Journal of Chromatography, 270 (1983) 402–406
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,083

Note

High-performance liquid chromatographic determination of bile acids involved in the synthesis of ursodeoxycholic acid

M. PACIOTTI* and L. PERINATI
Zambon Farmaceutici Research Laboratories, Bresso-Milan (Italy)
and
F. GORI and P. RAMPAZZO
Zambon Chimica S.p.A., Lonigo-Vicenza (Italy)
(Received June 7th, 1983)

The conventional synthesis of ursodeoxycholic acid involves the oxidation of chenodeoxycholic acid to 3-α-hydroxy-7-oxocholanic acid and the reduction of this to ursodeoxycholic acid. During the oxidative step a side reaction takes place which gives rise to the formation of 3-oxo-7-α-hydroxycholanic and 3,7-dioxocholanic acid. The reduction of 3-α-hydroxy-7-oxocholanic acid gives a mixture of ursodeoxycholic and chenodeoxycholic acids which have to be separated in order to obtain pure ursodeoxycholic acid.

To follow better the behaviour of these reactions it appeared useful to set up an high-performance liquid chromatographic (HPLC) method which did not require any derivatization step and which would permit the quantitation of all the chemical species involved.

There have been numerous investigations on the determination of conjugated bile acids[1–13], but few reports on the analysis of free bile acids[8–13] and none on the quantitative separation of all the compounds of interest to us.

EXPERIMENTAL

Samples

Cholic acid (CA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LA) were purchased from Fluka. 3-α-Hydroxy-7-oxocholanic acid, 3-oxo-7-α-hydroxycholanic acid, 3,7-dioxocholanic acid and the methyl esters of UDCA and CDCA (UDCMe and CDCMe respectively) were synthesized by Zambon Chimica.

Apparatus

The equipment used was a Hewlett-Packard Model 1084B high-performance liquid chromatograph equipped with a Model 79875A variable-wavelength UV detector (set at 195 nm) and a loop injector with an effective volume of 20 μl.

A reversed-phase column (Brownlee Labs., Santa Clara, CA, U.S.A.), 25 cm × 4.6 mm I.D., containing 5-μm RP-8, was used for analyzing all the bile acids and

0021-9673/83/$03.00 © 1983 Elsevier Science Publishers B.V.

esters. The column was maintained at room temperature (20 ± 1°C) or in a thermostatic oven at a controlled temperature.

Solvents

The mobile phases were prepared by mixing appropriate quantities of acetonitrile (LiChrosolv, Merck) and 0.02 *M* phosphate buffer (KH_2PO_4) adjusted to pH 3.0 with orthophosphoric acid.

Sample preparation

Methanolic solutions of the samples were prepared at concentrations of 5–10 mg/ml for hydroxy acids and 0.5–1 mg/ml for oxoacids and methyl esters.

RESULTS AND DISCUSSION

Fig. 1 shows a typical separation obtained with this method.

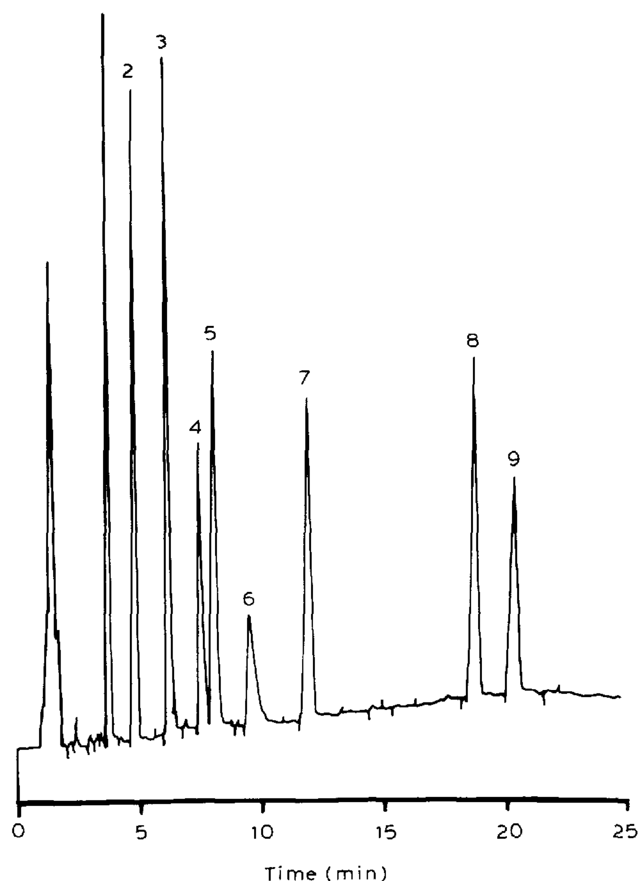

Fig. 1. HPLC separation of a synthetic mixture of bile acids. Conditions: Brownlee Labs. 5-μm RP-8 column maintained at 40°C. Mobile phase: A, 0.02 *M* KH_2PO_4 buffer adjusted to pH 3.0 with H_3PO_4; B, acetonitrile containing 2 ml/l of H_3PO_4 (2%, v/v). Gradient elution: 0–9 min, 47.5% B; 9–20 min, 47.5 to 55.0% B. Flow-rate: 2 ml/min. Peaks: 1 = CA; 2 = UDCA; 3 = 3-α-hydroxy-7-oxocholanic acid; 4 = 3,7-dioxocholanic acid; 5 = CDCA; 6 = 3-oxo-7-α-hydroxycholanic acid; 7 = UDCMe; 8 = CDCMe; 9 = LA.

