Tauro-7α,12α-dihydroxy-5β-cholanic acid as internal standard in the gas–liquid chromatographic analysis of bile acid methyl ester acetates

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Abstract Tauro-7α,12α-dihydroxy-5β-cholanic acid has been used as internal standard in the gas–liquid chromatographic analysis of bile acid methyl ester acetates. The advantage of this compound over other internal standards is that its use takes into account the hydrolysis rate of the bile acids. The entire procedure is monitored by thin-layer chromatography, gas–liquid chromatography, and by radioactivity measurement and zonal scanning.—**Ghoos, Y., P. Rutgeerts, and G. Vantrappen.** Tauro-7α,12α-dihydroxy-5β-cholanic acid as internal standard in the gas–liquid chromatographic analysis of bile acid methyl ester acetates. *J. Lipid Res.* 1983. **24:** 1376–1379.

Supplementary key words gas–liquid chromatography • bile acid methyl ester acetates

Bile acid methyl ester acetates are suitable for gas–liquid chromatography and further mass spectrometric analysis as they have a high stability and a low molecular weight, and do not introduce isotopic complexity (1). Hitherto however, only free bile acids were used as internal standards in the synthesis procedures (2–9). They do not take into account the hydrolysis steps by alkaline hydrolysis or enzymatic cleavage (cholylglycine hydrolase) that are currently used. But, alkaline hydrolysis destroys keto bile acids (10, 11) while enzymatic cleavage may be affected by unknown inhibitors (12). Therefore, we introduced tauro-7α,12α-dihydroxy-5β-cholanic acid as internal standard. By adding this internal standard to the biological sample, all steps of the bile acid methyl ester acetate synthesis and analysis can be monitored.

MATERIALS AND METHODS

Bile acids

TPhDCA, GPhDCA and PhDCA were purchased from Calbiochem-Behring Corp. (La Jolla, CA). All other bile acids (free and glyco- and tauro-conjugated CA, CDCA, UDCA, DCA, and LCA) were Steraloid products (Wilton, NH). The following labeled bile acids were purchased from the Radiochemical Centre (Amersham, England): tauro-[carboxyl-^{14}C]cholic acid, [1-^{14}C]glycocholic acid, [carboxyl-^{14}C]cholic acid, [carboxyl-^{14}C]lithocholic acid, and [carboxyl-^{14}C]-chenodeoxycholic acid. [^{3}H(G)]Glycochenodeoxycholic acid was obtained from New England Nuclear (Boston, MA). 7,12-DCA and 7,12-DCA conjugates were checked for purity by TLC in the solvent system propionic acid – isoamylacetate – propanol – water 15:20:10:5 (solvent A) (13). The other bile acids were also assayed by the 3α-hydroxysteroid dehydrogenase technique (14). The bile acids were at least 98% pure. Labeled bile acids were checked for chemical and radiochemical purity by TLC in solvent A and by subsequent zonal scanning (Berthold, model LB 2733; Wildbad, West Germany). The compounds were found to be more than 99% pure. UDCA was contaminated with CDCA as revealed by GLC. The corresponding correction was made. The bile acid methyl ester acetates were run by TLC in the solvent system heptane–diethylether 40:60 (solvent B) (15).

Study of 7α,12α-dihydroxy-5β-cholanic acid conjugates as internal standards during deconjugation

To determine whether the 7,12-DCA conjugates are deconjugated in the same way as the common bile acids, 40 mM, 20 mM, and 10 mM solutions of TCA, TCDCA, TDCA, and TLCA were prepared and mixed with the corresponding concentration of a tauro-7,12-DCA solution. To each solution 18.5 kBq (0.5 μCi) of ^{14}C-la-

Abbreviations and trivial names: LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid. Dihydroxy bile acid (2OH-BA) refers to all dihydroxy-5β-cholanic acids. The prefixes glyco (G) and tauro (T) are used for bile acids having glycine or taurine in the amide linkage at C-24. Me-Ac, bile acid methyl ester acetates; BA, bile acids; GLC, gas–liquid chromatography; IS, internal standard; RF, response factor; R_t, retention time; RWR, relative weight response; TLC, thin-layer chromatography.

