High pressure liquid chromatography solvent systems for studies of bile acid biosynthesis

Kristian Prydz, Bengt Frode Kase, and Jan I. Pedersen[1]

Institute for Nutrition Research, School of Medicine, University of Oslo, Oslo 3, Norway

Abstract Reversed phase high pressure liquid chromatography (HPLC) solvent systems have been developed for the separation of intermediates in the formation of bile acids and bile acid conjugates from cholesterol. Four different mobile phases (water–methanol, 10 mM acetate buffer (pH 4.37)–methanol, 30 mM trifluoroacetic acid (pH 2.9 with triethylamine)–methanol, and 50 mM potassium phosphate buffer (pH 7.0)–2-propanol) have been applied to obtain separation of all the main intermediates with use of the same reversed phase column (Zorbax ODS).—**Prydz, K., B. F. Kase, and J. I. Pedersen.** High pressure liquid chromatography solvent systems for studies of bile acid biosynthesis. *J. Lipid Res.* 1988. **29:** 532–537.

Supplementary key words cholic acid • chenodeoxycholic acid • bile acid CoA esters • conjugated bile acids

Cholesterol is converted to water-soluble bile acid conjugates through a series of reactions that take place in the liver (**Fig. 1**, ref. 1). The major pathway is initiated by 7α-hydroxylation. The steroid nucleus of 7α-hydroxycholesterol (compound II, Fig. 1), is further modified and di- or trihydroxylated 5β-cholestane is formed. 7α-Hydroxy-4-cholesten-3-one (III) is believed to be the main substrate for the 12α-hydroxylase (2), but in human liver, even 5β-cholestane-3α,7α,26-triol (VII) and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA, IX) may be 12α-hydroxylated (3).

Following the nuclear reactions, one of the terminal methyl groups is oxidized to give 5β-cholestane-3α,7α,26-triol (VII) or 5β-cholestane-3α,7α,12α-tetrol (VIII) (4). The side chain is further oxidized to a carboxylic acid and then undergoes chain shortening in a manner similar to the β-oxidation of fatty acids (5–7). This reaction sequence is initiated by activation of the carboxyl group with coenzyme A and terminated by the thiolase step giving chain shortened CoA esters of the primary bile acids, cholic and chenodeoxycholic. These bile acid-CoA esters are the immediate substrates for the amino acid-N-acyl transferases forming the peptide bonds to glycine or taurine (8).

HPLC has been used for the separation and quantitation of bile acids and their amino acid conjugates and sulfates (9–12). However, most reports dealing with the separation of bile acid precursors are with other techniques such as thin-layer chromatography and gas–liquid chromatography–mass spectrometry (2–4, 13–16).

In recent years we have used HPLC methods extensively in a number of in vitro and in vivo studies on bile acid formation (for a review see ref. 17). The present report describes isocratic HPLC solvent systems we have found

Abbreviations: DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high pressure liquid chromatography; ODS, octadecylsilane; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; TFA, trifluoroacetic acid.

[1]To whom correspondence should be addressed at: Institute for Nutrition Research, University of Oslo, P.O. Box 1046, Blindern, 0316 Oslo 3, Norway.

