Isotope derivative assay of human serum bile acids

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Summary A new method for the selective determination of the main serum bile acids has been developed. Serum samples with added ^{14}C-labeled bile acid were submitted to deproteinization, alkaline hydrolysis, methylation, and were then chromatographed on alumina before acetylation with 2 μl of [^{3}H]acetic anhydride. Excess reagent was eliminated by evaporation; elimination of residual tritiated contaminants and separation of the doubly labeled bile acid derivatives were obtained by thin-layer chromatography, column chromatography on Lipidex 5000, and crystallization. The sensitivity of the method is about 10 pmol of each bile acid. Analyses of seven sera with normal or elevated concentration of bile acids by the proposed method and gas–liquid chromatography showed a close correlation (r = 0.94; slope = 0.93).—**Pageaux, J. F., B. Duperray, M. Dubois, and H. Pacheco.** Isotope derivative assay of human serum bile acids. *J. Lipid Res.* 1981. **22:** 725–729.

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Determination of serum bile acid levels may afford sensitive and specific detection of hepatic or intestinal dysfunctions in man (1–4). At the present time, such a determination may be obtained by several methods, each attended by drawbacks. Enzymatic methods are neither very sensitive nor specific and permit, in general, only total bile acid determination (5); radioimmunologic methods are very sensitive but exhibit some lack of specificity (6, 7). Chromatographic methods are very specific and allow simultaneous determination of all serum bile acids but they lack sensitivity and hence require relatively large serum samples (8–12). Moreover, the bile acid levels determined by enzymatic methods do not agree very well with those obtained by chromatographic and radioimmunologic methods (13, 14). In this report we describe a double isotope derivative assay of human bile acids which is as selective and specific as chromatographic methods and as selective as radioimmunologic assays.

Abbreviations and trivial names: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; LC, lithocholic acid, 3α-hydroxy-5β-cholanoic acid; DC, deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; CDC, chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; C, cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid. The prefixes glyco (G) and tauro (T) are used for bile acids having glycine or taurine in amide linkage at C-24.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade. Bile acids were purchased from Steraloids Inc, Wilton, NH. Lipidex 5000 was purchased from Packard Instruments, Downers Grove, IL. Amberlite XAD-2, 20–50 mesh, was obtained from Fluka, A.G., Buchs, S.G., Switzerland. Silica gel 60 (70–230 mesh) and Silica gel G were purchased from Merck, A.G., Darmstadt, Federal Republic of Germany. [^{3}H]Acetic anhydride (sp act 100 mCi/mmol), [24-^{14}C]lithocholic acid (59 mCi/mmol), [24-^{14}C]deoxycholic acid (52 mCi/mmol), [24-^{14}C]chenodeoxycholic acid (50 mCi/mmol), and [24-^{14}C]taurocholic acid (54 mCi/mmol) were obtained from Radiochemical Centre, Amersham, England. Before use, [^{3}H]acetic anhydride was diluted with nonradioactive acetic anhydride and distilled under vacuum. The specific activity of each batch of [^{3}H]acetic anhydride was determined by acetylating samples of known mass of methylated [^{14}C]bile acid and determining the amount of ^{3}H incorporated into samples as described below.

Gas–liquid chromatography

Serum bile acids were analyzed, as acetate derivatives, according to a method described previously (15, 16). Three-ml serum samples were used.

Radioactivity determination

A Packard liquid scintillation spectrometer, model 3320, was used for simultaneous determination of ^{3}H and ^{14}C radioactivities, using Unisolve 1 (Kock-Light Lab.) as scintillator liquid.

Sample preparation

Serum samples (0.5 ml) received 0.5 to 2 nCi (10 to 40 pmol) of each ^{14}C-labeled bile acid, and they were then processed according to a method previously described (15, 16). Briefly, serum bile acids were extracted on a XAD-2 column, deconjugated by alkaline hydrolysis, methylated by diazomethane, and purified on a neutral alumina column. The eluate was evaporated to dryness. The dry samples were treated with 60 μl of the reagent mixture hexane–benzene–[^{3}H]acetic anhydride–dry pyridine 30:30:2:1 (v/v), at 85°C for 72 hr in a screw-capped tube.