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beled TCA, ^{14}C-labeled GCA, and ^{3}H-labeled GCDCA were added. Two hundred μl of the solutions was used for deconjugation. The degree of hydrolysis was monitored by TLC and by zonal radioactivity scanning. This control procedure was repeated for the corresponding glyco-conjugated bile acids.

Preparation and gas–liquid chromatography of bile acid methyl ester acetates

The bile acid methyl ester acetates were prepared as described previously (16). Gas–liquid chromatography was carried out in a Packard-Becker gas chromatograph, model 433 (Delft, The Netherlands). The apparatus was equipped with a flame ionization detector and the separation was achieved on a Gas-Chrom Q 100–120 mesh column, activated by 3% OV-225 (Applied Science Laboratories, State College, PA) (17). The inlet and outlet temperatures were kept at 260°C. The analysis was done isothermally at 250°C. Helium was used as the carrier gas at a flow rate of 20 ml per min. To test the internal standard (IS) we also tried QF-1 as the liquid phase under the following conditions: 1% QF-1 on Gas-Chrom Q 100–120 mesh; carrier gas, N_2 at 40 ml per min; inlet and outlet temperatures, 250°C; column temperature, 240°C for 20 min, increased to 250°C at a rate of 5°C per min.

Preparation of standard curves

The methyl ester acetates of LCA and DCA and 3β-hydroxy-5-cholenic acid were obtained from Steraloids. The acetates of 3β-hydroxy-5α-cholestane and 3β-hydroxy-5β-cholestane, and the methyl ester of 3-keto-5β-cholanic acid were also obtained from Steraloids. 7,12-DCA, CDCA, UDCA, and CA were prepared by methylation and acetylation of the free bile acids. The purity of the bile acid derivatives was determined by TLC and GLC and by zonal scanning.

Calibration and calculations

The R_f and RWR values (18) were determined for each bile acid. The amount of bile acid (μmol of BA) present in a sample was calculated by the following formula:

$$\mu\text{mol}_{BA} = \frac{\text{area}_{BA}}{\text{area}_{IS}} \times \frac{R_{f_{BA}}}{\text{RF}_{IS}} \times \mu\text{mol}_{IS} \text{ added.}$$

Application of the method to biological samples

A known quantity of bile acids was added to 2 ml of serum and also to 200 μl of duodenal aspirate. The bile acids were deconjugated, and the free bile acids were methylated and acetylated.

Comparison of tauro-7,12-DCA and 7,12-DCA as internal standards

To investigate if quantitative differences exist in using conjugated versus unconjugated internal standards, the following procedure was followed. To 200 μl of bile sample, 200 μl of 5 mM tauro-7,12-DCA and 200 μl of 5 mM 7,12-DCA were added. These analyses were repeated six times for patient 1 and three times for patient 2.

RESULTS

Hydrolysis rate of tauro- and glyco-7,12-DCA

The hydrolysis rate of 200 μl of tauro- and glyco-7,12-DCA (10 mM, 20 mM, and 40 mM) was compared with the hydrolysis rate of all other conjugated bile acids. In all instances there was complete hydrolysis after an overnight (16 hr) incubation at 37°C. An exception was for 40 mM TCA, which was only 91% hydrolyzed.

Gas–liquid chromatography of bile acids with tauro-7α,12α-dihydroxy-5β-cholanic acid methyl ester acetate as internal standard

In **Fig. 1** the GLC pattern of a mixture of bile acid standards is shown. Approximately equal concentrations of the bile acid methyl esters of LCA, 7,12-DCA, and DCA were chromatographed, whereas double concentrations of CDCA, UDCA, and CA were injected. Cholesterol, which is always present in bile samples, was chromatographed with the bile acids.