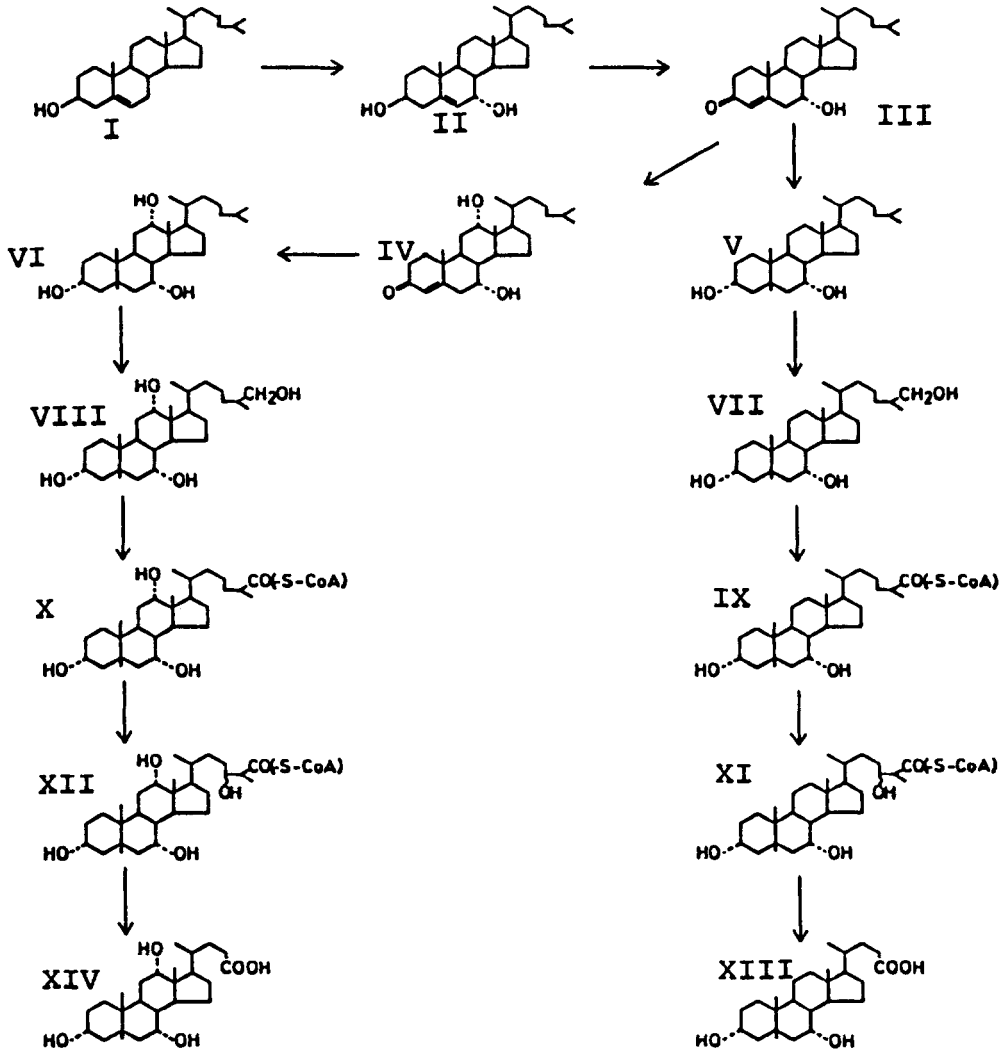

Fig. 1. Proposed major pathways for the biosynthesis of bile acids. I: Cholesterol, II: 7α-hydroxycholesterol, III: 7α-hydroxy-4-cholesten-3-one, IV: 7α,12α-dihydroxy-4-cholesten-3-one, V: 5β-cholestane-3α,7α-diol, VI: 5β-cholestane-3α,7α,12α-triol, VII: 5β-cholestane-3α,7α,26-triol, VIII: 5β-cholestane-3α,7α,12α,26-tetrol, IX: 3α,7α-dihydroxy-5β-cholestanoyl-CoA, X: 3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA, XI: 24-hydroxy-DHCA, XII: 24-hydroxy-THCA, XIII: chenodeoxycholic acid, XIV: cholic acid.

useful for the separation of a wide variety of possible intermediates in the conversion of cholesterol to the conjugated bile acids.

MATERIALS AND METHODS

Chemicals

Chemicals for solvent preparation were of HPLC or analytical grade. 2-Propanol was purchased from Rathburn Chemicals LTD, Scotland. Acetic acid, sodium acetate, methanol, KH_2PO_4, and K_2HPO_4 were purchased from Merck, Darmstadt. Trifluoroacetic acid and triethylamine were from Sigma Chemical Company, St. Louis, MO.

