1.45	12.30	1.38
lanosterol	12.63	1.43	16.20	1.54	12.47	1.40
β-sitosterol	13.74	1.56	17.69	1.69	13.89	1.56
coprostanone	16.38	1.86	10.36	0.99	8.93	1.01
cholestanone	17.90	2.03	11.24	1.07	10.27	1.16

*column temperature: QF-1. 220 °C; JXR, 222°C; SP 2250, 270°C

Table 2.11 Relative retention times (RR_t) of bile acid methyl esters and trifluoracetates (TFA) analysed on 3% QF-1 columns and compared with RR_t reported by Eneroth & Sjovall (1971)

Bile Acid	Substituents	Methyl Esters R_t (mins)	RR_t (found)	RR_t (ex Eneroth)	Trifluoracetates (TFA) R_t (mins)	RR_t (found)	RR_t (ex Eneroth)
ILC	3β	5.4	0.48	0.48	10.8	0.42	0.46
LC	3α	6.0	0.54	0.53	12.9	0.44	0.47
23-Nor-DC	3α, 12α	8.0	0.73	--	14.0	0.48	--
	3β, 12α	9.4	0.87	0.88	16.8	0.57	0.58
	3β, 7α	11.6	0.95	0.96	22.7	0.70	0.69
DC	3α, 12α	10.8	(1.00)	(1.00)	19.2	0.65	0.68
CDC	3α, 7α	12.2	1.13	1.13	24.8	0.84	0.83
UDC	3α, 7β	13.3	1.23	1.22	27.7	0.94	0.95
	3β, 12 keto	16.6	1.33	1.34	36.0	1.22	1.23
HDC	3α, 6α	15.8	1.46	1.47	27.1	0.92	0.93
	3α, 12 keto	16.6	1.54	1.57	40.4	1.37	1.38
	3β, 7α, 12α	19.4	1.80	1.81	28.0	0.95	0.95
C	3α, 7α, 12α	23.0	2.13	2.14	37.8	1.28	1.29

Relative retention times (RR_t) of bile acid methyl esters are calculated with respect to the retention time (R_t) of DC (i.e. $RR_{t(DC)} = 1.00$); $R_{t(DC)} = 10.80$ min; column temperature, 240°C for methyl esters and 230°C for TFA derivatives.

Table 2.12 Relative retention times (RR_t) of bile acid methyl esters and other derivatives analysed on non-polar JXR and SP 2250 columns

Bile Acid	Substituents	1% JXR Methyl Esters R_t (mins)	RR_t	'oxo' Derivatives R_t (mins)	RR_t	3% SP 2250 Methyl Esters R_t (mins)	RR_t	'oxo' Derivatives R_t (mins)	RR_t
LC	3α	7.2	0.66	6.2	0.56	6.1	0.57	4.3	0.40
23-Nor-DC	3α, 12α	7.8	0.72	6.8	0.62	7.8	0.73	5.2	0.49
DC	3α, 12α	10.9	(1.00)	9.2	0.84	10.7	(1.00)	6.0	0.56
CDC	3α, 7α	10.9	1.00	9.2	0.84	12.0	1.13	7.8	0.73
UDC	3α, 7β					11.3	1.06		
HDC	3α, 6α					13.9	1.28		
C	3α, 7α, 12α	19.0	1.74	12.0	1.10	21.0	1.97	11.9	1.11

Relative retention times (RR_t) are calculated with respect to the retention time (R_t) of DC (i.e. $RR_{t\ (DC)} = 1.00$); column temperature, JXR: 250°C for methyl esters and 240°C for 'oxo' derivatives; SP 2250: 280°C for methyl esters and for 'oxo' derivatives.

Table 2.14 Estimation of neutral sterol content of faeces analysed before and after oxidation

Subject	Neutral Sterol Content of Faeces (mg/g dry weight) Before Oxidation	After Oxidation
1	28.4	29.2
2	20.3	20.8
3	25.8	26.2
4	24.2	24.0
5	27.5	28.4
6	31.6	32.7
Mean ± SD =	26.3 ± 3.9	26.9 ± 4.2

Faecal extracts from 3-day stool used in Table 2.13 were analysed directly and after oxidation using JXR columns.

Table 2.18 Estimation of faecal bile acids before and after the preparation of 'oxo' derivatives

Bile Acid Content of Faeces (mg/g dry weight)		
Subject	Methyl Esters	'oxo' Derivatives
1	9.2	9.6
2	8.9	9.1
3	8.8	9.3
4	9.1	8.9
5	10.0	10.4
6	10.2	10.6
Mean ± SD =	9.4 ± 0.6	9.7 ± 0.7

3-day stools were collected from six subjects. Bile acid content of faeces was estimated as methyl esters on QF-1 columns and as 'oxo' derivatives on JXR columns.

TABLE 3.3 Data on subjects who completed fenfluramine study

Subject	Sex	Age (yrs)	Body Weight (kg) Actual	Normal *	% Overweight
1	F	25	65	60	
2	M	30	60	60	
3	F	24	59	60	
4	M	30	64	58	
5	F	24	89	59	50
6	F	40	96	60	60
7	F	25	63	59	
8D	M	28	134	74	80
9	F	23	60	60	
10	F	28	61	60	
11	F	25	75	65	15
12	M	60	107	82	30
13	F	60	71	59	20
14D	F	28	67	63	
15	F	36	90	60	50
16D	F	60	70	54	30
Mean ± SD =		34 ± 13.6	77 ± 21	62 ± 7	43 ± 22 (N=8)

*Normal weight calculated according to New Weight Standards for Men and Women (Statistical Bulletin, 1959). Numbers marked with D indicate subjects who had a diarrhoea when treated with fenfluramine.

Table 3.5 Comparison of bile acid and neutral sterol excretion in Men and Women with 'normal weight'*

Subject	Body Weight (kg)	Bile Acids	Neutral Sterols (mg/day)	Total Steroids	Bile Acids	Neutral Sterols (mg/kg/day)	Total Steroids
Men							
1	64	307	507	814	4.8	7.9	12.7
2	60	231	515	746	3.9	8.6	11.7
3	59	281	620	901	4.8	10.5	15.3
4	61	290	540	830	4.8	8.8	13.6
5	68	321	590	911	4.7	8.7	13.4
Mean ± SD =	62 ± 4.2	286 ± 34	554 ± 49	840 ± 68	4.6 ± 0.4	8.9 ± 1.6	13.3 ± 1.3
Women							
6	65	175	380	555	2.7	5.8	8.5
7	59	226	430	656	3.8	7.3	11.1
8	63	183	358	541	2.9	5.7	8.6
9	60	215	540	755	3.6	9.0	12.6
10	67	285	401	686	4.3	6.0	10.2
Mean ± SD =	63 ± 3.3	217 ± 44	422 ± 71	639 ± 90	3.5 ± 0.7	6.8 ± 1.4	10.2 ± 1.7

*The 'normal weight' for each subject was based on New Weight Standards for Men and Women (Statistical Bulletin, 1959)

Table 3.6 Bile Acid and neutral sterol excretion in subjects (Men and Women) with 'normal weight'*

Subject No.	Sex	Body Weight (kg)	Bile Acids	Neutral Sterols (mg/day)	Total Steroids	Bile Acids	Neutral Sterols (mg/kg/day)	Total Sterols
1	F	65	251	583	834	3.90	8.9	12.8
2	M	60	231	515	746	3.95	8.6	12.4
3	F	59	226	430	656	3.8	7.3	11.1
7	F	63	183	358	541	2.9	5.7	8.6
9	F	60	215	540	755	3.6	9.0	12.6
10	F	61	175	380	555	2.9	6.2	9.1
14	F	67	285	401	686	4.3	6.0	10.2
4	M	64	307	507	814	4.8	7.9	12.7
Mean ± SD =		62 ± 2.5	234 ± 40	464 ± 73	698 ± 97	3.8 ± 0.6	7.5 ± 1.1	11.2 ± 1.5

*The 'normal weight' for each subject was calculated as for Table 3.4

Table 3.7 Bile acid and neutral sterol excretion in 'overweight subjects'* (Men and Women)

Subject No.	Sex	Body Weight (kg)	% Overweight	Bile Acids	Neutral Sterols (mg/day)	Total Steroids	Bile Acids	Neutral Sterols (mg/kg/day)	Total Steroids
5	F	89	50	501	912	1413	5.6	10.2	15.87
6	F	96	60	397	728	1125	4.1	7.6	11.7
8	M	134	80	524	900	1424	3.9	6.7	10.6
11	F	75	15	289	672	961	3.8	9.0	12.8
12	M	107	30	357	825	1182	3.3	7.7	11.0
13	F	71	20	270	667	937	3.8	9.4	13.2
15	F	90	50	340	858	1198	3.8	9.5	13.3
16	F	70	30	348	565	913	5.0	8.1	13.0
Mean ± SD =		92.± 19	42 ± 22	378 ± 92	766 ± 114	1144±178	4.2 ± 0.7	8.5 ± 10	12.7 ± 1.5

*The 'normal weight' for each subject was calculated as for Table 3,4

Table 4.1 Retention times of bile salts analysed on Partisil-10 ODS and μ Bondapak FAA columns

Bile Salt	Partisil-10 ODS (HPLC System II) $R_{t(TDC)}$= 12.84 min R_t (min)	RR_t	μ Bondapak FAA (HPLC System III) $R_{t(TDC)}$= 18.20 min R_t (min)	RR_t
TUDC	6.68	0.52	10.19	0.56
TC	6.93	0.54	9.46	0.52
TCDC	11.56	0.90	15.65	0.86
TDC	12.84	1.00	18.20	1.00
TL	22.08	1.72	31.85	1.75
GUDC	13.61	1.06	19.29	1.06
GLYC	8.22	0.64	13.47	0.74
GCDC	15.41	1.20	26.21	1.44
GDC	16.95	1.32	30.90	1.70
GL	27.48	2.14	50.96	2.80

Relative retention times (RR_t) are calculated with respect to the retention time of TDC (i.e $RR_{t\ (TDC)}$ = 1.00) TUDC, tauroursodeoxycholate;TL,taurolithocholate;GUDC, glycoursodeoxycholate; GL, glycolithocholate.

