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FORMATION AND SEPARATION BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FLUORESCENT AND UV-ABSORBING BILE SALT DERIVATIVES

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SUMMARY

A method is described for the production of bile salt derivatives via the substituent hydroxyl groups on the steroid nucleus that permits derivatization of taurine and glycine conjugates. The method involves the oxidation of the hydroxyl groups at C-3α or C-7α on the nucleus by the action of hydroxysteroid dehydrogenases. The keto bile salts produced are isolated and reacted with 2,4-dinitrophenylhydrazine or Dns-hydrazine to yield ultraviolet absorbing or fluorescent derivatives, respectively. The bile salt hydrazones were separated using a reversed-phase C_{18} radial compression cartridge with a methanol–phosphate buffer gradient elution. Although the 3-keto derivatives of chenodeoxycholic and deoxycholic acids were not resolved, the 7-keto Dns-hydrazone of chenodeoxycholic acid was separated from 3-keto derivative of deoxycholic acid.

INTRODUCTION

The high-performance liquid chromatography (HPLC) of individual bile salts in biological samples of low bile salt content has been limited by the lack of a suitable method of detection. The most commonly used methods are UV absorbance at or below 210 nm of native bile salts[1–4], which, however, imposes limitations on the composition of the mobile phase, or the formation of UV-absorbing[5,6] or fluorescent derivatives[7]. The derivatization procedures used by these workers involved reaction with the carboxyl functional group on the bile salt side chain, and are therefore unsuitable for direct analysis of taurine conjugated bile salts. However, derivatization of a keto group on the steroid nucleus with Dns-hydrazine is possible[8] and in this report we present a method for the production of UV-absorbing or fluorescent hydrazones following enzymatic oxidation of the 3α- or 7α-hydroxyl groups of bile salts and their separation by HPLC on a reversed-phase radial compression column.

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EXPERIMENTAL

Reagents and materials

Dns-hydrazine Grade II, chromatographically purified 3α- and 7α-hydroxy steroid dehydrogenase and sodium taurochenodeoxycholic acid (TCDC) were from Sigma (Poole, Great Britain). The sodium salts of taurocholic acid (TC), taurodeoxycholic acid (TDC), taurolithocholic acid (TLiC), glycocholic acid (GC), glycochenodeoxycholic acid (GCDC), glycodeoxycholic acid (GDC), glycolithocholic acid (GliC), cholic acid (C), chenodeoxycholic acid (CDC), deoxycholic acid (DC), lithocholic acid (liC), 7α,12α-dihydroxy-3-keto-5β-cholanoic acid and its taurine conjugate, and the free acid of ursodeoxycholic acid (UDC) were obtained from Calbiochem (Bishops Stortford, Great Britain). Nicotinamide–adenine dinucleotide (oxidized; NAD) Grade II was from Boehringer (Lewes, Great Britain). HPLC-grade methanol was from Rathburn Chemicals (Walkerburn, Great Britain). All other solvents and reagents were AnalaR grade from BDH (Poole, Great Britain). Sep-Pak C_{18} reversed-phase cartridges were purchased from Waters Associates (Northwich, Great Britain). Solutions and aqueous solvents were prepared with ultra-pure water obtained from a Milli-Q System [Millipore (U.K.), London, Great Britain] fed from a reservoir of double-distilled water (Fistream, Fisons Scientific Apparatus, Loughborough, Great Britain). Solvents were filtered through a 0.45 μm PTFE filter (Gelman Sciences, Brackmills, Great Britain) and degassed in a sonic bath.

All the bile acids studied were 5β-cholan-24-oic acid derivatives with hydroxyl groups in the following positions: C, 3α, 7α, 12α; CDC, 3α, 7α; DC, 3α, 12α; LiC, 3α; UDC, 3α, 7β.

Preparation and isolation of keto bile salt derivatives

Keto derivatives of bile salt standards were prepared by incubation of bile salt solutions with 3α- or 7α-hydroxysteroid dehydrogenase and NAD for 1 h at 37°C in pyrophosphate buffer (pH 9.5)[9]. Following incubation the reaction mixture was acidified by the addition of 4 drops of concentrated hydrochloric acid and passed through a Sep-Pak C_{18} cartridge primed with water. The cartridge was then washed with 10 ml of water and the keto bile salts were eluted with 5 ml of methanol, and the extract taken to dryness under nitrogen prior to derivatization. UV-absorbing derivatives[10] were produced by reconstituting the dried extracts in 400 μl of 2,4-dinitrophenylhydrazine (DNPH; 1 mg ml^{-1} in ethanol) and 2 drops of concentrated hydrochloric acid. Fluorescent derivatives[8] were prepared by reconstitution in 200 μl of acid ethanol (650 μl of concentrated hydrochloric acid per litre ethanol) and 200 μl Dns-hydrazine (1 mg ml^{-1} in ethanol). In both cases bile salt hydrazones were obtained by heating the reaction mixtures at 60°C for 10 min. Samples were analysed by HPLC without further extraction.