Labeled and unlabeled steroids

Steroids labeled with tritium in 6β- or 7β-position (6–7 Ci/mol) and unlabeled steroids were synthesized as described (4) and purified by HPLC before use. 5β-Cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,26-tetrol were formed biosynthetically from 5β-cholestane-3α,7α,12α-triol (18). [24-^{14}C]Cholic acid and chenodeoxycholic acid (50 Ci/mol) were purchased from Amersham International plc, Amersham, England.

Hydrolysis and extraction

When preservation of intermediates with intact coenzyme A ester bond was desired, no hydrolysis was performed. Mild alkaline hydrolysis (50°C, 30 min, pH > 9)

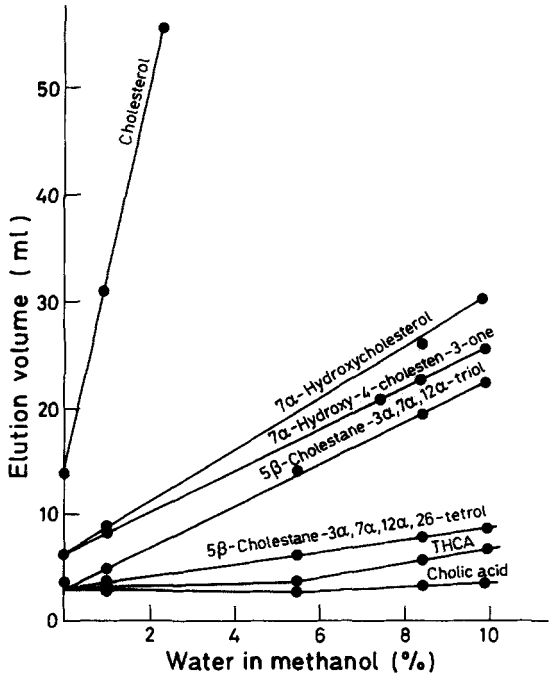

Fig. 2. *Variation in reversed phase HPLC elution volume of cholic acid and some bile acid precursors as a function of percentage water in methanol.*

results in complete disappearance of CoA esters. Amino acid conjugates were subjected to strong alkaline hydrolysis (120°C, 18 hr, pH > 9) to obtain the unconjugated bile acids. Incubation mixtures with coenzyme A esters were extracted with butanol (6 times) and evaporated in a Rotavapor (Buchi, Switzerland). Unesterified precursors and bile acid conjugates from incubations, serum, or urine were concentrated on SEP-PAK C_{18} cartridges (Waters Associates, Milford, MA) and eluted with methanol. Alternatively, the unesterified precursors were extracted with ethyl acetate, ether or with chloroform–methanol 2:1. Organic phases were evaporated with nitrogen and the samples were dissolved in the eluent.

Solvent systems

Four different solvent systems were used. I, water–methanol; II, 10 mM acetate buffer (pH 4.37)–methanol; III, 30 mM trifluoroacetic acid (pH 2.90 with triethylamine)–methanol; IV, 50 mM potassium phosphate buffer (pH 7.00)–2-propanol. The aqueous and organic components were mixed in the desired volumes as described under Results.

Chromatography

Isocratic elution was carried out with a single pump (Spectra-Physics Model 3500B, Spectra-Physics, Santa Clara, CA) equipped with a Rheodyne injector (Model 7129) and a Zorbax ODS column (4.6 × 250 mm, 6-μm particles). A flow rate of 1 ml/min was used. Fractions of 1 ml were collected and assayed for radioactivity in a Packard Tri-Carb 300C scintillation spectrometer after addition of an appropriate volume of Packard Insta-Gel II. Counting efficiency was about 60%, and the minimum amount of radioactivity that could be detected was of the order of 200 dpm. Recovery of radioactivity from the column was essentially complete. Since all analytes used in these experiments were radioactive, an ultraviolet detector or differential refractometer was not used.