Table 4.6 Reproducibility of 'peak height response' of Refractive Index (RI) detector

HPLC RUN: Bile Acid	Refractive Index detector response (peak height in mm) 1	2	3	4	5	6	Mean	± S.D.	% CV
LC	117	115	113	112	114	116	115	1.9	1.7
DC	82	84	85	86	83	88	85	2.2	2.6
CDC	60	62	61	64	62	63	62	1.4	2.3
C	30	29	32	29	31	30	30	1.2	4.1

Chromatographic conditions for Micro Pak columns as in text (4.6)
CV = coefficient of variation

Table 4.7 Reproducibility of retention times of bile acids analysed on Varian Micro Pak columns

Injection	Retention Times (R_t) of Bile Acids (min) LC	DC	CDC	C
1	1.82	3.72	4.21	7.55
2	1.78	3.72	4.26	7.53
3	1.80	3.80	4.22	7.60
4	1.75	3.76	4.28	7.54
5	1.84	3.74	4.30	7.56
6	1.76	3.78	4.24	7.52
Mean ± SD =	1.79±0.03	3.75±0.03	4.25 ± 0.04	7,55 ± 0.03
Coefficient of Variation =	1.7%	0.9%	1.0%	<1.0%

The details of chromatographic conditions are described in Chapter 4.6

Table 4.8 The effect of changes in mobile phase flow rate on the separation of bile acids on Varian Micro Pak columns

Bile Acid	Flow ml/min	R_t (min)	RR_t DC = 1.0	R_s DC:CDC	N_{DC}	HETP (H)
LC	1.50	2.20	0.52			
DC		4.20	1.00			
CDC		4.74	1.13	0.93	1128	0.22
LC	1.70	2.00	0.50			
DC		4.00	1.00			
CDC		4.40	1.08	0.88	1024	0.24
LC	1.83	1.70	0.46			
DC		3.70	1.00			
CDC		4.10	1.10	0.80	876	0.29
LC	2.00	1.50	0.42			
DC		3.60	1.00			
CDC		3.90	1.08	0.62	829	0.30

Column efficiency was measured in terms of 'plate number' (N) and HETP (H) using the equations i) $N = 16\left(\frac{R_t}{w}\right)^2$ where, N is the number of theoretical plates; and R_t, the retention time in minutes and w, base line peak width also in minutes; ii) HETP (H) = $\frac{L}{N}$ where, H is the height equivalent to a theoretical plate in mm; L, the length of column in mm (Baumann, 1972).

Table 4.9 The effect of changes in mobile phase composition on the separation of bile acids on Varian Micro Pak columns

Mobile Phase Ratio:	Bile Acid	R_t (min)	RR_t (DC = 1.0)	R_s DC:CDC	N_{DC}	HETP (H)
(Experiment 1) 47.5:47.5:5	LC	1.60	0.55			
	DC	2.90	1.00			
	CDC	3.20	1.10	0.67	538	0.47
(Experiment 2) 55:45:5	LC	1.70	0.47			
	DC	3.66	1.00			
	CDC	4.14	1,13	1.0	857	0.29
(Experiment 3) 60:35:5	LC	2.70	0.57			
	DC	4.70	1.00			
	CDC	6.40	1.36	2.6	1414	0.18

The resolution R_s (a measure of the 'degree of separation') of DC and CDC was calculated using the equation: $R_s = \frac{\Delta R_t}{w}$ where, ΔR_t is the difference in the retention time R_t of two peaks (i.e. $R_{t1}-R_{t2}$) and w, is the average peak width (i.e. $W_1 + W_2$) x ½ (Baumann, 1972). The column efficiency (N) and HETP, height equivalent to a theoretical plate were measured as in Table 4.7. The relative retention times (RR_t) are based on the retention time of DC.

Table 5.3A Concentration of bile salts present in bile obtained from gall bladders containing gallstone ('gallstone bile')

Sample No.	Taurine conjugates (mg/ml)				Glycine conjugates (mg/ml)				Total Bile Salts mg/ml
	TC	TCDC	TDC	Total T	GLYC	GCDC	GDC	Total G	
1	5.5	3.0	1.5	10.0	15.0	13.0	4.5	32.5	42.5
2	8.0	7.0	2.0	17.0	7.0	10.0	5.0	21.5	39.0
3	4.0	1.8	0.8	6.6	7.2	6.0	5.0	18.2	24.8
4	2.2	2.6	2.2	7.0	5.4	3.6	4.8	13.8	20.8
5	4.0	4.5	4.0	12.5	6.5	6.5	6.0	19.0	31.5
6	3.0	2.7	1.8	7.5	5.0	4.6	4.4	14.0	21.5
7	3.0	1.5	2.5	7.0	6.3	5.3	3.5	15.1	22.1
8	5.2	1.8	1.0	8.0	7.6	4.8	2.8	15.2	23.2
9	6.5	1.5	3.0	11.0	12.5	1.0	3.5	17.0	28.0
20	6.5	4.5	4.0	15.0	12.0	8.5	8.0	28.5	43.5
11	7.2	2.2	2.0	11.4	5.6	5.0	6.2	16.8	28.2
12	5.4	0.3	0.6	6.3	1.4	3.0	3.2	7.6	13.9
13	3.8	4.6	2.0	10.4	7.3	4.4	5.2	16.9	27.3
14	7.6	2.8	2.4	12.8	6.8	6.8	8.0	21.6	34.4
15	5.2	2.2	1.2	8.6	7.6	3.6	2.0	13.2	21.8
16	3.6	5.2	1.6	10.4	7.8	7.8	3.9	19.5	29.9
Mean =	5.0	3.0	2.0	10.0	7.6	5.9	4.8	18.2	28.2
SD =	±1.8	±1.7	±1.0	±3.1	±3.2	±2.9	±1.7	±5.9	±8.3
Range:	(2-8)	(0.3-7)	(0.6-4)	(6-17)	(1-15)	(1-13)	(2-8)	(7-33)	(13-44)

T = taurine conjugates; G.= glycine conjugates

Table 5.3B Concentration of bile salts present in bile obtained from gall bladders without gallstones (post mortem 'non-gallstone bile')

Sample No.	Taurine conjugates (mg/ml) TC	TCDC	TDC	Total T	Glycine conjugates (mg/ml) GLYC	GCDC	GDC	Total G	Total Bile Salts (mg/ml)
1	12.8	9.6	4.0	26.4	12.8	7.2	3.2	23.2	49.6
2	9.3	8.1	3.6	21.0	10.2	6.3	3.6	20.1	41.1
3	8.1	6.6	2.1	16.8	6.6	9.0	5.1	20.7	37.5
4	8.0	8.2	2.8	19.0	8.2	6.0	4.0	18.2	37.2
5	8.4	11.7	5.1	25.2	12.9	9.0	6.0	27.9	53.1
6	10.4	12.0	4.4	26.8	8.8	10.0	2.4	21.2	48.0
7	9.9	8.1	2.4	20.4	14.4	7.5	4.5	26.4	46.8
8	13.2	7.2	7.2	27.6	10.8	6.0	6.6	23.4	51.0
9	12.0	9.6	1.2	22.8	10.2	6.0	2.7	18.9	41.7
10	12.0	7.6	2.4	22.0	6.8	3.6	2.4	12.8	34.8
11	9.6	13.2	2.8	25.6	11.2	16.8	6.0	34.0	59.6
12	9.2	11.2	2.4	22.8	11.2	8.0	4.0	23.2	46.0
13	9.0	10.2	1.5	20.7	11.1	8.7	1.5	21.3	42.0
14	13.6	15.6	3.6	32.8	11.2	10.8	3.6	25.6	58.2
15	7.8	9.0	1.8	18.6	16.2	14.4	3.0	33.6	52.2
16	5.9	8.4	1.6	15.9	16.1	9.3	3.1	28.5	44.4
Mean =	9.9*	9.8*	3.1*	22.8*	11.2*	8.7*	3.8	23.8*	46.6*
SD =	±2.2	±2.4	±1.6	±4.4	±2.9	±3.3	±1.5	±4.9	±7.3
Range:	(6-14)	(7-16)	(1-7)	(16-33)	(7-16)	(4-15)	(2-7)	(13-24)	(35-60)
	P<0.001	P<0.001	P<0.05	P<0.001	P<0.01	P<0.02	N.S.	P<0.02	P<0.001

*Significantly different from the concentration found in 'Gallstone Bile' (Table 5.3A); N.S. = not significant

Table 5.4 Glycine to taurine (G:T) ratio of bile salts present in 'gallstone bile' and in 'non-gallstone bile'

Sample No.	'Gallstone Bile' G (mg/ml)	T (mg/ml)	G:T Ratio	Sample No.	'Non-gallstone Bile' G (mg/ml)	T (mg/ml)	G:T Ratio
1	32.5	10.0	3.25	1	23.2	26.4	0.88
2	21.5	17.0	1.27	2	20.1	21.0	0.96
3	18.2	6.6	2.76	3	20.7	16.8	1.23
4	13.8	7.0	1.97	4	18.2	19.0	0.96
5	19.0	12.5	1.52	5	27.9	25.2	1.04
6	14.0	7.5	1.87	6	21.2	26.8	0.80
7	15.1	7.0	2.16	7	26.4	20.4	1.29
8	15.2	8.0	1.90	8	23.4	27.6	0.85
9	17.0	11.0	1.55	9	18.9	22.8	0.83
10	28.5	15.0	1.90	10	12.8	22.0	0.58
11	16.8	11.4	1.47	11	34.0	25.6	1.33
12	7.6	6.3	1.21	12	23.2	22.8	1.02
13	16.9	10.4	1.63	13	21.3	20.7	1.03
14	21.6	12.8	1.70	14	25.6	32.8	0.78
15	13.2	8.6	1.54	15	33.6	18.6	1.81
16	19.5	10.4	1.90	16	28.5	15.9	1.79
Mean =			1.85*	N=16			1.07
SD =			± 0.53				±0.34
			P < 0.001				

