Glyco-7α,12α-dihydroxy-5β-cholanic acid as internal standard for high-pressure liquid chromatographic analysis of conjugated bile acids

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Summary Glyco-7α,12α-dihydroxy-5β-cholanic acid was tested as internal standard for high-pressure liquid chromatographic analysis of the five main glycine- and taurine-conjugated bile acids present in adult human serum and bile. When the standard is added to the samples before extraction, the recovery rate throughout the procedure is similar to that of other bile acids. For all bile acids studied, the response, relative to the internal standard, is linear at 205 nm. Baseline separation is observed between the internal standard and all other bile acids, both in artificial mixtures and extracts of biological samples. Thus, glyco-7α,12α-dihydroxy-5β-cholanic acid is a reliable internal standard for HPLC analysis of conjugated bile acids in serum and bile. – **Muraca, M., and Y. Ghoos.** Glyco-7α,12α-dihydroxy-5β-cholanic acid as internal standard for high-pressure liquid chromatographic analysis of conjugated bile acids. *J. Lipid Res.* 1985. **26:** 1009–1011.

Supplementary key words taurine-conjugated bile acids • glycine-conjugated bile acids

HPLC is being used increasingly for the analysis of conjugated bile acids in biological fluids (1–8). Quantitation of individual bile acids by HPLC in serum and bile gives results comparable to those obtained with well-established GLC procedures (6), and offers the additional advantage of quantifying separately the taurine and glycine conjugated species. Moreover, the chromatographic analysis can be performed with minimal pretreatment of the sample, when compared with the derivatization procedure required prior to GLC analysis. However, a convenient internal standard for HPLC analysis of bile acids has not yet been described. Recently, the taurine conjugate of 7,12-DCA has been proposed as internal standard for bile acid analysis by GLC (9). In the present work, the glycine conjugate of 7,12-DCA was studied and validated as an internal standard for HPLC analysis of conjugated bile acids.

MATERIALS AND METHODS

Apparatus

All the chromatographic equipment was from Waters Associates (Milford, MA). The liquid chromatograph consisted of the following components: a model U6K injector, a model 590 pump, a model 481 variable wavelength UV absorbance detector, and a model 730 plotter/integrator. The column was a Radial-PAK Cartridge, 100 × 8 mm, 5-μm particle size, subjected to radial compression with an RCM-100 module. A Guard-Pak C-18 pre-column was mounted in line between the injector and the analytical column.

Chemicals

Glycine- and taurine-conjugated CA, CDCA, UDCA, DCA, and LCA were obtained from Steraloids (Wilton, NH), while glycine- and taurine-conjugated 7,12-DCA were from Calbiochem-Behring Corp. (La Jolla, CA). Purity of the bile acids was checked by TLC with propionic acid–isoamylacetate–propanol–water 15:20:10:5 (10). The compounds were found to be at least 98% pure. HPLC-grade methanol was obtained from Merck (Darmstadt, West Germany).

Collection of biological samples

Serum samples were obtained from normal individuals and from patients with cholestasis due to extrahepatic biliary obstruction. Bile was obtained by duodenal intubation from patients with gallstones or with Crohn's disease.

Extraction of bile acids from biological samples and from artificial aqueous solutions

The internal standard was added to either fresh serum or bile and the taurine- and glycine-conjugated bile acids were extracted from the samples by reversed phase chromatography (6). A solution of glyco-7,12-DCA, 1.0 mmol/l, was prepared in the mobile phase (see Liquid chromatography). Serum (2 ml) or duodenal bile (0.2 ml, diluted to 2 ml with 0.5 M phosphate buffer, pH 7.4) were mixed with 1 ml of internal standard solution, giving a final standard concentration of 0.33 mmol/l. Then 13 ml of a mixture consisting of mobile phase–0.2 M NaOH 6:8 (v/v) was added, and the solution was passed through a Sep-Pak C-18 cartridge (Waters Associates), pre-activated according to the manufacturer's instructions. The cartridge was washed in sequence with 10 ml of water, 3 ml of 10% aqueous acetone, and a further 10 ml of water. Flow rate

Abbreviations and trivial names: 7,12-DCA, 7α,12α-dihydroxy-5β-cholanic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid. The prefixes glyco (G) and tauro (T) denote bile acids having glycine or taurine in the amide linkage at C-24. HPLC, high-pressure liquid chromatography; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; R_f, response factor; IS, internal standard.