RESULTS AND DISCUSSION

The different solvent systems we describe have all been used with the same Zorbax ODS column. The reported elution volumes may vary to some extent depending on the individual column. Other C_{18} columns have been tested, and the chromatograms are comparable to those reported. Minor adjustments of the proportions of aqueous and organic phase may be made to give the desired separation and elution volumes.

I. Water–Methanol

The retention behavior of the bile acid precursors in water–methanol systems is shown in **Fig. 2.** Cholesterol was separated from the bulk of metabolites in 0–1% water in methanol. The elution volumes in 8.5% water in methanol for the metabolites starting with 7α-hydroxycholesterol are shown in **Table 1.** This solvent gives good separation of most metabolites without a carboxyl group in the side chain.

II. 10 mM Acetate buffer (pH 4.37) in methanol

In addition to the major pathway for side chain oxidation involving mitochondrial 26-hydroxylation, a pathway starting with a microsomal 25-hydroxylation has been reported (19). The tetrols 5β-cholestane 3α,7α,12α,25-tetrol

TABLE 1. Elution volume for cholic acid, chenodeoxycholic acid, and some of their precursors with 8.5% water in methanol as solvent

Substance	Elution Volume
	ml
5β-Cholestane-3α,7α-diol	39
7α-Hydroxycholesterol	28
7α-Hydroxy-4-cholesten-3-one	22
5β-Cholestane-3α,7α,12α-triol	19
7α,12α-Dihydroxy-4-cholesten-3-one	16
5β-Cholestane-3α,7α,26-triol	13
3α,7α-Dihydroxy-5β-cholestanoic acid (DHCA)	11
5β-Cholestane-3α,7α,12α,26-tetrol	8
3α,7α,12α-Trihydroxy-5β-cholestanoic acid (THCA)	6
Chenodeoxycholic acid	4
Cholic acid	4

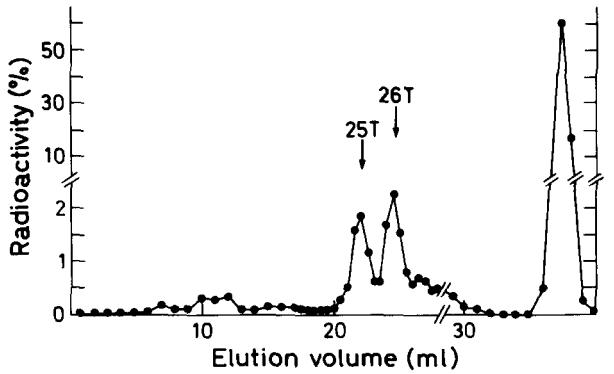

Fig. 3. Chromatogram of an incubation extract showing the separation of 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,26-tetrol, and 5β-cholestane 3α,7α,12α,25-tetrol eluted with 18.5% 10 mM acetate buffer (pH 4.37) in methanol. A liver biopsy homogenate from a patient with Zellweger syndrome (22) was incubated with 5β-cholestane-3α,7α,12α-triol and extracted as previously described (18).

and 5β-cholestane 3α,7α,12α,26-tetrol and their precursor 5β-cholestane 3β,7α,12α-triol may be separated by elution with 18.5% 10 mM acetate buffer (pH 4.37) in methanol (**Fig. 3**).

III. 30 mM Trifluoroacetic acid (pH 2.9 with triethylamine) in methanol

After introduction of a carboxylic acid group in the steroid side chain, separation is improved by lowering the pH of the solvent aqueous phase. 3α,7α-Dihydroxy-5β-cholestanoic acid (DHCA), 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA), chenodeoxycholic acid, and cholic acid all possess a terminal acid group in the side chain. Our