*Significantly different from theG:T ratio found in 'Non-gallstone Bile'

Table 5.6 Tri-hydroxy to Di-hydroxy (T:D) ratio of bile salts present in 'gallstone bile' and in 'non-gallstone bile'

'Gallstone Bile'				'Non-gallstone Bile'			
Sample No.	C (mg/ml)	CDC + DC	T:D Ratio	Sample No.	C (mg/ml)	CDC + C	T:D Ratio
1	20.5	22.0	0.93	1	25.6	24.0	1.10
2	15.0	24.0	0.63	2	19.5	21.6	0.90
3	11.2	13.6	0.82	3	14.7	22.8	0.65
4	7.6	13.2	0.58	4	16.2	21.0	0.77
5	10.5	21.0	0.50	5	21.3	31.7	0.67
6	8.0	13.5	0.59	6	19.2	28.8	0.67
7	9.3	12.8	0.73	7	24.3	22.5	1.10
8	12.8	10.4	1.23	8	24.0	27.0	0.89
9	19.0	9.0	2.10	9	22.2	19.5	1.14
10	18.5	25.0	0.74	10	18.8	16.0	1.16
11	12.8	15.4	0.83	11	20.8	35.4	0.59
12	6.8	7.1	0.96	12	20.4	25.6	0.80
13	11.1	14.2	0.78	13	20.1	21.9	0.92
14	14.4	20.0	0.72	14	24.8	33.6	0.74
15	12.8	9.6	1.33	15	24.0	28.2	0.85
16	11.4	18.5	0.62	16	22.0	22.4	0.98
Mean =			0.86*	N=16			0.88
SD =			±0.39				±0.18
Range:			(0.5-211)				(0.6-1.2)

*Not significantly different from the T:D ratio found in 'Non-gallstone Bile'

Table 5.8 Primary bile salt (C:CDC) ratio in 'gallstone bile' and in 'non-gallstone bile'

'Gallstone Bile'				'Non-gallstone Bile'			
Sample No.	C (mg/ml)	CDC	C:CDC	Sample No.	C (mg/ml)	CDC	C:CDC
1	20.5	16.0	1.28	1	25.6	16.8	1.52
2	15.0	17.0	0.88	2	19.5	14.4	1.35
3	11.2	7.8	1.44	3	14.7	15.6	0.94
4	7.6	6.2	1.23	4	16.2	14.2	1.14
5	10.5	11.0	0.96	5	21.3	20.7	1.03
6	8.0	7.3	1.10	6	19.2	22.0	0.87
7	9.3	6.8	1.37	7	24.3	15.6	1.56
8	12.8	6.6	1.94	8	24.0	13.2	1.82
9	19.0	2.5	7.60	9	22.2	15.6	1.42
10	18.5	13.0	1.42	10	18.8	11.2	1.68
11	12.8	7.2	1.78	11	20.8	30.0	0.69
12	6.8	3.3	2.06	12	20.4	19.2	1.06
13	11.1	9.0	1.23	13	20.1	18.9	1.06
14	14.4	9.6	1.50	14	24.8	26.4	0.94
15	12.8	5.8	2.20	15	24.0	23.4	1.03
16	11.4	13.0	0.88	16	22.0	17.7	1.24
Mean =			1.80*	N=16			1.20
SD =			±1.60				±0.32
Range:			(0.9-8)				(0.7-2)

*Not significantly different from the C:CDC ratio found in "Non-gallstone Bile'

Table 5.10 Concentration of C, CDC and DC Bile salts in 'gallstone bile' and in 'non-gallstone bile'

Sample No.	"Gallstone Bile' C	CDC (mg/ml)	DC
1	20.5	16.0	6.0
2	15.0	17.0	7.0
3	11.2	7.8	5.8
4	7.6	6.2	7.0
5	10.5	11.0	10.0
6	8.0	7.3	6.2
7	9.3	6.8	6.0
8	12.8	6.6	3.8
9	19.0	2.5	6.5
10	18.5	13.0	12.0
11	12.8	7.2	8.2
12	6.8	3.3	3.8
13	11.1	9.0	5.2
14	14.4	9.6	10.4
15	12.8	5.8	3.8
16	11.4	13.0	5.5
Mean =	12.6*	8.9*	6.7
SD =	±4.1	±4.2	±2.4
	P<0.001 (45%)	P<0.001 (31%)**	(24%)**

Sample No.	'Non-gallstone Bile' C	CDC (mg/ml)	DC
1	25.6	16.8	7.2
2	19.5	14.4	7.2
3	14.7	15.6	7.2
4	16.2	14.2	6.8
5	21.3	20.7	11.1
6	19.2	22.0	6.8
7	24.3	15.6	6.9
8	24.0	13.2	13.8
9	22.2	15.6	3.9
10	18.8	11.2	4.8
11	20.8	30.0	5.4
12	20.4	19.2	6.4
13	20.1	18.9	3.0
14	24,8	26.4	7.2
15	24.0	23.4	4.8
16	22.0	17.7	4.7
N = 16	21.2	18.4	6.7
	±3.1	±5.0	±2.7
	(46%)	(40%)	(14%)

* Significantly different from the concentration found in 'Non-gallstone Bile'

** Significantly different from the percentage composition found in 'Non-gallstone Bile'

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THE APPLICATION OF HIGH PRESSURE LIQUID CHROMATOGRAPHY TO THE ANALYSIS OF BILE SALTS IN HUMAN BILE

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Summary

Two high pressure liquid chromatography (HPLC) systems have been evaluated for the separation of conjugated bile salts from human bile. Partisil-10 ODS with mobile phase of methanol/water pH 2 (55 : 45) at 200 nm, 0.1 AUF UV detection gave only partial separation of bile salts. However, a μ Bondapak fatty acid analysis column using isopropanol/8.8 mmol/l potassium phosphate pH 2.5 (32 : 68) as the mobile phase and 193 nm, 0.1 AUF UV detection separated all the six conjugated bile salts in bile. The limit of detection ranged from 0.1 μg for sodium taurocholate to 0.2 μg for sodium glycodeoxycholate.

The reproducibility and the application of the method to the analysis of conjugated bile salts was demonstrated using bile from five patients. Its application to the studies of hepato-biliary disease is discussed.

Introduction **

The metabolism of bile salts has attracted a great deal of attention over the last two decades in connection with hepato-biliary disease. There is increasing evidence that the physiochemical properties of bile salts are extremely important in the production of a lithogenic bile, and in the pathogenesis of cholesterol gallstones [1].

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** The following abbreviations are used in the text: TC = taurocholate; TUDC = tauroursodeoxycholate; TCDC = taurochenodeoxycholate; TDC = taurodeoxycholate; TLC = taurolithocholate; GC = glycocholate; GUDC = glycoursodeoxycholate; GCDC = glycochenodeoxycholate; GDC = glycodeoxycholate; GLC = glycolithocholate; LC = lithocholic; CDC = chenodeoxycholic; DC = deoxycholic; C = cholic; UDC = ursodeoxycholic; T = trihydroxy bile acid; D = dihydroxy bile acid; HPLC = high pressure liquid chromatography; GC = gas chromatography; TLC = thin layer chromatography.
The bile salts used in this study refer to the sodium salts of 5β-cholan-24 oic acids and the prefixes glyco and tauro refer to glycine and taurine conjugates, respectively.

The possible therapeutic uses of bile salts to dissolve gallstones has been of interest to clinicians. Chenodeoxycholic acid therapy to dissolve cholesterol gallstones in vivo has been used for several years [2]. More recent reports describe the use of ursodeoxycholic acid, a dihydroxy bile acid found in small amounts in human bile, to dissolve cholesterol stones [3]. It is suggested that continued oral administration of either of these bile salts over a period of two years transforms a "lithogenic bile" to a "litholytic bile".

This recent revival of interest both in the pathophysiology of bile salts and their therapeutic use, indicates the importance of detailed analysis and characterisation of conjugated bile salts and their distribution in bile in different pathological states.

The present study was initiated in an attempt to evolve a method for investigating the biliary bile acid conjugates present in patients with cholesterol gallstones. Of the various methods available, gas chromatography, although a sensitive technique, cannot be applied to the analysis of conjugated bile salts, as it involves the hydrolysis of the conjugates and subsequent derivatisation [4,5]. The disadvantages of such destructive procedures in the form of degradative losses, long reaction time and the formation of non-bile acid artefacts [6,15] may be eliminated to some extent by employing the enzymic (non-destructive) methods for the cleavage of bile acid conjugates [7], although derivatisation is still necessary to complete the analysis. Thin-layer chromatography (TLC) has been extensively used for the analysis of bile salt conjugates [5] but suffers from inadequate resolution between isomeric dihydroxy bile acids [8] and quantification may pose a problem because of interference from other compounds or due to difficulty of eluting bile salts from the adsorbent [5].

Enzymic quantitation (EQ) has been applied to the determination of bile salt conjugates [7,9], and recently another enzymatic method, also using hydroxysteroid dehydrogenase [10], has been introduced but these have not received wide clinical application in bile acid determinations despite their potential value. Radioimmunoassay (RIA) is a highly sensitive method and good specificity has been reported for conjugated cholate and conjugated chenodeoxycholate [11], but not between different conjugates. Despite its diagnostic value, this technique has not been widely used, mainly because of the difficult technology required for its measurement [12]. Furthermore, we have found no reports describing the RIA of ursodeoxycholate and the RIA of deoxycholate is not yet established.

