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MEASUREMENT OF FAECAL BILE ACID SULPHATES

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SUMMARY

A method is described for the measurement, by difference, of the sulphate fractions of faecal bile acids. A solvolysis step (for the deliberate hydrolysis of the bile acid sulphates) was added to the procedure of sample homogenisation, extraction, enzymatic hydrolysis and thin-layer chromatography. The bile acids were quantitated by gas—liquid chromatography of their methyl ester and trifluoroacetate methyl ester derivatives on 3% QF-1 columns. The total bile acid excretion in 15 control subjects was 603 ± 71 mg/24 h ($\bar{x}$ ± S.E.M.). The major bile acid peaks (mg/24 h) were: lithocholic acid, without solvolysis 118 ± 26 and including solvolysis 175 ± 30; deoxycholic acid 60 ± 8 and 90 ± 18 and chenodeoxycholic acid 13 ± 7 and 15 ± 7. It was concluded that bile acid sulphates may form a considerable proportion of the total bile acids excreted in man.

INTRODUCTION

For several substances hepatic sulphation provides an effective detoxifying mechanism. Many potentially toxic drugs, for example, are rendered harmless by sulphation in the liver which ensures their secretion in the bile and faeces. Although the major primary and secondary bile acids are largely conserved within the entero-hepatic circulation by intestinal and hepatic transport mechanisms, they are also partially sulphated in the human liver [1,2]. First reported by Palmer [3] in 1967, sulphation has been shown to enhance the excretion of bile acids, particularly lithocholic acid [4,5]. This hepatic sulphation of bile acids probably influences the physiology of the major bile acids in health but it becomes particularly important in cholestasis and during chenodeoxycholic acid treatment of gallstones. In cholestasis the urinary excretion of bile acids becomes the major route for their elimination from the body [4]. During chenodeoxycholic acid therapy, increased amounts of its principal bacterial metabolite, lithocholic acid, form in and are absorbed from the intestine. In man, sulphation of lithocholic acid prevents its accumulation within

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the liver and hence the tissue damage which might well ensue; in animals where hepatic sulphation is less well developed, lithocholate accumulation does occur and is associated with changes in liver structure and function [6].

Despite this, surprisingly little is known about the excretion of faecal bile acid sulphates in man [7,8] and to date no methods for measuring faecal bile acid sulphates have been published. We have modified an established procedure [9] for faecal bile acid determination based on a technique for the analysis of urinary bile acid sulphates [10], to enable us to quantitate the proportion of faecal bile acids excreted as sulphate esters.

METHOD

The method used was modified from technique of Grundy et al. [9] for measuring faecal bile acids. The procedure is illustrated schematically in Fig. 1. Details of the steps involved were as follows.