HPLC

A Waters Associates HPLC system consisting of a Model 660 solvent programmer, two Model 6000A solvent delivery systems and a Model U6K universal liquid chromatographic injector was used throughout this study. Chromatographic separation was performed using a Waters Associates radial compression module (RCM 100) fitted with a Radial-Pak C_{18}, 10 μm, (8 mm I.D.) reversed-phase car-

tridge. A Waters Associates Model 440 absorbance detector was used for absorbance detection at 340 nm and an SFR 100 ratio-recording spectrofluorimeter fitted with a special 15-μl HPLC flow cell (Baird Atomic, Braintree, Great Britain) was used for on-line fluorescence detection. Peak areas were determined with an on-line computer and chromatography software (Triton 3 data collection and analysis system, Trivector Scientific, Sandy, Great Britain).

2,4-Dinitrophenylhydrazone derivatives were separated using a 7-min linear gradient of methanol–10 m*M* potassium dihydrogen orthophosphate (from 40:60 to 90:10) at a flow-rate of 2.0 ml min^{-1} with absorbance detection at 340 nm.

Dns-hydrazones were separated using a 13-min linear gradient of methanol–10 m*M* potassium dihydrogen orthophosphate buffer (pH 3.4) with acetic acid (from 60:40 to 85:15) at a flow-rate of 2.0 ml min^{-1} with fluorescence detection at 510 nm on excitation at 360 nm.

RESULTS

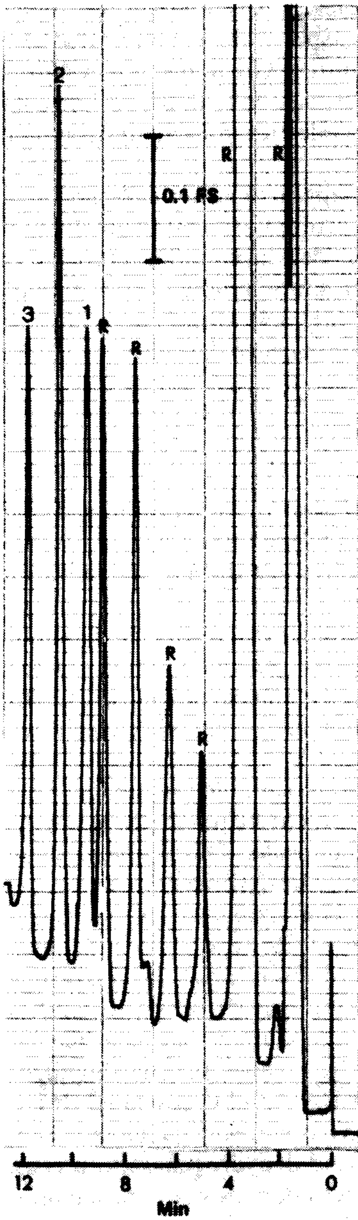
Although good separation of bile salt 2,4-DNPH derivatives was obtained (Fig. 1) with a detection limit of 20 pmoles, the production of isomers as a result of reaction of both nitro groups of DNPH with certain keto bile acids during derivatization tended to limit this technique.

The Dns-hydrazine derivative of 7α,12α-dihydroxy-3-keto-5β-cholanoic acid appeared as a single peak on HPLC with a simple methanol–water gradient system, was well separated from reagent peaks and gave a linear response over the range 30 pmoles to 5 nmoles. Similar results were obtained when cholic acid was taken through the complete derivatization procedure.

Although methanol–water gradient systems proved suitable for the less polar bile salt 3α-Dns-hydrazones, the increase in water content required to retain the taurine conjugates also increased the retention of reagent peaks. This problems could be avoided by the addition of excess pyruvate after the Dns-hydrazine reaction and incubating at room temperature for 20 min, thereby converting the excess reagent into Dns-pyruvate which eluted close to the solvent front. However, if 10 m*M* phosphate buffer was used in the mobile phase, retention of the bile salt 3α-Dns-hydrazones was increased without affecting the position of the reagent peaks. Separation of taurine and glycine conjugates was possible and a mixture of thirteen bile salt 3α-Dns-hydrazones was resolved into nine peaks (Figs. 2 and 3). The dihydroxy isomers were not resolved under these conditions and UDC and C co-eluted. It was possible to differentiate chenodeoxycholic from deoxycholic acid by using 7α-hydroxysteroid dehydrogenase instead of 3α-hydroxysteroid dehydrogenase to prepare 7-keto derivatives of the former (Fig. 4). Since the 7-keto derivatives were the more polar an increase in the aqueous content of the mobile phase was required.