The majority of methods available for isolation and quantification of conjugated bile salts involve multiple steps including extraction, purification and separation, but a rapid method for identification and determination of individual conjugated bile salts without alteration of the steroid structure is still required.

Several methods for the separation of bile salts by high pressure liquid chromatography (HPLC) have been published [13,14]. Recently, reverse-phase chromatography has been applied to the analysis of bile salts and preliminary results have been encouraging [15,16].

In the present study, HPLC was applied to the analysis of bile and two systems were evaluated.

Materials

Apparatus: The equipment used in this study consisted of a Du Pont model 840 liquid chromatograph and a Cecil model 2012 variable UV spectrophotometer equipped with a 8-μl flow cell of 10-mm path length.

The columns used were a Whatman Partisil-10 ODS column (250 mm × 4.6 mm × 6.35 mm) and a Waters μ Bondapak Fatty Acid Analysis column (300 mm × 4.6 mm × 6.35 mm). The columns were fitted with a 60 mm × 2 mm × 6.35 mm pre-column packed with CO : PELL ODS (Whatman). A 10-μl Hamilton syringe was used to inject the solutions via a Whatman model LIB septum injector. The areas of the peaks were calculated using a Varian electronic integrator, model CDS 111A. All chromatographic data was recorded on a 10-mV recorder (Smiths). Bile sample solutions were centrifuged in 110 mm × 15 mm centrifuge tubes in an MSE (model MULTEX) centrifuge. A pH meter (Radiometer, Copenhagen) was used for pH determinations.

Solvents: The water used to prepare the mobile phases was glass distilled, de-ionised and acidified to pH 2—2.5 with 50% phosphoric acid. This water was used for the preparation of mobile phase A and for dissolving the potassium phosphate used in mobile phase B. All other solvents and reagents were of analytical grade. The mobile phases were freshly prepared, filtered through a 1 micron porosity membrane filter (Sartorius) and finally degassed by connecting to a water pump for half a minute.

The mobile phase A comprised of methanol/water, pH 2 (55 : 45) and the mobile phase B, comprised of isopropanol/8.8 mmol/l potassium phosphate, pH 2.5 (32 : 68).

Bile salts: The sodium salts of conjugated bile salts used in this study were supplied by Sigma Chemicals Co., Dorset, U.K., with the exception of glycoursodeoxycholate and tauroursodeoxycholate obtained from International Enzymes Ltd., Windsor, U.K., and had ca. 99% purity specifications. The bile salts used as standards were purified as necessary on TLC (20 cm × 20 cm × 0.25 mm) plates, pre-coated with silica gel 60 (E. Merck). The solvent mixture comprised chloroform/methanol/water (70 : 25 : 3). Bile salts were detected with iodine vapour.

Methods

Collection of bile: 2—5 ml of bile was aspirated from the gallbladder in patients undergoing elective cholecystectomy for gallstones. This was stored at —20°C until analysed.

Preparation of bile sample: To 5 ml ethanol in a 25-ml conical flask, 0.5 ml gallbladder bile was added slowly with agitation. The deproteinised mixture was centrifuged at 1000 × *g* for 5 min to precipitate the proteins. The clear supernatant was transferred into a test tube. The precipitate was washed twice with ethanol (2 × 2 ml), each time the resulting supernatants pooled with the original supernatant. The total supernatant was filtered through a 1-micron filter and evaporated to dryness. The mixture was dissolved in 0.5 ml of the appropriate mobile phase and 2—3 μl of this solution was used for HPLC.

High pressure liquid chromatography

Partisil-10 ODS column: Mobile phase A was used at a flow rate of 1.0 ml/min, and mobile phase B was used at a flow rate of 1.2 ml/min. The bile salts were monitored at 200 nm for mobile phase A and 193 nm for mobile phase B; the detector sensitivity was set at 0.1 AUF.

μ Bondapak fatty acid analysis column: Mobile phase A was used at a flow rate of 1.2 ml/min and mobile phase B was used at a flow rate of 1.0 ml/min. The bile salts were monitored at 200 nm for mobile phase A and 193 nm for mobile phase B, and the detector sensitivity was set at 0.1 AUF.

All the separations were carried out at ambient temperature and at constant flow rates.

Analysis of bile sample: A reference mixture of bile salts containing conjugates of TC, TCDC, TDC, GDC, GC, GCDC, was prepared and chromatographed to establish retention time characteristics. This was then followed by the injection of 2—3 μl aliquots of the bile sample solution and analysed under identical chromatographic conditions to those for the reference mixture. The amount of each bile salt present in the sample solution was determined by comparison of peak areas with those from the respective standards.

Results

Table I lists the retention times of ten bile salts relative to TDC analysed on Partisil-10 ODS using mobile phase A and B and on the μ Bondapak fatty acid analysis column using the two mobile phases. The order of elution is as expected in reverse-phase chromatography, the polar trihydroxy conjugates elute first, followed by the elution of the less polar dihydroxy bile salts.

In all the systems studied, the elution of the taurine conjugates was generally before the glycine conjugates except for the relative position of GC which was

TABLE I

RELATIVE RETENTION TIMES (RRts) OF 10 CONJUGATED BILE SALTS WHICH MAY BE PRESENT IN BILE, EXPRESSED WITH RESPECT TO TDC (FOR CHROMATOGRAPHIC CONDITIONS SEE TEXT)

	Partisil-10 ODS		μ Bondapak fatty acid analysis column	
	Mobile phase A	Mobile phase B	Mobile phase A	Mobile phase B
Bile salt	$RRt_{(TDC)}$ = 12.80 min	$RRt_{(TDC)}$ = 12.84 min	$RRt_{(TDC)}$ = 18.20 min	$RRt_{(TDC)}$ = 16.44 min
TUDC	0.48	0.52		0.56
TC	0.53	0.54	0.67	0.52
TCDC	0.94	0.90	0.96	0.86
TDC	1.00	1.00	1.00	1.00
TLC	1.76	1.72		1.75
GUDC	1.08	1.06		1.06
GC	0.64	0.64	1.06	0.74
GCDC	1.22	1.20	1.65	1.44
GDC	1.27	1.32	1.82	1.70
GLC	2.17	2.14		2.82

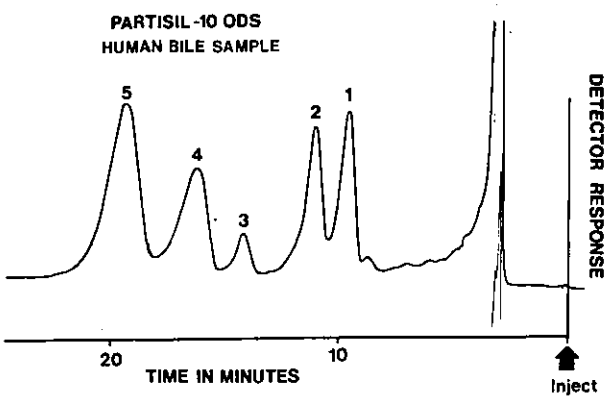

Fig. 1A. HPLC separation of biliary bile salts from a patient with cholesterol gallstones, on Partisil-10 ODS using mobile phase A. Peak 1, TC 2, GC 3, non bile acid peak 4, TCDC + TDC 5, GCDC + GDC.

significantly affected by the pH of the mobile phase. Optimum separation of the six commonly found conjugates in bile was obtained using μ Bondapak fatty acid analysis column and solvent B.

Figs. 1A and 1B represent the analysis of conjugated bile salts in the same human bile sample using Partisil-10 ODS, mobile phase A and Bondapak FAA with mobile phase B respectively. In the former system, TCL (peak 1) and GC (peak 2) elute as single separate peaks but TCDC and TDC elute as a single peak (peak 4) as also do GCDC and GDC (peak 5). Peak 3 was detected in bile samples only on the Partisil-10 ODS column; and was not detected when the ethanolic extract of bile was further extracted with petroleum ether (2 X 5 ml) after addition of 4 ml of water. The μ Bondapak fatty acid analysis column using mobile phase B separated all the major six conjugates present in bile into individual peaks (Fig. 1B, peak 1—6).

When known quantities of bile salts were added to the bile sample before extraction with ethanol, subsequent chromatography demonstrated no alteration in the relative retention times of the conjugated bile salts present in the sample. Recovery of the added bile salts was in the range of 80—90%.

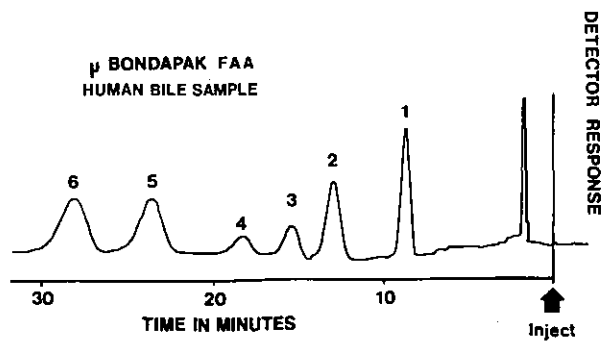

Fig. 1B. HPLC separation of the same bile sample as in Fig. 1A on μ Bondapak fatty acid analysis column using mobile phase B. Peak 1, TC 2, GC 3, TCDC 4, TDC 5, GCDC 6, GDC.

Quantitative analysis

Calibration curves showing the relationship between detector response and the amount of bile salt conjugate injected are shown in Figs. 2A and 2B. For amounts of bile salts ranging from 0.1—5.0 μg the detector response as measured by peak area was linear.

