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Note

Improved method for measuring the glycine and taurine conjugates of bile salts by high-performance liquid chromatography with tauro-7α,12α-dihydroxy-5β-cholanic acid as internal standard

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High-performance liquid chromatography (HPLC) on a reversed-phase (RP) column with an ultraviolet (UV) detector has been used by many workers for separating and quantifying the glycine and taurine conjugates of bile salts (BS)[1–7]. The methods described are generally easy to perform and offer more detailed information than those obtained by gas–liquid chromatography (GLC) or enzyme assay. However, these methods suffer from the lack of a conveneient internal standard (I.S.). The recent introduction of glyco-7α,12α-dihydroxy-5β-cholanic acid as an I.S. has made the RP-HPLC technique suitable and reliable for the quantification of BS conjugates in biological samples[8].

In this paper we describe the use of an alternative internal standard, the taurine conjugate of 7α,12α-dihydroxy-5β-cholanic acid (T-7,12-DCA), previously described as an I.S. for GLC analysis[9], which offers the advantage of a shorter retention time, exactly in the centre of the chromatogram. The simultaneous use of a high-speed RP-HPLC column allows baseline separations of all the physiological BS conjugates, which are quantified within only 12 min.The method described is particularly recommended for the analysis of samples that do not contain glycine-conjugated BS (such as most animal biles), where a 5-min run is required.

EXPERIMENTAL

Chemicals

Glycine and taurine conjugates, including the I.S., were purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.). All the standards were found to be at least 97% pure by HPLC. HPLC-grade solvents were obtained from Carlo Erba (Milan, Italy).

Apparatus

The chromatographic apparatus (Gilson Medical Electronics, Middleton, WI, U.S.A.) was equipped with a spectrophotometric UV detector, set at 205 nm, and a computerized data station for peak integration and subsequent I.S. method calcu-

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lations. A Hypersil 3ODS (3 μm) column (100 mm × 4.6 mm I.D.), supplied by HPLC Technology (Macclesfield, U.K.), was kept at 35 ± 0.1°C in a Clar 055 electronic thermostat oven (Violet, Rome, Italy).

Preparation of solvent and standards

The I.S. and the glycine and taurine conjugates of sodium cholate (C), ursodeoxycholate (UDC), chenodeoxycholate (CDC) and deoxycholate (DC) were individually dissolved in methanol. Four calibration mixtures were prepared by mixing the BS with the I.S. in ratios of 0.5, 1.0, 1.5 and 2.0:1.0. These solutions were evaporated to dryness under nitrogen and the residues were dissolved in the mobile phase so that 25 μg of total BS were present in the volume injected (20 μl). The mobile phase was methanol–20 m*M* phosphate buffer (pH 2.5)–water–acetonitrile (150:50:20:20) a flow-rate of 1.0 ml/min.

Sample preparation

Human gall blabber bile samples, obtained at surgery by needle aspiration, were immediately poured into test-tubes containing isopropanol (1:9, v/v), shaken for 10 min and centrifuged at 500 *g* for 10 min. The isopropanol extracts were stored at −20°C until analysed. An aliquot of the supernatant was used for the enzymatic analysis of total BS by the 3α-hydroxysteroid dehydrogenase method (E. Merck, Darmstadt, F.R.G.). The remainder was dissolved in the mobile phase and the solution was filtered through an FHLP 013 filter (Millipore, Molsheim, France) and injected into the HPLC column.

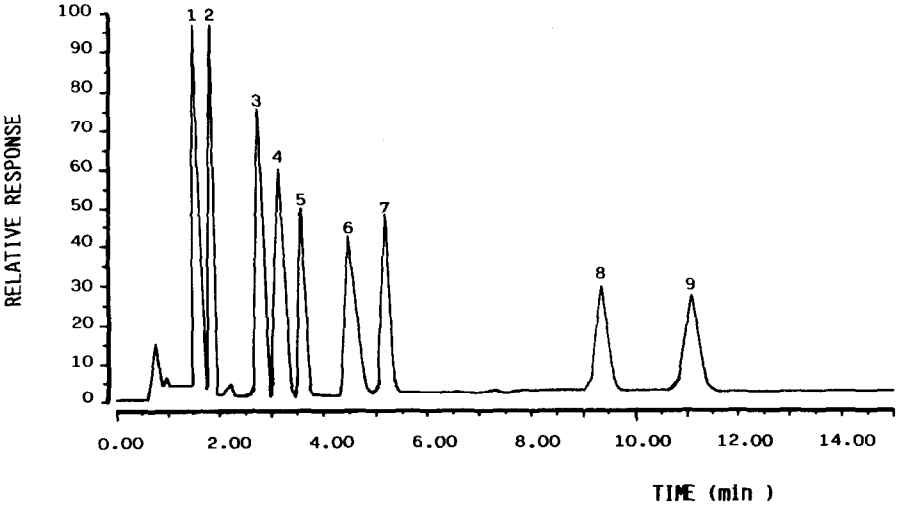

Fig. 1. Elution pattern of pure BS conjugates in a calibration mixture. Peak identities (abbreviations and *rk'* in parentheses): 1 = tauroursodeoxycholate (TUDC, 0.19); 2 = taurocholate (TC, 0.27); 3 = taurochenodeoxycholate (TCDC, 056); 4 = taurodeoxycholate (TDC, 0.63); 5 = glycoursodeoxycholate (GUDC, 0.77); 6 = tauro-7α,12α-deoxycholate (I.S., 1.00); 7 = glycocholate (GC, 1.19); 8 = glycochenodeoxycholate (GCDC, 2.32); 9 = glycodeoxycholate (GDC, 2.79).