DISCUSSION

Bile salt derivatization has been achieved using the hydroxyl groups on the steroid nucleus to produce UV-absorbing or fluorescent derivatives irrespective of the side chain conjugation present. The technique involves initial oxidation of the hydroxyl groups at C-3α or C-7α with a hydroxysteroid dehydrogenase, isolation



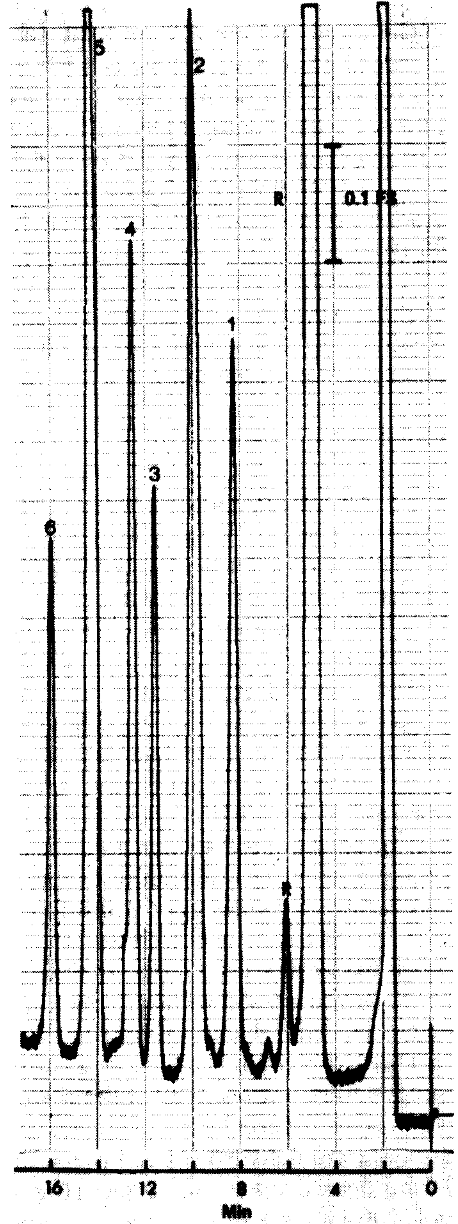


Fig. 1. Separation of bile salt 3α-2,4-dinitrophenylhydrazones (200 ng of each). Peaks: R = reagent; 1 = TC; 2 = TCDC + TDC; 3 = TLiC.

Fig. 2. Separation of bile salt 3α-Dns-hydrazones (200 ng of each). Peaks: R = reagents; 1 = TC; 2 = TCDC + TDC; 3 = TLiC; 4 = GC; 5 = GCDC + GDC; 6 = GLiC.

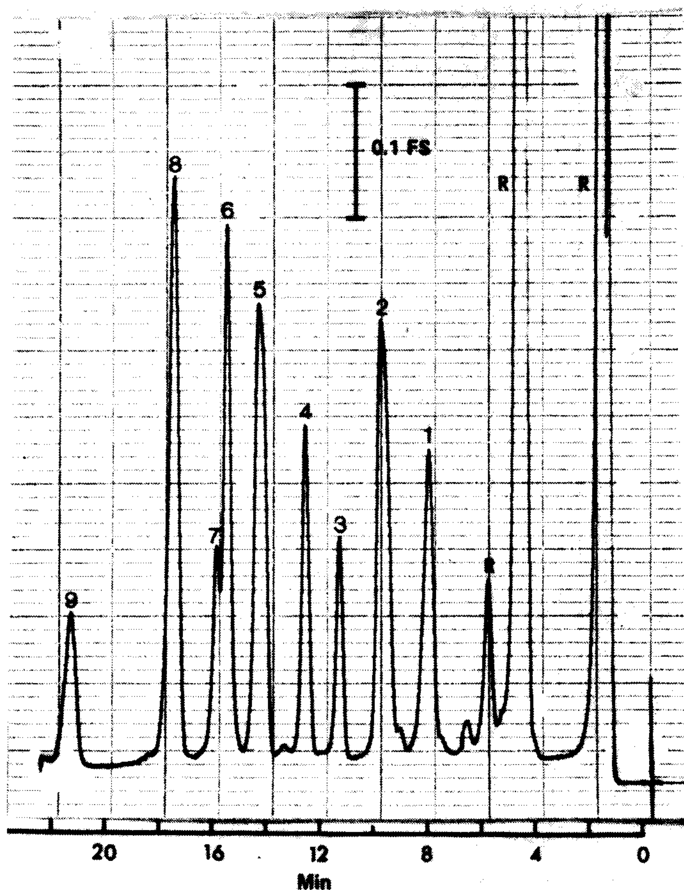


Fig. 3. Separation of bile salt 3α-Dns-hydrazones (400 ng of each). Peaks: R = reagent; 1 = TC; 2 = TCDC + TDC; 3 = TLiC; 4 = GC; 5 = GCDC + GDC; 6 = UDC + C; 7 = GLiC; 8 = CDC + DC; 9 = LiC. Lower sensitivity setting than other chromatograms.