Reproducibility of the analysis and sensitivity

The reproducibility of the analysis was tested by replicate analysis of a bile

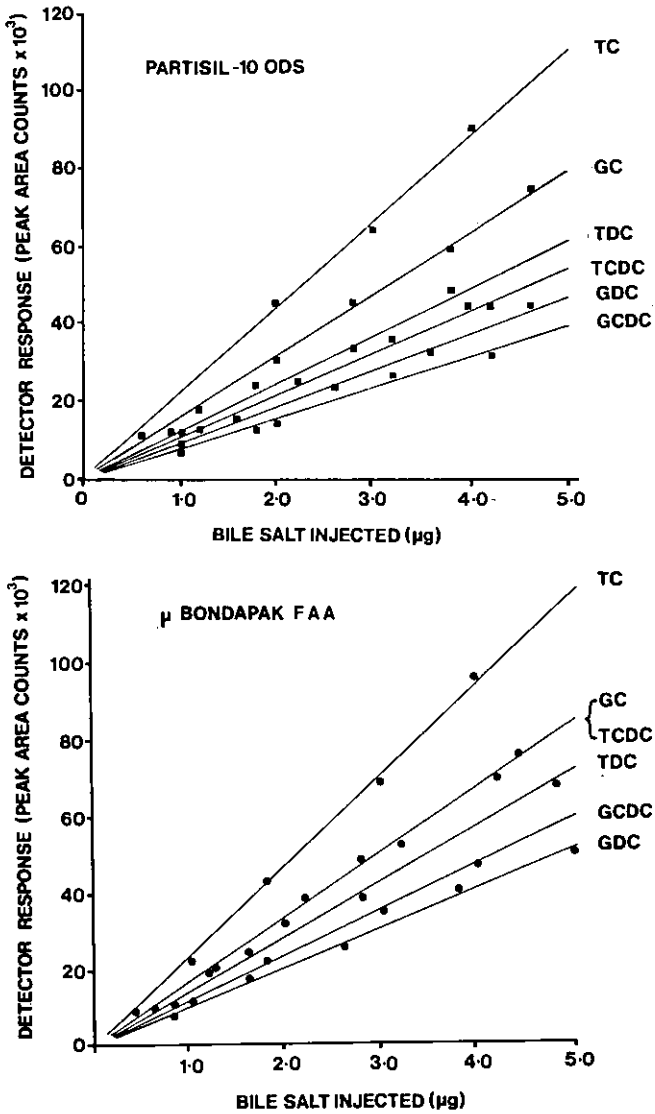

Fig. 2A and 2B. Relationship between the amount of conjugated bile salts injected and the peak area response of the UV detector for Partisil-10 ODS column using mobile phase A (Fig. 2A) and for μ Bondapak fatty acid analysis column using mobile phase B (Fig. 2B). Correlation coefficient of linear regression ranged from 0.995 to 0.999. Each point is a mean of 3 determinations.

TABLE II

REPRODUCIBILITY OF THE PRESENT METHOD FOR THE DETERMINATION OF CONJUGATED BILE SALTS IN HUMAN BILE AS ANALYSED ON THE PARTISIL-10 ODS AND μ BONDAPAK FATTY ACID ANALYSIS COLUMN

Bile salt	Partisil-10 ODS (g/l)		μ Bondapak (F.A.A.) (g/l)	
	Mean	S.D.	Mean	S.D.
TC	1.35	0.06	1.53	0.07
TCDC	2.75	0.13	1.40	0.09
TDC			1.19	0.05
GC	2.95	0.28	3.38	0.35
GCDC	5.40	0.29	2.61	0.06
GDC			2.75	0.20
TBBS	12.45 $n = 4$	0.35	12.86 $n = 4$	0.30

TBBS = total biliary bile salts (g/l).

sample analysed on the two columns on different days. The mean concentration of four analyses of total biliary bile salts was 12.45 g/l (range, 12.16—13.15 g/l) as detected with Partisil-10 ODS column and 12.86 g/l (range, 12.5 - 13.30 g/l) with μ Bondapak fatty acid analysis column for the same bile sample. The results are presented in Table II.

The lower limit of detection for TC at 193 nm, 0.05 AUF Bondapak fatty acid was 0.1 μg and for GDC 0.2 μg with a minimum peak height of 10% of full-scale.

Analysis of human bile

The results of duplicate analysis of five samples of bile analysed on the μ Bondapak fatty acid analysis column are shown in Table III. The distribution of the major conjugates is expressed as the mean of two determinations. The mean of five bile samples was 12.03 g/l (range, 10.2—13.52 g/l).

TABLE III

RESULTS OF DUPLICATE ANALYSIS OF THE SIX PRINCIPAL CONJUGATES IN HUMAN BILE AS ANALYSED ON μ BONDAPAK FATTY ACID ANALYSIS COLUMN USING MOBILE PHASE B (FOR CHROMATOGRAPHIC CONDITIONS SEE TEXT)

Patient	TC	TCDC	TDC	Total T	GC	GCDC	DGC	Total G	TBBS g/l
A	1.25	0.85	0.65	2.75	3.40	3.30	1.80	8.50	11.25
B	1.90	1.70	0.60	4.20	1.90	2.60	1.50	6.00	10.20
C	2.00	0.90	0.40	3.30	3.80	3.00	2.50	9.30	12.60
D	1.35	1.68	1.24	4.27	3.10	3.15	3.00	9.25	13.52
E	1.20	1.60	1.20	4.00	3.00	2.70	2.90	8.65	12.60
Mean	1.54	1.35	0.82	3.70	3.04	2.95	2.34	8.33	12.03

TBBS = total biliary bile salts; Total T = total taurine conjugates; Total G = total glycine conjugates.

TABLE IV

THE DISTRIBUTION OF G/T, T/D RATIOS IN FIVE PATIENTS AS DETERMINED BY THE TWO COLUMNS AND C:CDC:DC RATIO ON μ BONDAPACK FATTY ACID ANALYSIS (F.A.A.) COLUMN

Patient	G/T Ratio		T/D Ratio		C:CDC:DC μ Bondapak (F.A.A.)
	Partisil-10 ODS	μ Bondapak (F.A.A.)	Partisil-10 ODS	μ Bondapak (F.A.A.)	
A	3.28	3.09	0.72	0.71	1.12:1.00:0.59
B	1.40	1.42	0.59	0.59	0.88:1.00:0.49
C	2.56	2.82	0.87	0.85	1.49:1.00:0.74
D	1.90	2.10	0.54	0.47	0.92:1.00:0.88
E	2.30	2.20	0.48	0.50	0.98:1.00:0.95
Mean	2.29	2.33	0.64	0.63	1.08:1.00:0.73

Glycine to taurine ratio (G/T)

The mean values for the glycine conjugates to taurine conjugates, setting the latter at unity, was 2.29 for the five samples with a range of 1.40—3.28 as determined with the Partisil-10 ODS; and 2.33 with a range of 1.42—3.09 on the μ Bondapak fatty acid analysis column. The distribution of the G/T ratio in the five patients is presented in Table IV.

Trihydroxy to dihydroxy ratio of bile salts (T/D)

The distribution of the T/D ratio in five patients is also presented in Table IV. The mean T/D ratio as determined using the Partisil-10 ODS, was 0.64 (range, 0.48—0.87) and 0.63 (range, 0.49—0.85) using the μ Bondapak fatty acid analysis column.

The mean ratio of C : CDC : DC in the five samples as obtained on the μ Bondapak fatty acid analysis column, was 1.08 : 1.00 : 0.63 and these results are shown in Table IV.

Discussion

The evaluation of two HPLC columns is presented. The system of Shaw et al. [16] using the μ Bondapak fatty acid analysis column gave the best separation of six bile salts in human bile, all the six bile salts being well separated. A high degree of reproducibility was obtained on each column, using a method which unlike alternative destructive procedures, does not require preliminary hydrolysis and derivatisation of free bile acids.

Preliminary work with bile extracts indicated that the relative positions and elution of bile salts in bile did not alter when known quantities of bile salts were introduced before extraction. Recovery of the added bile salts was in the range of 80—90%. This work also indicated that it was important to remove neutral lipids from bile before HPLC. Peak 3, (Fig. 1A) was found to be a non bile acid peak which was not found in the sample after extraction with petroleum ether.

Throughout we found it was essential to use pre-columns to protect the main columns. There was a slight loss in resolution but the analysis time did not

increase significantly. After every 6—8 weeks of continuous use the first centimeter of the packing material (CO : PELL ODS) needed replacing and the column regeneration by passing known quantities of solvents recommended by the manufacturer.

Initially the detection of bile salts in the eluate from the HPLC columns was monitored using a Du Pont 845 Refractive Index detector (results not shown). Although the sensitivity of this detector was sufficient for work with bile, the detector was found to be unstable and difficult to optimise. Therefore a UV detector was substituted; this was found to be more stable and had greater sensitivity.

Difficulty arose when the separations with mobile phase A, containing methanol could not be monitored below 200 nm and 0.05 AUF sensitivity because of higher absorption of UV light by methanol. For this reason all separations using mobile phase A were monitored at 200 nm and those using isopropanol at 193 nm.

HPLC-mass spectrometry [19] has indicated that the six principal bile salts in bile were TC, TCDC, TDC, GC, GCDC and GDC. Examination of Table I and Fig. 2B demonstrates that the μ Bondapak fatty acid analysis column using mobile phase B provides the optimum separation of these six bile salts. The conjugates found in bile together with the G/T and T/D ratios (Table IV) obtained with both columns are similar to those obtained by other authors [16—18].