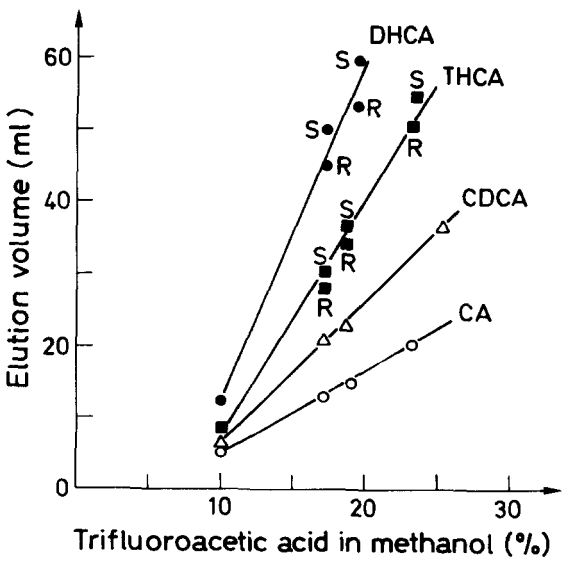

Fig. 4. Variation in reversed phase HPLC elution volume of DHCA, THCA, cholic acid, and chenodeoxycholic acid as a function of percentage 30 mM trifluoroacetic acid (pH 2.9 with triethylamine) in methanol.

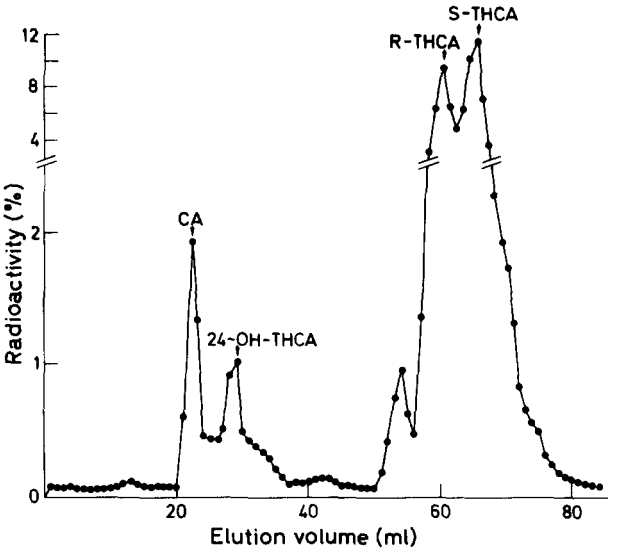

Fig. 5. Chromatogram of an incubation extract showing the separation of S-THCA, R-THCA, 24-hydroxy-THCA, and cholic acid eluted with 23% 30 mM trifluoroacetic acid (pH 2.9 with triethylamine) in methanol. Rat liver peroxisomes and THCA were incubated and extracted as described (7).

first separations of these substances were performed with 10 mM acetate buffer (pH 4.37) in methanol (6). Better separation was achieved with 25 mM phosphate buffer (pH 3.4) in methanol (20, 21). This was subsequently replaced by 30 mM trifluoroacetic acid (pH 2.9 with triethylamine) in methanol (22). This solvent is less detrimental to the packing material of the columns than phosphate buffer, which causes rapid peak broadening and reduction in elution volume. **Fig. 4** shows the elution volumes of DHCA, THCA, cholic acid, and chenodeoxycholic acid as a function of solvent composition. **Fig. 5** shows the separation of cholic acid from THCA in an incubation extract eluted with 23% trifluoroacetic acid in methanol. A corresponding separation was obtained for chenodeoxycholic acid and DHCA with 17% TFA in methanol (7). The peak at 29 ml in Fig. 5 was identified by GLC-MS as 24-OH-THCA (6). The TFA-methanol system has been used for the separa-

TABLE 2. Elution volume for THCA and the primary bile acid and their respective amino acid conjugates with 20% 30 mM trifluroacetic acid (pH 2.9 with triethylamine) in methanol as mobile phase

Compound	Elution Volume
	ml
THCA	26
Cholic acid	11
Glycocholic acid	7.5
Taurocholic acid	5
Chenodeoxycholic acid	20
Glycochenodeoxycholic acid	10.5
Taurochenodeoxycholic acid	6

tion of THCA, cholic acid, glycocholic acid, and taurocholic acid (23). **Table 2** shows the elution volumes for THCA, cholic acid, glycocholic acid, taurocholic, chenodeoxycholic acid, glycochenodeoxycholic acid, and taurochenodeoxycholic acid with 20% 30 mM TFA in methanol as eluent. This system is well suited for separation of cholic acid and chenodeoxycholic acid from their respective conjugates.