Purification of doubly labeled bile acid derivatives

After cooling, 1 ml of ethanol was added and the sample was held at laboratory temperature for 30 min. Solvents and reagents were evaporated under vacuum at 40–45°C. This procedure was repeated. The dry residue was dissolved in 5 ml of diethyl ether and washed once with 2 ml of 0.1 N NaOH and then twice with 5 ml of water; the solvent was then evaporated under a stream of nitrogen. The residue was dissolved in 0.2 ml of acetone containing, as carrier, 100 μg of each methylated and acetylated derivative of the bile acids under study. This solution was chromatographed on Silica gel G plates (0.2 mm thick) developed in isooctane–ethyl acetate–acetic acid 60:39:1 (v/v).

Each assay was run between two samples of 100 μg of carriers used as position standards. Standards were located by spraying an ethanolic solution of 2,7 dichlorofluorescein. The bands corresponding to the doubly labeled bile acid derivatives were scraped off the plates together and eluted with 2 × 5 ml of hot methanol–ethyl acetate 1:4 (v/v).

The solvent of the combined eluates was evaporated under nitrogen. The residue, dissolved in isooctane, was applied on a Lipidex 5000 column (30 × 1.4 cm id, 0.1 ml/min flow rate) with isooctane as eluting solvent. The fractions containing the doubly labeled derivatives were collected separately. The standard elution volumes were 1.27, 1.73, 1.97, and 3.02 for the methylated and acetylated derivatives of LC, DC, CDC, and C, respectively. To each fraction, 25 mg of the corresponding unlabeled carrier was added. The solvent was evaporated under nitrogen and residues were recrystallized from hexane. The crystals were washed with cold hexane, and dissolved in 10 ml of liquid scintillator and their radioactivities were measured. Suitable corrections were made for background and crossover of the two labels. The amount (μmol) of each bile acid contained in the serum sample was calculated according to Eq. 1:

$$m = \frac{Y}{A}\frac{X}{X'}\frac{2}{n} - M \qquad \text{Eq. 1}$$

Y: net counting rate (cpm) for ^{3}H in the first channel.

X′: net counting rate (cpm) for ^{14}C in the second channel.

X: net counting rate (cpm) for ^{14}C obtained in the second channel with M μmol of ^{14}C-labeled standard in the counting vial.

A: acetic anhydride specific activity (cpm × μmol^{-1}).

n: number of hydroxyl groups per bile acid molecule.

M: amount (μmol) of ^{14}C-labeled standard added to serum sample.

Validation of the method

It is important to verify that all the serum extractable forms of a given bile acid are recovered in the same proportions after they have all been converted into free bile acids. The recoveries were determined using ^{14}C-labeled bile acids (60 pmol) and unlabeled bile acids (12 nmol) added to 0.5 ml normal serum samples. Acetylation yield was determined using GLC and TLC analysis of the reaction products of different experiments with unlabeled or labeled standards and pathologic or normal serum samples.

Radiochemical purity of doubly labeled bile acid derivatives

Doubly labeled derivatives of CDC and C obtained from a serum sample were recrystallized three times after their separation on Lipidex 5000. The $^{3}H/^{14}C$ ratio was determined after each crystallization. Doubly labeled crystals of LC obtained from a serum sample were chromatographed on a Silica 60 column (15 × 1.5 cm id) and eluted with isooctane–ethyl acetate 15:1 (v/v). $^{3}H/^{14}C$ ratio was determined in each 5-ml eluate fraction (**Fig. 1**).

To test the mutual contamination of doubly labeled derivatives of DC and CDC, mixtures of 100 μg of each compound were separated on Lipidex 5000, and the fractions were crystallized twice; aliquots were counted at each stage.

Mixtures of the two derivatives, only one of them being ^{14}C-labeled, were recrystallized twice after addition of 25–130 mg of the unlabeled carrier corresponding to the unlabeled acid in the mixture. After each crystallization, aliquots of crystals and supernatants were counted. The amount of DC and CDC resulting from mutual residual contamination can be estimated by assuming that recovery rates of the two dihydroxylated acids are essentially identical at the end of TLC step.