of keto bile salts by Sep-Pak C_{18} extraction, and subsequent derivatization via the keto groups formed. This technique should be applicable to other substituent hydroxyl groups providing the initial oxidation step can be carried out (*e.g.* with 12α-hydroxysteroid dehydrogenase).

HPLC of bile salt Dns-hydrazones has been performed on a Radial-Pak C_{18} cartridge with methanol–phosphate buffer (pH 3.4) gradient elution. The various bile salts studied chromatographed in a similar manner to the reversed-phase separation of native bile salts[11], with an elution order of trihydroxy, dihydroxy, monohydroxy and taurine conjugates eluting before glycine conjugates and free bile salts. Unlike native bile salts, Dns-hydrazones of the dihydroxy isomers CDC and DC have not been resolved. However these bile salts can be differentiated by oxidation of the hydroxyl group at the C-7α position of CDC with 7α-hydroxysteroid dehydrogenase. The 7-keto-dansyl hydrazone of CDC can then be separated from the 3-keto derivative of DC. This approach may be applicable to other bile salts.

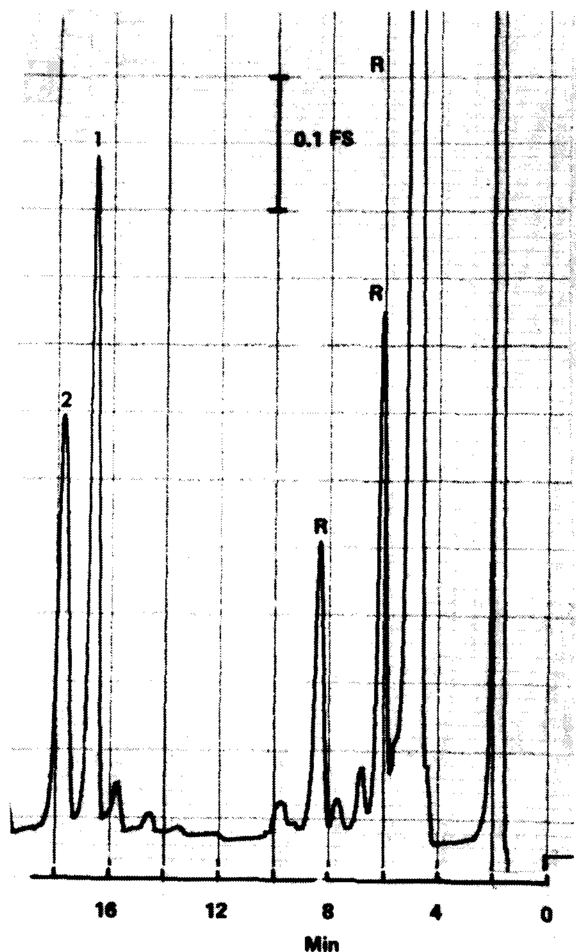


Fig. 4. Separation of 7α-Dns-hydrazone of CDC (1) and 3α-dansyl hydrazone of DC (2). R = reagent peaks (200 ng of each).

Using the Baird Atomic spectrofluorimeter with the HPLC flow-cell attachment, fluorescence detection allowed 30 pmoles of bile salt to be quantitated. A lower detection limit of 10–20 pmoles was obtained for some bile salts by Stellaard *et al.*[6] using a Model 440 absorbance detector (254 nm) to detect bile acid phenacyl esters. Shaikh *et al.*[5] also prepared derivatives via the carboxyl group on the bile salt side chain, this time to *p*-nitrobenzyl esters, but obtained a detection limit of 200 pmoles using a Chromatronix dual channel UV absorbance detector (254 nm). In addition these workers reported the preparation of esters by derivatization via hydroxyl groups on the steroid nucleus but considerable sample clean-up was required prior to HPLC analysis, and no information was given on the application of this method to the derivatization of conjugates. Improved sensitivity for the bile salt Dns-hydrazones can be achieved with the dedicated fluorescence detector, specifically designed for HPLC, which we are now using (unpublished observation).

The method described should prove suitable for the analysis of bile salts present in low concentrations in biological materials, including serum and tissues. Naturally occurring keto bile salts such as those in faeces, could be derivatized directly without the initial oxidation step, and analysis of bile salt sulphate esters, provided a free hydroxyl group is available, should also be possible.

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