The limitation of the Partisil-10 ODS column and mobile phase A was the incomplete resolution obtained between glycine and taurine conjugates of CDC and DC. The separation was improved by substituting isopropanol as the mobile phase B. The μ Bondapak fatty acid analysis column using mobile phase B separates the bile sample into six distinct peaks, resolving the glycine and taurine conjugates of CDC and DC completely. HPLC of a standard mixture of bile salts containing TUDC, GUDC, TLC and GLC demonstrated distinct peaks for TLC and GLC but the conjugates of UDC were not fully separated (Table I), and none of these bile salts were detected in the bile samples analysed. However, it should be possible to detect these conjugates when present in significant quantities, which may be of use in the clinical or therapeutic situation. The μ Bondapak fatty acid analysis column provides a method for an efficient separation of bile into its principal constituent bile salts. The analysis occurs in a practical period of time, 30 min, which can be further reduced by solvent programming.

Applications and final conclusions

The major attraction of the procedure described is the ability to analyse and quantitate simultaneously bile salts as they occur naturally in bile. This information is not obtained using the conventional methods involving hydrolysis. Other methods which can separate conjugates are either more complicated or less accurate.

Detailed information about G/T, T/D ratios and the concentrations of individual bile salts may be important in the determination of a lithogenic bile; and possibly the detection of bile salts not normally present in bile may be of clinical significance in the pathology of cholesterol gallstones. The procedure

may also be useful for monitoring CDC and UDC levels in bile in patients undergoing CDC or UDC therapy for cholesterol gallstones.

The method can be easily adapted for analysis of bile salts in duodenal aspirates or other biological samples containing bile salts. With an improved sensitivity of detection this method can be applied to the analysis of serum bile salts. Therefore the procedure described provides more precise information about the presence and concentration of bile salts which may be important clinically.

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Bile acid, neutral sterol and faecal fat excretion in subjects treated with fenfluramine and its relationship to fenfluramine-induced diarrhoea

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Summary

Bile acid, neutral sterol and faecal fat excretion was studied over a period of 9 weeks in a group of 16 healthy subjects before, during and after administration of fenfluramine. Statistical analysis revealed a significant increase in bile acid excretion during the drug phase ($P<0.02$); and during recovery period of 3 weeks ($P<0.05$). Faecal neutral sterol, as the total of coprostanol and cholesterol elimination was also enhanced after fenfluramine. Coprostanol was replaced by cholesterol in 12 subjects. Faecal fat was studied in 6 subjects, the excretion increased during the drug phase ($P<0.05$), and remained elevated during the post-drug period ($P<0.01$). The composition of the bile acids remained unaltered in all the subjects except 3 who had a fenfluramine-induced watery diarrhoea; and these excreted chenodeoxy and cholic together with smaller amounts of secondary bile acids. A higher excretion of bile acids was found in the 8 overweight subjects ($P<0.01$) before administration of fenfluramine.

These results are discussed in an attempt to correlate the effect of fenfluramine with changes in bile acid and neutral sterol excretion, and its relationship to fenfluramine-induced diarrhoea.

Introduction

Obesity presents a major nutritional and medical problem. Its complications are well documented such as increased morbidity and mortality due to cardiovascular disorders, hypertension, diabetes and cholelithiasis. Dietary restriction on its own by personal effort is not always effective in the management of simple, exogenous obesity. Controlled trials on the use of appetite suppressants such as amphetamines have shown a variety of undesirable side effects including central nervous stimulation, addiction, toxicity, euphoria and even ineffectiveness. The use of amphetamines has been discouraged but fenfluramine, an analogue of amphetamine, has become widely used as an anti-obesity agent devoid of central nervous stimulation (Munro, Seaton and Duncan, 1966; Le Douarec and Niven, 1970). There are reports of its beneficial effects on hyperlipidaemia (Pawan, 1969; Bliss, Kirk and Newall, 1972), on hypercholesterolaemia (Tomlinson, Lines and Greenfield, 1975), and on hyperglycaemia (Turtle, 1972). However it has been reported that some patients on the drug complain of frequency of bowel motions amounting to diarrhoea. The incidence of diarrhoea reported by Munro *et al.* (1966), Hollingsworth and Amatruda (1969) and Sedgwick (1972) was 40, 44 and 67% respectively. The cause of this diarrhoea consecutive to the treatment with fenfluramine is obscure. No direct correlation has been reported between the onset of diarrhoea and serum levels of fenfluramine. Dietetic survey in people with frequency of bowel motions suggest an interaction between nutrients (fish) and the drug. Studies by Duhault and Verdavainne (1967) suggested that diarrhoea may be related to the decreased absorption of triglycerides in the intestinal tract, to the release of serotonin or to the alteration in gut motility. Some of these views were supported by Garattini (1971) who reported that the lipid-lowering effect of fenfluramine was related to the inhibition exerted by the drug on gastric and intestinal motility. In another study Dannenburg and Ward (1971) demonstrated that, *in vitro*, fenfluramine inhibited pancreatic lipase and they suggested that this reduces the intestinal absorption of fat. But fat balance studies carried out by Evans *et al.* (1975) revealed that the apparent digestibility of fat in healthy students was unimpaired, and there was no evidence of reduction of intestinal fat absorption.

One line of study which interests the present authors is the examination of the effect of fenfluramine on bile acid metabolism, and in this investigation they set out to examine the effect of fenfluramine on total faecal bile acid and neutral sterol excretion in healthy subjects before, during and after administration of fenfluramine and to evaluate the changes in bile acid excretion to fenfluramine-induced diarrhoea. The second purpose of the study was to examine the effect of fenfluramine on the

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composition of faecal bile acids and neutral sterols excreted by the subjects before and after a course of fenfluramine.

Subjects, experimental design and methods

Subjects

Sixteen healthy subjects who were members of the hospital staff kindly participated in the study.

Design of study

The study was conducted over a period of 9 weeks, consisting of 3 consecutive periods of 3 weeks in the sequence of: 1 pre-drug, 2 drug, 3 post-drug. Each subject was his or her own control to minimize intersubject variation. In an attempt to eliminate the influence of dietary change, all subjects were asked to make no deliberate alteration in their diet. The subjects were seen once a week when they were supplied with tablets for the following week.

After an initial pre-drug period, oral fenfluramine was instituted at a dose of 40 mg/day in the first week, 60–80 mg/day in the second week, and reduced to 40 mg/day during the third week before stopping.

In order to observe the pattern of faecal bile acid excretion in the overweight individuals as compared to subjects of normal weight, the 16 subjects were divided into 2 groups. Eight subjects were within 10% of their ideal body weight for age, sex and height according to the Statistical Bulletin (1959). Eight subjects were between 11 and 80% above their ideal body weight.

Analytical methods

Collection and processing of faeces

Total stool collections were made over 3-day periods in 6 subjects during the 3 phases of the study. The remaining 10 subjects collected faeces from one day at a time and provided 9 collections over the study period. Faeces were collected into plastic containers and stored at −20°C until analysed. Before analysis the stool was thawed and homogenized. A 50-g aliquot was freeze-dried for all analyses.

Faecal fat analysis

Faecal fat was analysed for the subjects with 3-day stool collections by the method of Van de Kamer, Huinink and Weijers (1949). Freeze-dried faeces were used because it has been shown that such samples yield more consistent results (Weijers and Van de Kamer, 1953).

Analysis of bile acids and neutral sterols

The freeze-dried samples of faeces were extracted using a method based on that of Evrard and Janssen (1968), with subsequent analysis of bile acids and neutral sterols by gas chromatography (Eneroth, Hellstrom and Sjovall, 1968). The degree of conjugation of bile acids was determined using thin layer chromatography (Huang and Nichols, 1975).

Statistical analysis

Student's *t*-test was used to compare the bile acid and neutral sterol excretion in the 16 subjects during the 3 phases of the study.

The reproducibility of bile acid analysis was studied in one individual over the 9-week experimental period. The coefficient of variation of replicate analysis ($n=15$) was 7·6% during the pre-drug period. The mean faecal bile acid excretion during this period was $9·3 \pm 0·4$ mg/g dry weight faeces (range 8·9–9·9). After the administration of the drug the coefficient of variation was 9·8% and the mean bile acid excretion was $11·0 \pm 0·5$ mg/g dry weight faeces (range 10·3–11·7).

Results

Bile acids

The mean bile acid excretion for the 16 subjects during the pre-drug period was $9·3 \pm 3·1$ mg/g dry weight faeces compared to $12·1 \pm 4·5$ mg/g dry weight during the drug period ($P<0·02$). The bile acid excretion remained elevated after the withdrawal of fenfluramine ($11·6 \pm 3·4$ mg/g dry weight) ($P<0·05$). There was no statistically significant difference between the excretion during the drug phase and the post-drug phase ($12·1 \pm 4·5$ mg/g dry weight compared to $11·6 \pm 3·4$ mg/g dry weight). This implies that the drug effect persisted for at least 3 weeks after the withdrawal of fenfluramine.

Bile acid excretion in the subjects with normal body weight (Fig. 1a) was compared with the excretion in the overweight subjects (Fig. 1b). During the period before the administration of fenfluramine the bile acid excretion in the overweight subjects ($10·8 \pm 3·8$ mg/g dry weight) is significantly higher ($P<0·01$) than the bile acid excretion in those of normal weight ($7·7 \pm 0·7$ mg/g dry weight). The excretion remained high in the overweight subjects ($13·7 \pm 5·3$ compared to $10·5 \pm 3·1$ mg/g dry weight) during the drug period ($P<0·01$); and also during the post-drug period ($12·9 \pm 3·8$ mg/g dry weight compared to $10·3 \pm 2·6$ mg/g dry weight; $P<0·01$).