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through the cartridge was kept at 10 ml/min. Bile acids were then extracted from the Sep-Pak with 3 ml of methanol, and the eluate was dried under a stream of nitrogen at 37°C.

Liquid chromatography

The residue was dissolved in 0.5 ml of the mobile phase. Solubilization was accelerated by sonicating the tube for about 30 sec. The solution was then passed through a disposable filter unit of 0.45-μm pore size (Millex-HV_4, Millipore, Bedford, MA), and 5-15 μl of the filtrate was injected into the liquid chromatograph. The mobile phase was prepared as described by Ruben and van Berge-Henegouwen (6) and consisted of methanol-water 75:25 (v/v) containing 2.5 mM KH_2PO_4 and 20 mM NaOH, and adjusted to pH 6.0 with 85% H_3PO_4. The flow rate was 1.0 ml/min and detection was performed at 205 nm; peak areas were integrated electronically.

Calibration standards

One volume of a solution containing 1.0 mmol/l of glyco-7,12-DCA was added to equal volumes of serial dilutions of aqueous solutions of the bile acids in the concentration range of 2.05-2.35 mmol/l. The mixture then underwent the described chromatographic procedure.

Recovery

One ml of the internal standard solution was added to 2 ml of normal serum and extracted as described above. The residue was redissolved in 0.5 ml of the mobile phase, and 10 μl was injected into the liquid chromatograph. Integrated peak areas corresponding to the standard were

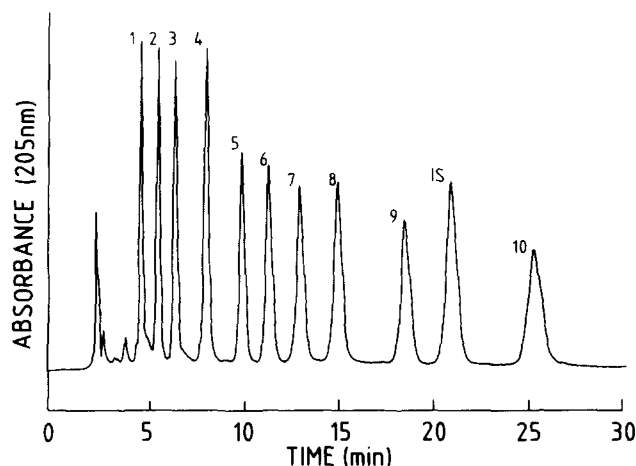

Fig. 1. Chromatogram of the reference compounds. R_f values are given in parentheses. 1. TUDCA (0.718); 2. GUDCA (0.767); 3. TCA (0.865); 4. GCA (1.102); 5. TCDCA (0.814); 6. TDCA (0.906); 7. GCDCA (0.890); 8. GDCA (1.031); 9. TLCA (0.894); 10. IS, glyco-7,12-DCA (1.000); 10. GLCA (1.004).

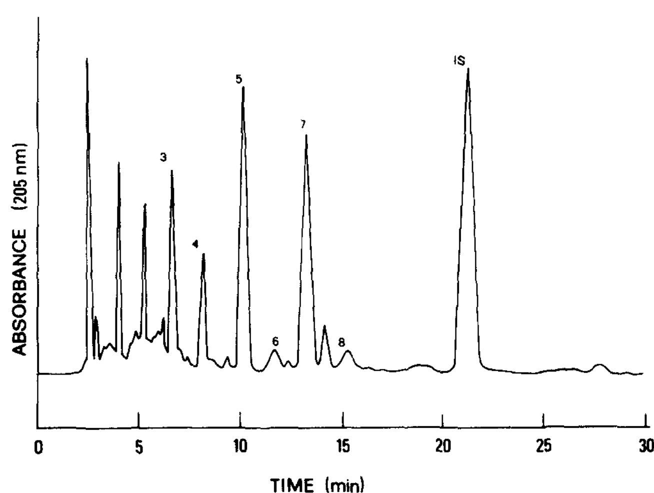

Fig. 2. Chromatogram of bile acids extracted from serum of a patient with liver cirrhosis, containing 23.2 μmol/l of CA, 57.6 μmol/l of CDCA, and 8.1 μmol/l of DCA. Total glycine to taurine conjugates ratio = 1.6:1. Bile acid peaks are denoted as in Fig. 1.

compared with a calibration curve, constructed by direct injection of 2-20 nmol of the standard into the liquid chromatograph.