IV. 50 mM Phosphate buffer (pH 7.00) in 2-propanol

Phosphate buffer (50 mM, pH 7.00) in 2-propanol has been applied as solvent for the separation of coenzyme A esters of 5β-cholestanoic acids from the corresponding free acids. The system was modified from that reported by Abbott et al. (24) for separation of bile acid CoA esters with 24 carbon atoms. A chromatogram for the separation of DHCA, DHCA-CoA, and chenodeoxycholic acid with 38.5% 2-propanol in 50 mM phosphate buffer (pH 7.00) as eluent is shown in **Fig. 6. Table 3** shows the solvent mixtures applied for the separation of DHCA, THCA, chenodeoxycholic acid, and cholic acid and their CoA esters.

The amounts of steroids routinely separated by the HPLC systems described above were of the order of 1–10 μg. Up to 100 μg could be injected without appreciable loss of separation. The systems were developed with radioactively labeled compounds. Unlabeled compounds may be detected by a differential refractometer.

Compounds III and IV (Fig. 1) absorb UV-radiation and may be detected at 254 nm (25). Since all material injected may be quantitatively recovered, the methods described can easily be combined with gas–liquid chromatography–mass spectrometry for identification of product peaks or for quantitation by selective ion monitoring (18).

TABLE 3. Elution volume for C_{24} and C_{27} bile acids and their CoA derivatives with 50 mM phosphate buffer (pH 7.0) in 2-propanol as solvent

Compound	Elution Volume		
	Solvent A[a]	Solvent B[a]	Solvent C[a]
		ml	
Choloyl-CoA	9		
Cholic acid	29	11	
Chenodeoxycholoyl-CoA		15	
Chenodeoxycholic acid		23	13
DHCA-CoA			19
DHCA			35
THCA-CoA		19	
THCA		34	

[a]

Solvent	50 mM Phosphate Buffer	2-Propanol
A	69.5%	30.5%
B	66.5%	33.5%
C	61.5%	38.5%

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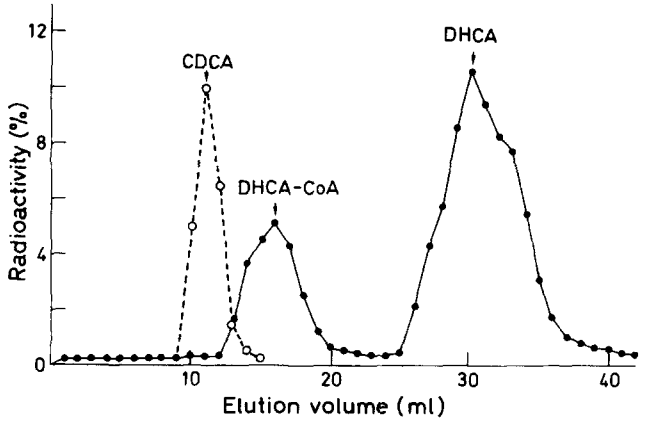

Fig. 6. Chromatogram of an incubation extract showing the elution of DHCA, DHCA-CoA, and chenodeoxycholic acid eluted with 38.5% 2-propanol in 50 mM phosphate buffer (pH 7.00). DHCA and DHCA-CoA were isolated from an incubation extract; chenodeoxycholic acid was the internal standard. Rat liver microsomes were incubated with tritium-labeled DHCA, CoA, ATP, and Mg^{2+}, and extracted with butanol as described in Methods (Prydz, K., B. F. Kase, I. Björkhem, and J. I. Pedersen, unpublished results).

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