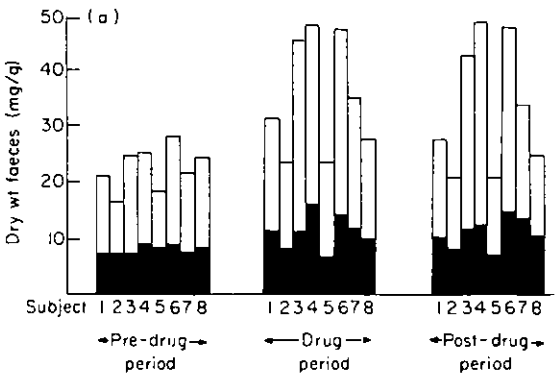
In 7 subjects bile acid excretion reached a peak by the end of the second week on the drug. In 4 subjects excretion dropped during the first week and then increased in the second and third week of medication. Three subjects had diarrhoea, and limited quantitative data only were obtainable because of the problem of 24-hr faecal collection in these subjects.

(a)

■ Bile acids
□ Neutral sterols
} Total faecal steroids
Dry wt faeces (mg/g)

	Pre-drug mean	±s.d.	Drug mean	±s.d.	Post-drug mean	±s.d.
■	7·7	0·7	10·5	3·1	10·3	2·6
□	14·4	3·4	24·0	7·7	21·7	8·8
Total	22·1	3·9	34·5	10·4	32·0	10·8

Level of significance (P) <0·01 (pre- v. drug mean) <0·01 (pre- v. post-drug mean)



(b)

	Pre-drug mean	±s.d.	Drug mean	±s.d.	Post-drug mean	± s.d.
■	10·8	5·4	13·7	5·3	12·9	3·8
□	19·0	3·8	22·6	10·0	26·6	9·3
Total	29·8	7·6	36·3	11·7	39·5	11·6

Level of significance (P) <0·02 (pre- v. drug mean) <0·01 (pre- v. post drug mean)

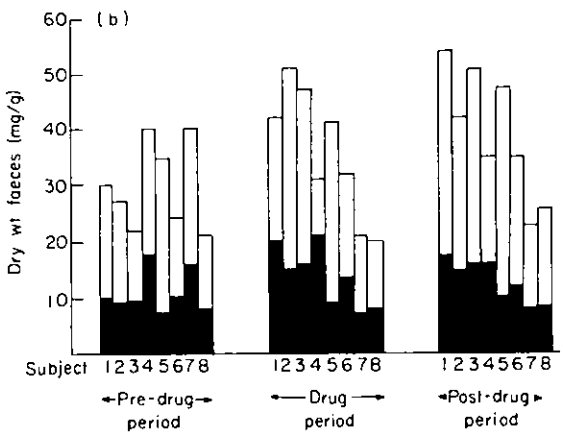


FIG. 1. (a) Total faecal steroid excretion during 3 phases of the study in 8 subjects with weights within 10% of ideal body weight.

(b) Total faecal steroid excretion during 3 phases of the study in 8 subjects overweight between 10 and 80% above ideal body weight.

Neutral sterols

The neutral sterol excretion, as the total of coprostanol and cholesterol, also indicated a similar increase. The mean neutral sterol excretion for the 16 subjects was 16·7±4·9 mg/g dry weight during the pre-drug period. The excretion increased to 23·3±8·6 mg/g dry weight during the drug period (P<0·02). The excretion remained elevated at 24·1±9·0 mg/g dry weight during the post-drug period (P<0·01).

Total faecal steroids (bile acids and neutral sterols)

The mean excretion of total faecal steroids as the sum of bile acids and neutral sterols was 26·0±7·0 mg/g dry weight during the pre-drug period. The excretion increased to 35·2±10·7 mg/g dry weight during the drug period (P<0·01), and to 36·7±11·5 mg/g dry weight during the post-drug period (P<0·01) (Fig. 1a and b).

The excretion of total faecal steroids in the overweight subjects was 29·8±7·6 mg/g dry weight as compared with 22·1±3·9 mg/g dry weight in the subjects of normal weight. This failed to reach the 5% level of significance.

Faecal fat

The mean faecal fat excretion in 6 subjects during the pre-drug period was 2·61±0·39 g/day. There was a significant increase in the excretion during the drug period (3·86±1·09 g/day; P<0·05): and also during the post-drug period (3·14±0·46 g/day; P<0·01), (Fig. 2).

Qualitative analysis of faecal bile acids and neutral sterols

Bile acids

The qualitative composition of faecal bile acids in the subjects without diarrhoea remained basically unaltered. The major components were secondary bile acids lithocholic, isolithocholic, deoxycholic and smaller quantities of other dihydroxy bile acids and bacterial oxidation products as shown in Table 1.

The subjects with fenfluramine-induced diarrhoea excreted increased amounts of primary bile acids, i.e. chenodeoxycholic (3α, 7α) and cholic acid (Tri-OH) (Fig. 3). In these subjects the excretion of primary bile acids increased from 2·8±1·5 mg/g dry weight, during the period before the administration of fenfluramine, to 7·3±5·6 mg/g dry weight during the drug period (P<0·05).

Thin layer chromatography of faecal bile acids revealed that bile acids excreted before and after the administration of fenfluramine were in the unconjugated form (Fig. 4). However, if there were conjugated bile acids in the faeces, these would be present in quantities less than 1 mg/100 g wet faeces

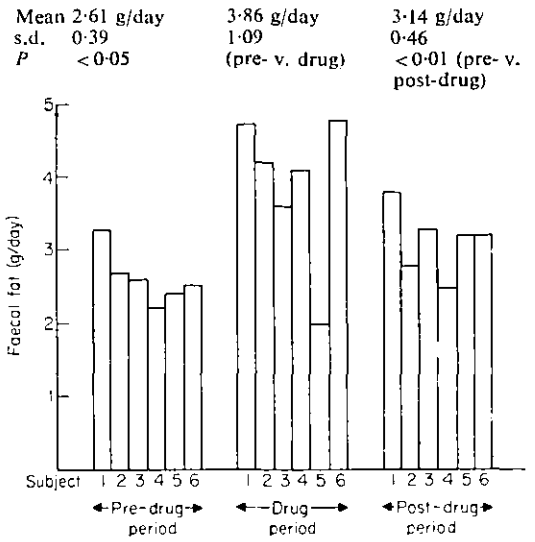


FIG. 2. Faecal fat excretion in 6 subjects during the 3 phases of the study. Faecal fat during the pre-drug period differs significantly from the drug period ($P<0·05$) and from the post-drug period ($P<0·01$).

if the sensitivity of the TLC system is taken into consideration.

Neutral sterols

The analysis of faecal neutral sterols revealed that coprostanol was the major neutral sterol excreted during the pre-drug period (Fig. 5). The pattern of neutral sterols excreted was markedly altered during the drug period. Coprostanol was replaced by cholesterol which then became the predominant sterol in the faeces of the 12 subjects. Cholesterol elimination in the faeces was prolonged in 3 subjects with fenfluramine-induced diarrhoea.

Discussion

Alterations in total faecal steroid patterns

In the present study, the authors have attempted to correlate the effect of fenfluramine with changes in total faecal steroids, individual bile acids and neutral sterol excretion. Results of the study reveal an increased elimination of faecal bile acids after fenfluramine, which coincides with elevated excretion of neutral sterols and faecal fat.

The pattern of faecal bile acid excretion in the subjects without fenfluramine-induced diarrhoea consisted mainly of deoxycholic, lithocholic and isolithocholic acids. These are secondary bile acids present in normal faeces and are produced in the gastrointestinal tract as a result of bacterial degradation of primary bile acids (Hill and Drasar, 1968). The primary bile acids are composed of chenodeoxycholic and cholic acid derived from cholesterol metabolism in the liver.

The pattern of bile acid excretion in the subjects with fenfluramine-induced diarrhoea consisted mainly of primary bile acids with smaller amounts of secondary bile acids (Fig. 3) and cholesterol replacing coprostanol. Studies by Rubulis, Rubert and Faloon (1970) revealed similar observations and reported an increased excretion of primary bile acids, cholesterol and faecal fat in patients treated with antibiotics. They suggested that the replacement of secondary bile acids by primary bile acids and the appearance of cholesterol in place of coprostanol may simply reflect increased transit through the bowel with less exposure of bile acids and cholesterol to bacterial action. Similar findings were also reported by Mitchell and Eastwood (1972) in patients with ileal dysfunction.

Studies carried out by Kirwan *et al.* (1975) have demonstrated a direct relationship between the colonic motility and faecal bile acid excretion. They reported chenodeoxycholic to be the principal factor

TABLE 1. Qualitative analysis of faecal bile acids in subjects treated with fenfluramine

Bile acid detected	Normal subjects	Subjects with diarrhoea
3β-hydroxy-5β-cholanoic	++	+
3α-hydroxy-5β-cholanoic	++	+
3β, 12α-dihydroxy-5β-cholanoic	+	+
3β, 7α-dihydroxy-5β-cholanoic	+	+
3α, 12α-dihydroxy-5β-cholanoic	++	+
3α, 12β-dihydroxy-5β-cholanoic	trace	+
3α, 7α-dihydroxy-5β-cholanoic	trace	++
3α, 7β-dihydroxy-5β-cholanoic	trace	+
3α, 6α-dihydroxy-5β-cholanoic	trace	trace
3β, 17 keto-5β-cholanoic	trace	trace
3α, 7α, 12α-trihydroxy-5β-cholanoic	trace	++

'trace' represents a GC peak of <4% full scale deflection on GC recorder.
+ represents a GC peak of 4–20% full scale deflection on GC recorder.
++ represents a GC peak of >20% full scale deflection on GC recorder.