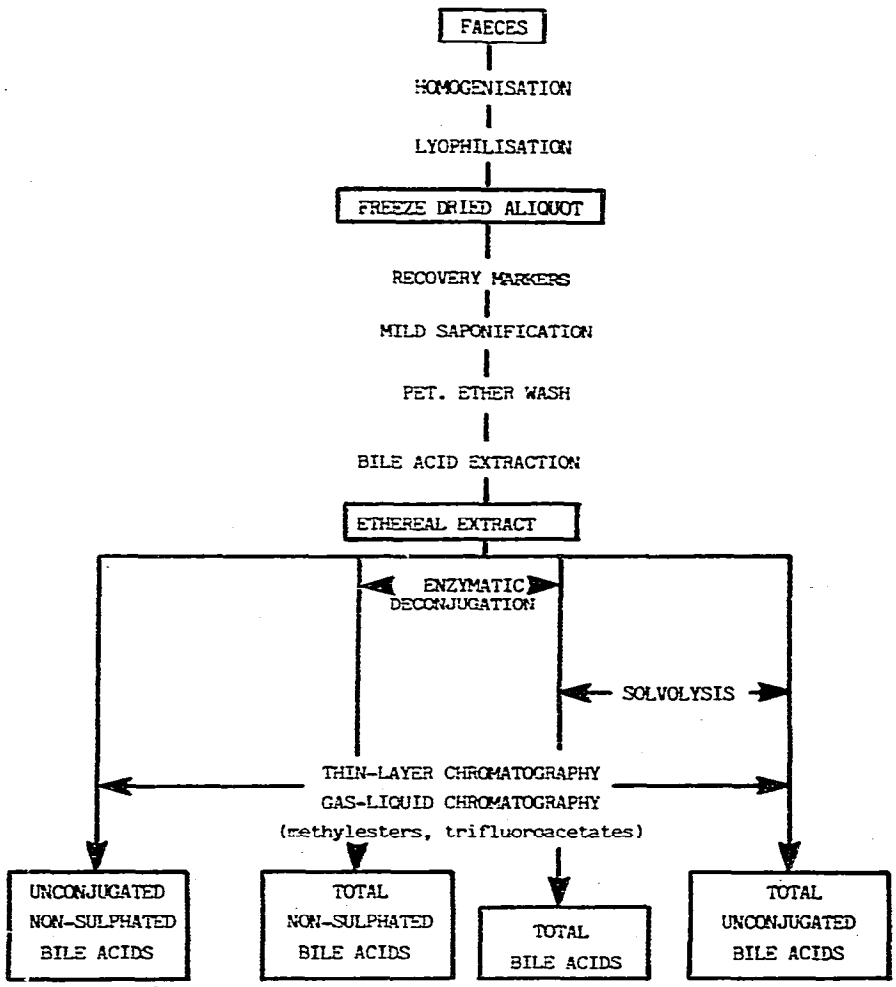

Fig. 1. Scheme of the steps involved in the separation procedure.

Homogenisation

The faecal samples (usually 72-h collections) were homogenised at 4°C in distilled water, the final weights of homogenate being adjusted to 2500 g. Aliquots (25 ml) of the homogenate were then freeze-dried and weighed.

Mild saponification

To approximately 800 mg freeze-dried material [2,4-^{14}C]chenodeoxycholic acid (10^4 dpm) and [^{3}H]cholesterol (10^4 dpm) were added as recovery markers of acidic and neutral sterols together with 80 ml 1 *M* sodium hydroxide (in 90% ethanol). The mixture was refluxed with continuous stirring for 2 h and then centrifuged at 3500 *g* for 10 min. The resulting pellet was washed with 20 ml 1 *M* sodium hydroxide (in 50% ethanol) and the washings pooled with the original supernatant.

Petroleum ether wash

The pooled supernatant was transferred to a separating flask and 20 ml water with 150 ml petroleum ether (b.p. 00—00°C) added. Following vigorous shaking by hand during 1 min the lower phase, which contained the bile acids was kept, and the upper phase washed with 20 ml 1 *M* sodium hydroxide (in 50% ethanol). The ethanolic washings were pooled. The upper phase, which contained the [^{3}H]cholesterol was sampled for scintillation counting.

Bile acid extraction

The pooled aqueous ethanol fraction was evaporated to approximately 50 ml and the mixture then acidified to pH < 1 with 11.5 *M* hydrochloric acid. This acidic phase was then promptly extracted with diethyl ether (3 × 150 ml). The ethereal washings were pooled, washed with water and divided into 8 equal volumes, each of which was evaporated to dryness under air in a fume cupboard, the test tubes being placed in a water bath at 50°C to hasten the process. The samples were then subjected to one of the four pathways illustrated in Fig. 1, each being carried out in duplicate.

Enzymatic deconjugation

The method of deconjugation used was essentially that of Nair et al. [11]. The ethereal residue was dissolved in 0.6 ml methanol and 0.066 *M* phosphate buffer (5 ml) was added. The pH of the mixture was adjusted to 5.6 and the test tubes placed in a water bath at 37°C for 10 min. *Clostridium welchii* solution (Sigma, St. Louis, MO, U.S.A.; 0.5 ml) was then added and the incubation period continued for 4 h. A standard aqueous 2 m*M* taurocholate solution was carried through with each batch of analyses to ensure completeness of the enzymatic conjugation. The pH was lowered to < 1 with 11.5 *M* hydrochloric acid and the bile acids rapidly extracted with 3 × 15 ml diethyl ether. The ether extracts were washed with water and then evaporated to dryness.

Solvolysis

The method used for solvolysis was essentially that of Burstein and Lieberman [12]. The ethereal residue was dissolved in 1 ml absolute ethanol and 0.5 ml 2 *M* hydrochloric acid added. The samples were subjected to three 10-sec

bursts of ultrasonic agitation. Acetone (2 ml) was then added and the solutions incubated at 37°C for 2 days. Following evaporation to dryness, the residue was dissolved in 4 ml of 5% methanolic potassium hydroxide and refluxed for 1.5 h. The solution was evaporated to dryness under vacuum. The residue was dissolved in 1 ml water, the solution acidified to pH < 1 and the bile acids extracted into diethyl ether (3 × 10 ml).

Thin-layer chromatography

All fractions were subjected to thin-layer chromatography (TLC) on 0.5-mm silica gel G. The plates were first developed in hexane—ethyl acetate (9:1) and then in isooctane—ethyl acetate—acetic acid (10:10:2) [13]. Following location with iodine, the unconjugated bile acid areas of each plate were scraped into a column fitted with a sintered glass disc and the bile acids were then eluted with 40 ml chloroform—methanol (2:1).