RESULTS AND DISCUSSION

The reliability of the proposed method lies in two main conditions. First, ^{14}C specific activity of bile acids must not undergo any modification during the sample preparation; and second, the doubly labeled derivatives must be radiochemically pure. The first condition is satisfied if all the forms (free, glyco-, or tauro-

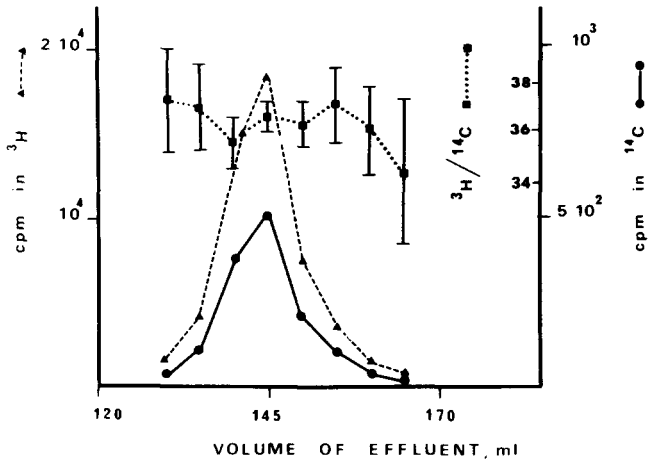

Fig. 1. Silica column chromatography of the doubly labeled lithocholic acid derivative. Each fraction was assayed for ^{14}C and ^{3}H by scintillation counting. The $^{3}H/^{14}C$ ratio was determined with its error (vertical bars).

conjugates) of a given bile acid display equal losses (in percent) during all the procedures preceding hydrolysis. The results shown in **Table 1** agree well with this first condition; no significant difference could be found between recoveries of the different forms of a given bile acid. Furthermore, for a given form of a bile acid, recovery did not depend upon the serum concentration.

The second condition is satisfied if: *i*) incompletely acetylated ^{14}C-labeled bile acid is not present (However under our conditions, acetylation is complete for standard methylated bile acid in amounts equal to or lower than 1.27 μmol. No incompletely acetylated bile acid was detected by GLC or TLC when pathological or normal serum samples were acetylated.); *ii*) the separation of the ^{3}H, ^{14}C-labeled bile acid is complete; and *iii*) the elimination of any tritiated molecule without ^{14}C-label is obtained.

The complete separation of DC and CDC derivatives is very difficult to obtain. After Lipidex chromatography the DC fraction was contaminated by 8 to 12% with CDC, and the CDC fraction was contaminated by 10% with DC. However, experiments performed with DC and CDC mixtures showed that the first crystallization reduces by 10- to 20-fold the residual contamination of one acid by the other. Taking into account the measured losses during each purification step, the above results permit the determination of the systematic error of DC and CDC concentrations. The error of CDC determination is always negligible (<2%). The relative error of DC concentration only becomes important (>10%) in the case of CDC/DC ratio higher than 10, which, in fact, always corresponds to a very low DC level. However, in all cases, results may be corrected by calculation.

The elimination of any tritiated substances without ^{14}C-label is fairly easy if acetic anhydride is distilled first before use. **Table 2** shows the variations of $^{3}H/^{14}C$ ratio of CDC and C, obtained from human serum, during purification steps following tritiation. **Fig. 2** illustrates the $^{3}H/^{14}C$ ratio of silica column eluate fractions of crystallized LC derivative. No significant variation of $^{3}H/^{14}C$ ratio was found. Radioactivity was never found outside the LC derivative peak. In both experiments the tritium contamination was null after the first crystallization. The proposed method displays a great specificity because of the independence of chemical purity of the final products.

Available acetic anhydride specific activity is high enough not to limit the sensitivity of the proposed method. The sensitivity depends only on ^{14}C-labeled bile acid specific activity which determines the minimum amount to be added to serum (8 pmol). The sensitivity of the method is therefore about 10 pmol of each bile acid. The reproducibility of the method was evaluated with a standard sample of [^{14}C]deoxycholic acid. The coefficient of variation was 2.7% and 6.9% when the samples contained, respectively, 127 and 6.5 nmol of bile acid (n = 6).