RESULTS

Chromatography of the standard bile acid mixture showed baseline separation between all the peaks corresponding to the taurine- and glycine-conjugates of individual bile acids, including glyco-7,12-DCA (**Fig. 1**). The taurine-conjugated 7,12-DCA overlapped partially with GDCA and thus was not studied further. When both normal and cholestatic serum or bile samples were analyzed, no interfering compounds were found in the region of the chromatogram corresponding to the retention time of glyco-7,12-DCA. A clear baseline separation among individual bile acids was also observed in extracts obtained from biological samples (**Fig. 2**). The lowest amount of bile acid that can be measured in serum using 2 ml of sample is about 3 μmol/l at a signal-to-noise ratio of about 3:1. In comparison with the original procedure (6), the new 5-μm particle size column improved the separation among all bile acids, and especially between GUDCA and TCA. This allowed quantitation of the small amounts of GUDCA normally present in bile. A good resolution among the various bile acids was still maintained at a flow rate of 1.5 ml/min, which shortened the analysis time from 26 to 17 min. Recovery of glyco-7,12-DCA from the Sep-Pak extraction procedure was 96% ± 5 (n = 6). Such a recovery is in close agreement with the data that have been reported for the naturally occurring bile acids by Ruben and van Berge-Henegouwen (6).

For each bile acid, a linear relationship was found between the amount injected and the detector response

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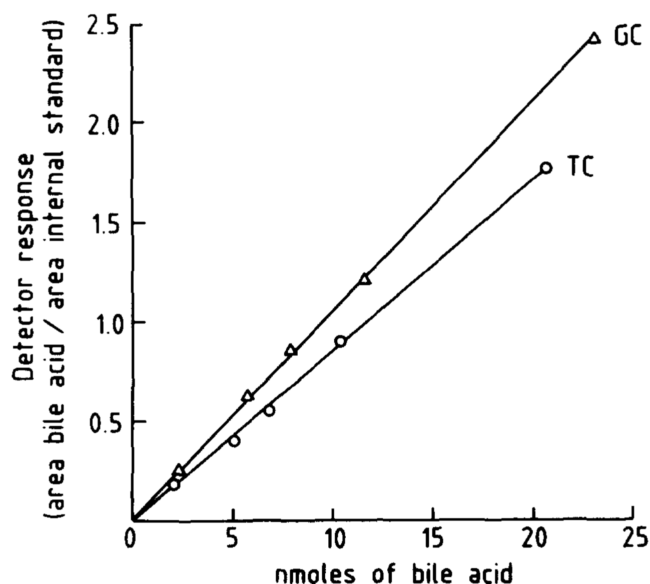

Fig. 3. The response ratio GCA/glyco-7,12-DCA and TCA/glyco-7,12-DCA is shown. Similar curves were obtained for the other bile acids.

relative to the internal standard (**Fig. 3**). Relative response factors calculated for individual bile acids are given in the legend for Fig. 1.

DISCUSSION

Several methods have recently been proposed for HPLC analysis of conjugated bile acids (1-8). We used the procedure described by Ruben and van Berge-Henegouwen (6) because it is simple and rapid. The mobile phase is compatible with the use of a UV detector, which provides a higher sensitivity in comparison with differential refractometers. Furthermore, we have found that better separations and shorter analysis times can be achieved by using the new Radial-Pak 5-μm particle size columns.

Dexamethasone (6), estriol (3, 6), and testosterone acetate (4) have been proposed as internal standards for liquid chromatographic analysis of bile acids. These compounds cannot be added to the samples prior to extraction since they can be lost during this step, and thus they do not allow monitoring of the whole procedure. Moreover, dexamethasone and estriol were found to overlap with other compounds in the chromatographic system. The use of the internal standard proposed here obviates these inconveniences. Glyco-7,12-DCA meets the most important criteria for a suitable standard: *1*) it is absent in biological material; *2*) it can be added to the sample prior to extraction, thus monitoring all steps of the procedure; *3*) it elutes in proximity of the other bile acids, while exhibiting baseline separation; *4*) the UV detector response is linear (Fig. 3) and is similar to the responses for the other bile acids tested (Fig. 1); and *5*) the standard is commercially available in a highly purified form.

In conclusion, these characteristics make glyco-7,12-DCA a convenient and accurate standard for HPLC analysis of conjugated bile acids. ■

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