In **Table 1** the RWR values are expressed in relation to the values obtained for the internal standard, taken as 1. Table 1 also shows the retention times of the bile

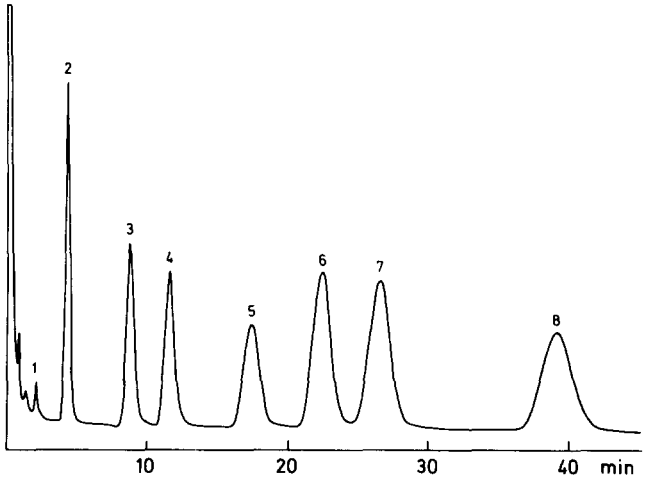

Fig. 1. The GLC pattern of a mixture of bile acids and cholesterol standard on 3% OV-225-column. 1, Unidentified compound; 2, cholesterol; 3, LCA; 4, 7,12-DCA; 5, DCA; 6, CDCA; 7, UDCA; 8, CA.

TABLE 1. The RWR values and the retention times of the bile acid Me-Ac and related compounds

Bile Acid	RWR		R_t	
	3% OV-225	1% QF-1	3% OV-225	1% QF-1
Cholesterol	1.15	1.20	0.24	0.26
LCA	1.08	1.04	0.50	0.46
7,12-DCA	1.00	1.00	0.67	0.78
DCA	1.01	0.94	1.00	1.00
CDCA	1.08	1.13	1.29	1.26
UDCA	0.97	0.85	1.53	1.42
CA	1.02	0.94	2.27	2.38
3β-Hydroxy-5β-cholestane	1.12	1.15	0.22	0.25
3β-Hydroxy-5α-cholestane	1.11	1.13	0.26	0.29
3β-Hydroxy-5-cholenic acid	1.07	1.04	0.61	0.53
3-Keto-5β-cholanic acid	1.03	0.99	0.69	0.63
3α-Hydroxy-7-keto-5β-cholanic acid	1.01	0.94	1.73	1.47

acid methyl ester acetates and related compounds, relative to DCA taken as 1.

In **Fig. 2,** a typical chromatogram response is shown for the CA concentration versus the ratio CA/7,12-DCA. Similar curves were obtained for the other bile acids, i.e., LCA, DCA, CDCA, and UDCA.

Recovery and reproducibility of bile acids added to biological fluids

The recoveries of LCA, DCA, CDCA, and CA, added as 200 μl or 100 μl of 10 mM solution to a bile sample were 98.9% ± 1.1 and 99.6% ± 1.3, respectively.

The recovery of 200 μl of a 0.10 mM solution added to serum was 97.2% ± 2.1. All recoveries are expressed as mean ± SD; n = 9.

The reproducibility of GLC analysis of a bile acid sample is shown in **Table 2.**

The recoveries of the bile acids from the same duodenal aspirate, whether related to tauro-7,12-DCA or to 7,12-DCA as internal standard are given in **Table 3.**