TABLE I

REGRESSION COEFFICIENTS (a,b) AND CORRELATION COEFFICIENTS (r) OF THE CURVES, (AREA BS/AREA I.S.) = a (AMOUNT BS/AMOUNT I.S.) + b FOR THE BS CONJUGATES IN THE CALIBRATION MIXTURES

The calibration factors, F = (amount BS/amount I.S.) · (area I.S./area BS) are expressed as means ± standard deviations of 12 separate determinations.

*BS**	*a*	*b*	*r*	*F*
TUDC	0.94	$9.1 \cdot 10^{-1}$	0.98	1.01 ± 0.05
TC	0.94	$8.9 \cdot 10^{-2}$	0.99	1.01 ± 0.05
TCDC	0.98	$9.9 \cdot 10^{-2}$	0.99	0.93 ± 0.04
TDC	0.98	$9.9 \cdot 10^{-2}$	0.99	0.92 ± 0.04
GUDC	0.64	$6.5 \cdot 10^{-2}$	0.99	1.50 ± 0.07
GC	0.79	$7.0 \cdot 10^{-2}$	0.99	1.20 ± 0.04
GCDC	0.61	$7.9 \cdot 10^{-2}$	0.99	1.50 ± 0.06
GDC	0.71	$9.0 \cdot 10^{-2}$	0.99	1.29 ± 0.05

* See Fig. 1 for abbreviations.

RESULTS AND DISCUSSION

Fig. 1 shows the elution pattern of the BS conjugates following the injection of a calibration mixture. All the major BS are completely resolved and show good peak simmetry. The retention times (t_R) and the capacity factors (k') of the various BS showed between- and within-day fluctuations as reported by Shaw *et al.*[10]. The k' values relative to the I.S. (rk'), as suggested by these authors, are, however, fairly constant and may be useful for the identification of the peaks as shown in Figs. 1

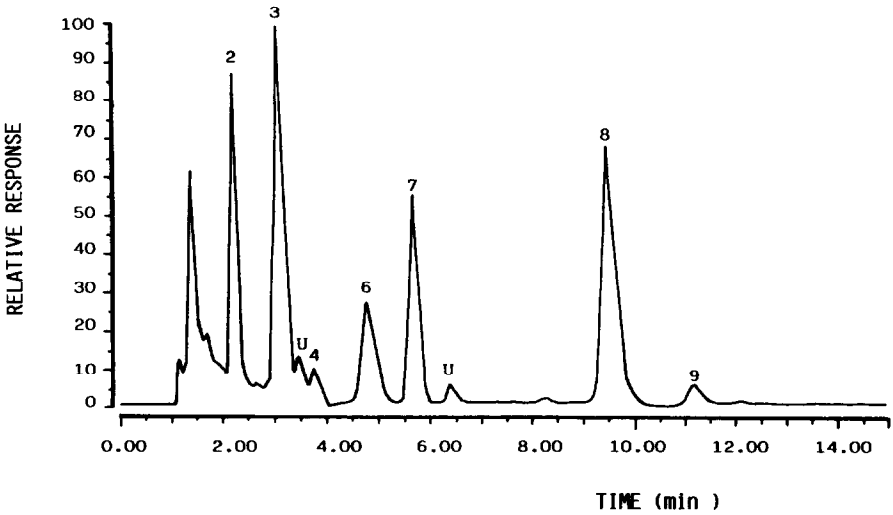

Fig. 2. Elution profile of the BS conjugates in a sample of human gall bladder bile. Peak identities (rk' in parentheses): 2 = TC (0.27); 3 = TCDC (0.54); 4 = TDC (0.67); 6 = I.S. (1.00); 7 = GC (1.21); 8 = GCDC (2.28); 9 = GDC (2.76).

and 2. The detector response is linear, as seen in Table I from the correlation coefficient of the regression peak area of BS/peak area of I.S. (y) *versus* amount of BS/amount of I.S. (x) relative to each compound. It should also be noted that the slopes (coefficient a) of the lines are similar within each type of conjugation (higher for taurine than for glycine conjugates) and the intercepts (coefficient b) are very low for all the BS tested. This indicates that the detector response is higher for taurine conjugates and that both the resolution and integration of the peaks is adequate.

The amount of the individual conjugates can be readily calculated from the following equation: amount of BS = (peak area of BS/peak area of I.S.) · amount of I.S. · F. The calibration factors [F = (amount of BS/amount of I.S.) · (peak area of I.S./peak area of BS)], calculated from the calibration mixtures, are also shown in Table I.

Fig. 2 shows the elution profile of the BS conjugates in a sample of human gall bladder bile. The I.S. did not appear to inferfere with the compounds present in bile samples.

Five replicate runs of the bile sample indicate, at most, a 5% variation in the evaluation of a single peak and a 3% variation in the determination of total BS concentration (the sum of all the peaks). The determination of the total BS concentration by the present method on six samples of human bile resulted in good agreement with the enzymatic evaluation by the 3α-hydroxysteroid dehydrogenase method ($r = 0.99$; $p < 0.001$), thus validating this method in comparison with that generally used for measuring the total BS concentration in bile.

In conclusion, the proposed method offers a rapid means of determining the total and individual glycine- and taurine-conjugated biliary BS.

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