Gas—liquid chromatography

The bile acid methyl esters were prepared with diazomethane [14]. The apparatus used was a Pye GCV gas—liquid chromatograph fitted with an autoinjection system (Pye Unicam, Cambridge, Great Britain). A preliminary chromatogram was obtained from each sample in order to select the most appropriate internal standard (such as 7-ketocholanoic acid, hyodeoxycholic acid, or hyocholic acid). The solution of bile acid methyl esters was evaporated to dryness and the internal standard added (200 μg in 200 μl methanol). A further 200 μl methanol was added and the solution Vortex mixed. An aliquot (1 μl) was then injected onto a 3% QF-1 column, temperatures: injection 250°C; column 240°C and detector 260°C. The remaining solution was evaporated to dryness and the trifluoroacetate derivatives of the bile acid methyl esters prepared [15]. Automatic injection was made onto the same column, temperatures: injection 230°C; column 230°C and detector 250°C. Identification was thus made using the peak shift technique [14].

RESULTS

Recovery experiments

(i) Recoveries of ^{14}C-labelled bile acids added as aqueous solutions (2 m*M*) to faecal samples before homogenisation and subjected to the whole procedure were (mean ± S.D., n = 6 in each case): lithocholic acid 102 ± 2.9%; deoxycholic acid 99 ± 2.1%; chenodeoxycholic acid 99 ± 4.0%; cholic acid 97.5 ± 2.5%; glycocholic acid 84.2 ± 6.8%; glycochenodeoxycholic acid 84.2 ± 6.7%; taurocholic acid 68.9 ± 10.3%.

The poor recovery of taurocholic acid is probably explained by the fact that although its enzymatic deconjugation was maximum at 4 h, it was only 75.2 ± 5.6% complete. The degree of deconjugation obtained with glycocholic acid was 92.1 ± 2.5% and with glycochenodeoxycholic 89.1 ± 4.1%. Conversion of conjugated bile acid to unconjugated bile acid because of inadvertent hydrolysis during reflux was approximately 12% for glycochenodeoxycholic and glycocholic acids but only 5% for taurocholic acid.

(ii) Recovery of [3-$^{35}SO_4$]-lithocholic acid [16] added in aqueous solution to the faecal samples before homogenisation was 85.0 ± 5.2%. The solvolysis procedure was applied to standard preparations of the 3α-sulphates of lithocholic, chenodeoxycholic, deoxycholic, cholic and taurolithocholic acids and to the 3,7-disulphate derivative of chenodeoxycholic acid. After solvolysis and using the TLC system of Cass et al. [17] only the parent acid of each preparation was detected.

(iii) Completeness of extraction of bile acids was estimated by comparing the amount of radioactivity recovered from a faecal sample (containing ^{14}C-bile acids) in a patient who had ingested [^{14}C]chenodeoxycholic acid (whilst on a normal solid diet), using the above procedure with that obtained by direct oxidation in an Intertechnique sample oxidiser. The recovery after 1 h reflux was 91 ± 0.4% ($\bar{x}$ ± S.D.; n = 3) and after 2 h reflux 99 ± 0.1% of that obtained by sample oxidation.

Reproducibility studies

(i) The results obtained by replication (n = 6) of the analysis of a sample, each replicate being the mean of duplicate determinations are given in Table I.

(ii) The recovery of [^{14}C]chenodeoxycholic acid carried through with each analysis was 77 ± 6% ($\bar{x}$ ± S.E.M.; n = 124).

TABLE I

BILE ACID FRACTIONS OF ONE SAMPLE ANALYSED IN DUPLICATE SIX TIMES

Figures denote $\bar{x}$ ± S.D. (mg/24 h) values.

	Total	Total non-sulphated	Unconjugated non-sulphated	Total unconjugated
Lithocholic	41.4 ± 1.9	37.9 ± 3.0	14.1 ± 1.3	16.9 ± 1.6
Deoxycholic	61.2 ± 3.0	49.3 ± 1.1	38.0 ± 3.2	48.3 ± 3.0
Chenodeoxycholic	66.8 ± 1.4	65.1 ± 2.9	47.1 ± 3.1	47.8 ± 2.8
Ursodeoxycholic	38.1 ± 1.1	35.5 ± 2.7	25.0 ± 1.7	27.2 ± 2.2
Cholic	31.0 ± 1.6	23.2 ± 2.0	18.7 ± 1.3	22.4 ± 1.6

Qualitative demonstration of the presence of bile acid sulphates in human faeces

To date, no means of measuring bile acid sulphates directly in biological samples has been described and all the techniques used require solvolysis, i.e. hydrolysis of the sulphate ester followed by estimation of the parent acid. Although the approach used in the present study was similar to that previously used for bile [18], serum [19] and urine [10] in that the sulphate contribution was determined by difference, it was considered necessary to provide some direct demonstration of the presence of bile acid sulphates. This was done as follows:

(i) Six ethereal extracts (Fig. 1) prepared from faecal samples as described above were, before solvolysis, subjected to TLC using hexane—ethyl acetate (9:1) and isooctane—ethyl acetate—acetic acid (10:10:2) [13]. The unconjugated bile acids were visualised in iodine. The chromatogram was further developed in chloroform—methanol—acetic acid—water (65:24:10:5) [20] when the unconjugated bile acids moved towards the solvent front and several

additional bands were seen, which corresponded in R_F to the published values for bile acid sulphates. Lithocholic acid sulphate was identified by repeating the third development after including taurolithocholic, lithocholic and lithocholic acid sulphate as standards. The presence of lithocholic acid sulphate was confirmed by the use of two-dimensional TLC. The solvent system chloroform—methanol—acetic acid—water (65:24:15:9) [17], was used in the first direction and ethyl acetate—butanol—acetic acid—water (8:6:3:3) [21] at right angles to the first direction. Lithocholic acid sulphate was included as standard for the second development. Lithocholic acid sulphate was clearly demonstrated.

(ii) Four of the ethereal extracts were subjected to Sephadex LH-20 column chromatography as described by Stiehl et al. [22]. Four fractions were eluted with chloroform—methanol (1:1) and four with methanol. Each fraction was subjected to TLC examination for unconjugated bile acids and sulphated bile acids using the methods described above. Sulphated bile acids were found only in the last two fractions eluted and solvolysis followed by gas—liquid chromatography confirmed the presence of lithocholic, deoxycholic and chenodeoxycholic sulphates.

Normal values

The mean total faecal bile acid excretion in 15 control subjects (8 males, 7 females, mean age 41 ± S.E.M. 5.8 years, range 17—84) was 603 (± S.E.M. 71) mg/day. The amounts of the major bile acid fractions are shown in Table II.

TABLE II

CONCENTRATIONS OF MAJOR BILE ACID FRACTIONS FOUND IN CONTROL SUBJECTS

n = 15; bile acid fractions (mg/24 h, $\bar{x}$ ± S.E.M.).

Bile acid	Total	Total non-sulphated	Total unconjugated	Unconjugated non-sulphated
Lithocholic	175 ± 30	118 ± 26	122 ± 29	97 ± 27
Deoxycholic	90 ± 18	60 ± 8	59 ± 10	47 ± 8
Chenodeoxycholic	15 ± 7	13 ± 7	6 ± 3	5 ± 2

DISCUSSION

None of the methods previously described for faecal bile acid measurement in man have deliberately included a solvolysis step. Our results and those of other workers [8] indicate that bile acid sulphates may form a considerable proportion of the total bile acids excreted in man and should be either knowingly included or excluded in such determinations.

Without the aid of mass spectrometry it is questionable whether one can confidently analyse the complex mixture of bile acids found in human faeces [23]. Studies using gas chromatography with mass spectrometry [23,24] have shown that whilst deoxycholic and lithocholic acids are quantitatively the most important bile acids there are a large number of minor components which together make a significant contribution to the total amount of faecal bile acids. In normal subjects these minor bile acids have been reported to account for as

much as 40% total faecal bile acids [25]. In the present study the problem of accurate quantitation without the use of mass spectrometry is further complicated by the inclusion of bile acid sulphate determination.

The retention times of the different bile acids were established with standards; where standards were not available the values given in the literature were used for comparison.

The difficulties of accurate sulphate determination are considerable and it must be borne in mind that:

(1) in vitro solvolysis by faecal bacteria may occur during homogenisation [26],

(2) acid catalysed solvolysis [27] may occur in the presence of most organic solvents including diethyl ether [28] and

(3) low pH (pH < 1) is essential for quantitative extraction of sulphates from aqueous solutions. Thus any step for extraction of bile acid sulphates must be performed at low pH and with speed if inadvertent solvolysis is to be avoided. It is probable that methods which use prolonged acid extraction procedures [29] include measurement of bile acid sulphates as their parent acids.

(4) Although enzymatic hydrolysis of conjugated bile acid sulphates has been described by other workers [21,22] to-date no detailed studies have been made of either the degree of sulphation on the rate of hydrolysis or of the nature of products.

There is considerable variation in the reported normal ranges for faecal bile acids in man. One important factor in this variation must be methodological differences. For example, results for the synthesis of bile acids (input into pool) obtained using isotopic methods are usually higher than the results obtained by chemical analysis of faecal bile acids (output from pool [30]). The findings in the present study are similar to the results obtained using isotopic techniques. If, however, the sulphate contribution is omitted then the results are similar to those found by many workers using chemical analyses.

The above procedure, therefore, whilst it provides no information on the type of bile acid sulphate present, does allow one by means of a simple addition to a well established procedure, to measure all the fractions which constitute faecal bile acid excretion in man.

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