In spite of clear advantages, this method is time-consuming (8 working days for 20 serum samples), quite expensive (in equipment investment), and it requires a well-trained technical assistant. For those reasons, it cannot be proposed as a routine method in clinical use; however, the procedure may prove efficient for research purposes in cases where a precise and

TABLE 1. Serum bile acid recoveries (%) at the end of the sample purification procedure on an alumina column

	[^{14}C]LC	GLC	CDC	[^{14}C]CDC	TCDC	[^{14}C]DC	GDC	TDC	C	GC	TC	[^{14}C]TC
Recovery[a]	82.5 ± 1.6	79.5 ± 2.4	84.9 ± 1.3	83.4 ± 1.4	82.4 ± 1.7	82.7 ± 1.8	81.4 ± 1.3	80.2 ± 1.9	78.6 ± 1.4	83.7 ± 2.4	84.1 ± 2.1	81.1 ± 0.8
Number of determinations	16	8	8	16	6	6	8	4	8	4	6	36

[a] Mean ± SEM.
Unlabeled bile acids (12 nmol) or ^{14}C-labeled bile acids (60 pmol) were added to 0.5 ml serum samples.

TABLE 2. Variations of $^3H/^{14}C$ ratio of serum chenodeoxycholic and cholic acids during purification steps after acetylation

Step	Chenodeoxycholic Acid			Cholic Acid		
	^3H	^{14}C	$^3H/^{14}C$[a]	^3H	^{14}C	$^3H/^{14}C$[a]
	cpm			*cpm*		
TLC	126,786	1,318	96.2 ± 1.9	102,404	1,724	59.4 ± 1.1
Lipidex chromatography	82,730	1,021	81.0 ± 1.9	68,009	1,241	54.8 ± 1.1
1st crystallization	57,379	719	79.8 ± 2.4	41,380	806	51.3 ± 1.3
2nd crystallization	41,231	527	78.2 ± 2.5	25,112	482	52.1 ± 1.8
3rd crystallization	24,918	315	79.1 ± 3.1	11,742	228	51.5 ± 2.5

[a] The $^3H/^{14}C$ ratio is given with the corresponding counting rate error for ^3H and ^{14}C considered as $\sqrt{N/n}$ (N, net counting rate; n, number of determinations).

No deoxycholic nor lithocholic acids were detected in this serum sample.

specific measure of many bile acids from small amounts of biological fluids with low bile acid concentrations is needed (e.g., chronic studies on small animals, or primary bile acid secretion by isolated hepatocytes). Serum concentrations of the main bile acids measured by the proposed method in seven specimens (range 0.12–52.7 μmol/l) were linearly correlated with the respective values obtained by a GLC method ($r = 0.94$, $P < 0.05$). The slope (0.93) was not significantly different from 1 (Fig. 2). This demonstrates the high specificity of our chromatographic method. The proposed method is more sensitive than most commonly used chromatographic procedures (8–12) except when they are coupled with mass spectrometry detection (17). GLC has the advantage of detecting unsuspected bile acids but it is important to note that those chromatographic methods are not always able to measure lithocholic acid.

Our method is as sensitive as radioimmunologic methods (18, 19) but it is more specific (6, 7) and allows the simultaneous determination of the main serum bile acids. Except for the time required for analysis, our method is superior to any enzymatic method.

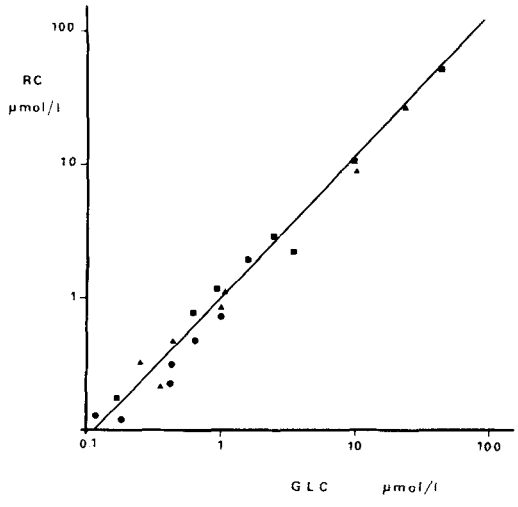

Fig. 2. Comparison of radiochemical (RC) and GLC determinations of serum bile acids in seven sera. For convenience a log-log scale has been used. Lithocholic acid was not measured by GLC. Regression line: $y = 0.93x + 0.05$ ($r = 0.94$; $n = 21$). (●) Deoxycholic acid; (■) chenodeoxycholic acid; (▲) cholic acid.

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