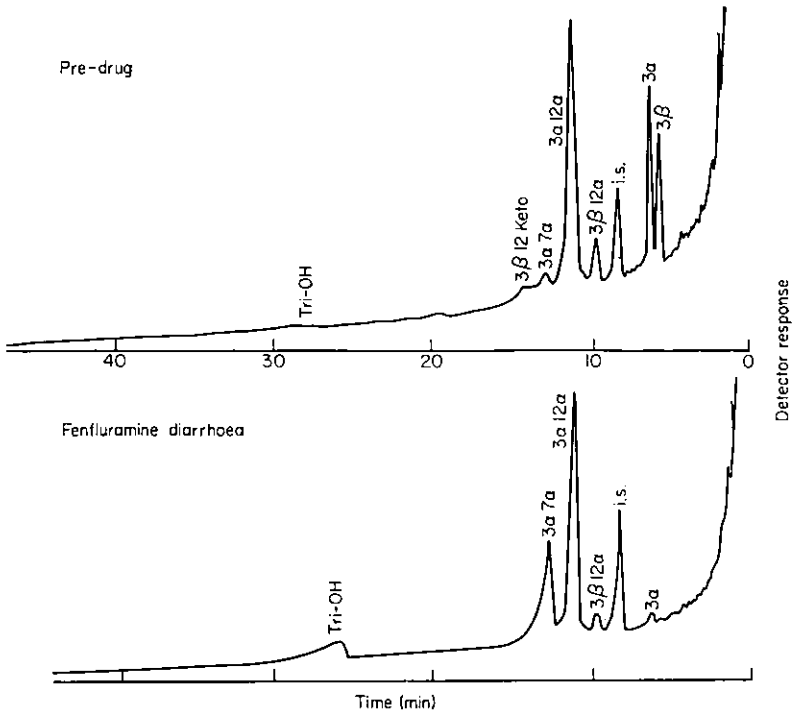


FIG. 3. Gas chromatographic separation of bile acids as methyl esters. Peak identification of main bile acids: secondary bile acids, 3β = isolithocholic; 3α = lithocholic; 3β, 12α = dihydroxy bile acid; 3α, 12α = deoxycholic. Primary bile acids: 3α, 7α = chenodeoxycholic; tri-OH = cholic; i.s. = internal standard (23-nor deoxycholic). During the pre-drug period secondary bile acids are predominant in the faeces. During the period of fenfluramine-induced diarrhoea the excretion of primary bile acids increases ($P < 0.05$).

responsible for the changes in motility. In perfusion studies of the human colon Mekhjian, Phillips and Hofmann (1971) demonstrated that chenodeoxycholic was a potent inhibitor of water and electrolytes from the colon and had a greater effect on diarrhoea.

The pattern of neutral sterols in the faeces was altered after the administration of fenfluramine. Normally coprostanol is the predominant neutral sterol with small amounts of cholestanol and plant sterols. These neutral sterols are metabolites formed from cholesterol in the gastrointestinal tract by the action of intestinal micro-organisms (Eneroth, Hellstrom and Ryhage, 1964). These metabolites are poorly absorbed from the gut and, therefore, constitute major neutral sterols in normal faeces. This form of pattern of neutral sterols was observed in the present study during the pre-drug period. After the administration of fenfluramine the excretion of coprostanol was replaced by cholesterol in the faeces (Fig. 5). Similar observation was made by Meihoff and Kern (1968) in patients with mannitol-induced diarrhoea. They reported a decrease in intestinal transit from a mean of 26 hr before the study to 4·6 hr during the period of diarrhoea and demonstrated alterations in faecal steroid patterns together with an increase in bile acids, neutral sterols and faecal fat excretion in their subjects. They suggested that rapid transit through the gastrointestinal tract was the major factor which decreases the normal absorption of bile acids, neutral sterols and faecal fat, hence the excretion of these increases.

Bile acid excretion pattern in the overweight subjects

Bile acid excretion was significantly higher in the overweight subjects. This observation is in agreement with the findings of Miettinen (1971) who reported a higher bile acid and neutral sterol excretion in obese normolipidaemic subjects as compared to non-obese normolipidaemic subjects and demonstrated that the greater the body weight the higher was the excretion of bile acids and neutral sterols.

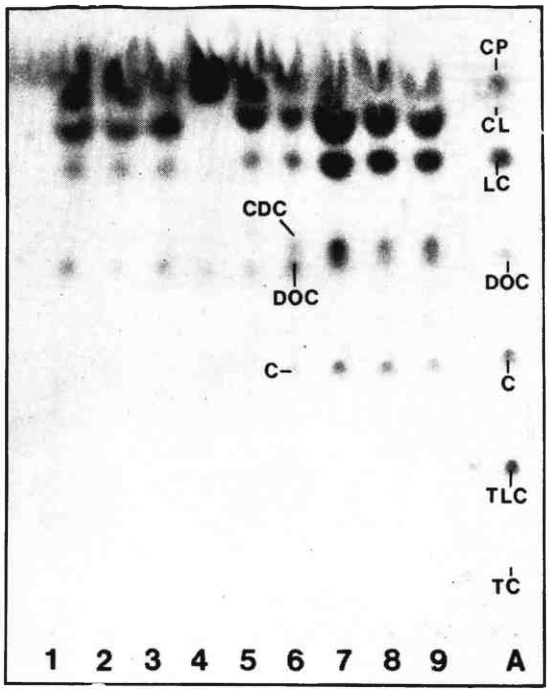


FIG. 4. Thin layer chromatographic separation of faecal bile acids in a subject without fenfluramine-induced diarrhoea. For TLC conditions see text. A = authentic bile acids and neutral sterols. TC = taurocholic; TLC = taurolithocholic; LC = lithocholic; CL = cholesterol; CP = coprostanol. Primary bile acids are not detected in the pre-drug faecal samples (1, 2 and 3) but are detected in samples (5 and 6) during the drug period and also during the post-drug period (7, 8 and 9).

The value of 7·7 ± 0·7 mg/g dry weight for bile acid excretion in the subjects with normal weight is similar to that of 6·13 mg/g dry weight for 20 subjects reported by Aries *et al.* (1971) and that of 8·72 mg/g dry weight for 5 normals reported by Evrard and Janssen (1968). Similar observations were also reported by Nestel and Hunter (1974) in studies of bile acid excretion in the overweight.

The pattern of bile acid excretion in the overweight subjects compared to those of normal weight differed only in the levels of cholic acid excretion. This was 5% by weight of the bile acid excretion in the over-weight group compared with 2% in the normal group ($P < 0.05$).

Bile acid and neutral sterol excretion (cholesterol-lowering effect)

Results of the present study reveal a significant increase in excretion of bile acids, neutral sterols and faecal fat after fenfluramine. In studies of cholesterol production in obesity Miettinen (1971) reported a direct relationship between body weight and serum cholesterol and faecal excretion of bile acids and neutral sterols. In later studies using the sterol balance technique Miettinen (1973) also reported that the removal of cholesterol as bile acids in the faeces is the primary factor which determines the level of serum cholesterol. Rubulis *et al.* (1970) studied the effect of neomycin and colchicine on serum cholesterol and reported an increase in excretion of bile acids, neutral sterols and faecal fat. Coincident with these changes there was a significant decrease in serum cholesterol and lipids. Therefore the observation of increased excretion of bile acids and neutral sterols after fenfluramine suggests an increased catabolism of cholesterol and possibly a decrease in the level of serum cholesterol pool.

The lipid-lowering effect of fenfluramine has been reported to be related to the inhibition exerted by the drug on gastric and intestinal motility (Garattini, 1971). The lipid-lowering effect of the drug is of particular interest in the obese patient where endogenous production of cholesterol is normally increased (Miettinen, 1971). The present results are consistent with the previous observation made from this laboratory, that fenfluramine caused a small but significant decrease in serum cholesterol (Bliss *et al.*, 1972). The cholesterol-lowering effect of fenfluramine was also observed by Dannenburg and Chremos (1971), and by Tomlinson *et al.* (1975) who also reported a decrease of 14% in the level of serum cholesterol in hypercholesterolaemic patients within 2 weeks.

From their study it appears to the authors that the increased faecal bile acid and neutral sterol excretion observed after administration of fenfluramine suggests enhanced removal of cholesterol. This could be beneficial in obesity and related metabolic disorders.

Acknowledgments

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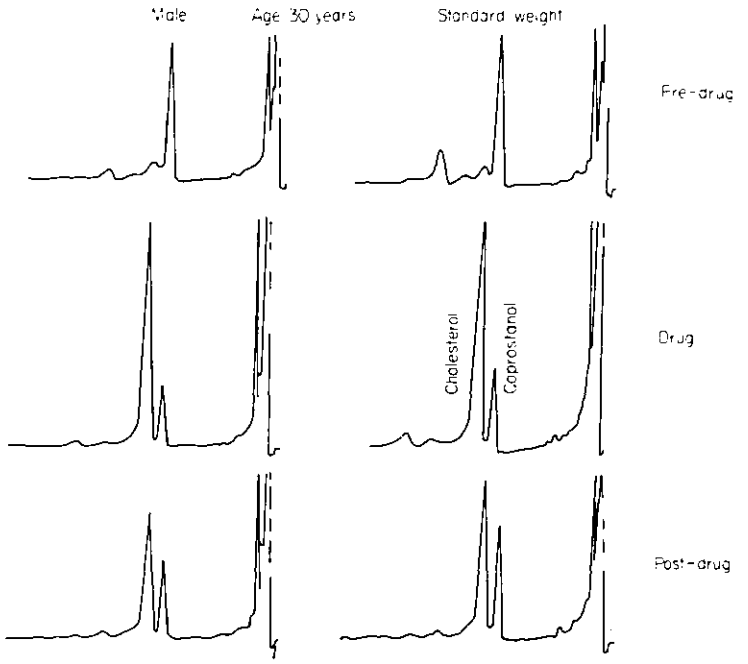

FIG. 5. Gas-chromatographic separation of neutral sterols excreted by a subject without fenfluramine-induced diarrhoea. Coprostanol is the predominant faecal neutral sterol during the pre-drug period. Excretion of coprostanol decreases during the drug period and that of cholesterol increases instead. The altered pattern of excretion of neutral sterols also persists during the post-drug period.

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