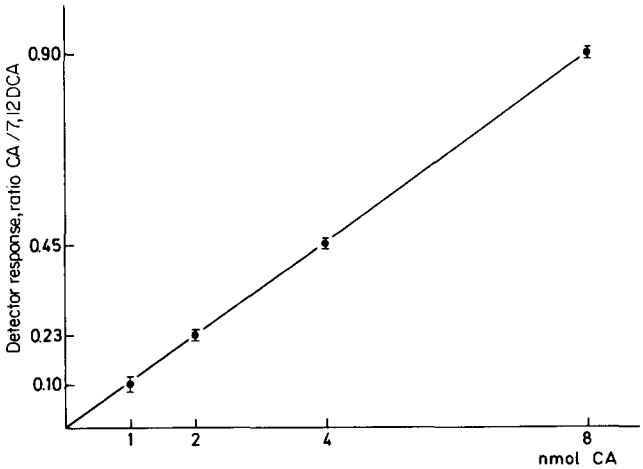

Fig. 2. The response ratio CA/7,12-DCA in relation to the CA concentration (mean ± SD, n = 6).

DISCUSSION

For chromatographic analysis of bile acids, the hydrolysis of the conjugated bile acids is always required. Hitherto no internal standard was available that also included the hydrolysis step. Thus we studied the suitability of tauro-7α,12α-dihydroxy-5β-cholanic acid as internal standard for GLC analysis of bile acids. The bile acid was used as a tauro- rather than a glycoconjugate, because the tauro-form is less susceptible to enzymatic cleavage (12). Tauro-7α,12α-dihydroxy-5β-cholanic acid may be added to the biological sample prior to any other derivation step. For a bile acid derivative to qualify as an accurate standard, it should meet several criteria. *1*) The internal standard should undergo all steps the biological sample is undergoing. Table 3 indicates that the conjugated 7,12-DCA, used in conjunction with the free 7,12-DCA, is able to indicate losses of bile acids, due to the hydrolysis or to the separation procedure. These losses are very small. Nevertheless, in all cases where the free 7,12-DCA was used as internal standard, there was a decrease in bile acid recovery. This means that this systematic loss may be overcome by using the conjugated internal standard. It may certainly be useful under conditions in which the cholylglycine hydrolase is inhibited (12). *2*) The internal standard should be completely separated from other

TABLE 2. Reproducibility of GLC analysis of a bile acid sample (n = 6; mean ± SD)

Bile Acid	Amount
	mmol/l
DCA	0.545 ± 0.027
CDCA	5.077 ± 0.020
UDCA	0.405 ± 0.009
CA	7.565 ± 0.068

TABLE 3. The recoveries of bile acids related to tauro-7,12-DCA and to 7,12-DCA as internal standards (mmol/l; mean ± SD)

	Patient 1 (6 samples)	Patient 2 (3 samples)
Tauro-7,12-DCA	13.593 ± 0.110	4.943 ± 0.101
7,12-DCA	13.104 ± 0.220	4.700 ± 0.064
Level of significance[a]	$0.005 < P < 0.01$	$0.005 < P < 0.01$

[a] Paired *t* test.

components in the sample. Fig. 1 shows that the 7,12-DCA methyl ester acetate is well separated from the bile acids present in biological samples. *3*) The internal standard should elute close to the other components; it should be present in approximately the same amount, and it should have a similar detector response as the other bile acids. 7,12-DCA methyl ester acetate elutes close to litho- and deoxycholic acid methyl ester acetates and is also separated from important cholestane compounds and keto bile acids. The detector response of 7α,12α-dihydroxy-5β-cholanic acid methyl ester acetate is in the same range as the other bile acids and Fig. 2 shows that the detector response is linear and accurate in the concentration range studied. *4*) The internal standard should not occur in any of the samples to be analyzed. 7,12-DCA and its glyco- and tauroconjugates are unusual bile acids, which (until now) have never been found in any biological sample. *5*) The internal standard should be highly pure. The purity of the 7,12-DCA we used was checked by GLC and TLC and found to be highly pure.

As 7α,12α-dihydroxy-5β-cholanic acid meets these criteria, it is a reliable internal standard for the bile acid methyl ester acetates that are used for the GLC analysis of bile acids in biological samples.

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