Fig. 2 shows the capacity factors (k') of each cholanic acid or cholanic ester as a function of the amount of acetonitrile in the mobile phase when the column is maintained at room temperature (20 ± 1°C).

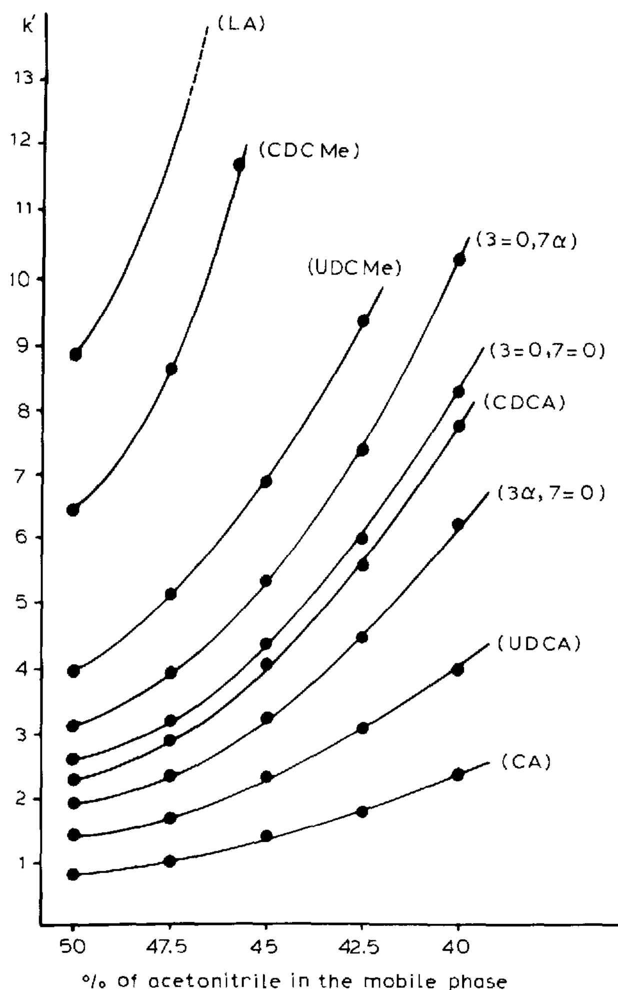

Fig. 2. k' values of each bile acid or ester *versus* acetonitrile content in the mobile phase (20 ± 1°C). (3α, 7 = O) = 3-α-hydroxy-7-oxocholanic acid; (3 = O, 7 = O) = 3,7-dioxocholanic acid; (3 = O, 7α) = 3-oxo-7-α-hydroxycholanic acid.

This method offers many advantages with respect to previously used methods. It avoids the derivatization of bile acids, is very rapid and so allows the quantitation of each component of a reaction mixture in a short time (10–15 min).

This method is also suitable for determining small amounts of CDCA in UDCA with good precision. Table I shows the results obtained from the analysis of a UDCA sample to which known quantities of CDCA were added, and Fig. 3 shows typical chromatograms of UDCA containing CDCA.

TABLE I

ANALYSIS OF MIXTURES OF KNOWN AMOUNTS OF UDCA AND CDCA

Amount of UDCA (mg)	*Amount of CDCA added (mg)*	*mg of CDCA found (mean ± S.D.) (n= 5)*
99	1	0.98 ± 0.02
98	2	1.90 ± 0.03
97	3	2.96 ± 0.02

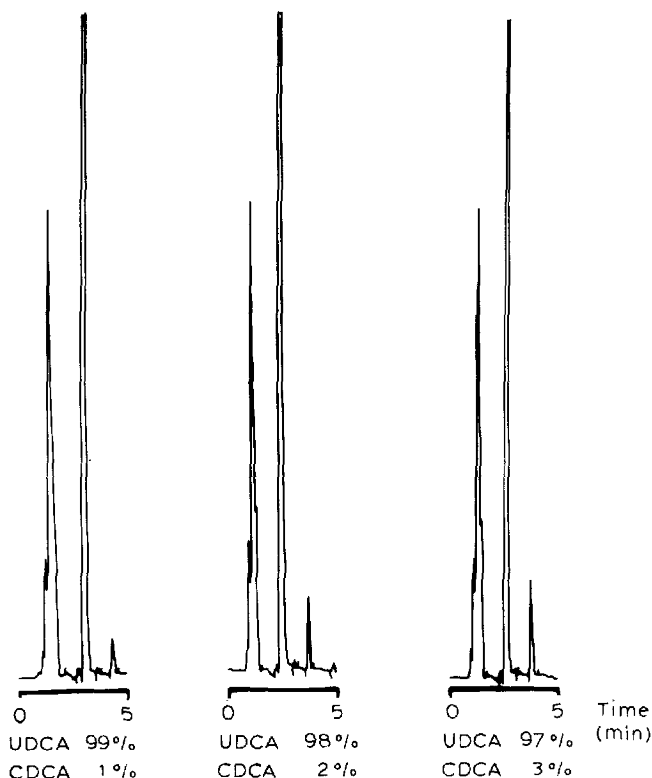

Fig. 3. HPLC determination of CDCA in UDCA samples. Conditions: Brownlee Labs. 5-μm RP-8 column maintained at 40°C. Mobile phase: acetonitrile 0.02 M KH_2PO_4 (55:45) adjusted to pH 3.0. Flow-rate: 2 ml/min.

The only difficulty found was in the separation of CDCA from 3,7-dioxocholanic acid. At certain fixed temperatures the two acids show equal retention times, while by changing the column temperature good separations are obtained. Fig. 4 shows the k' behaviour of CDCA and 3,7-dioxocholanic acid at temperatures ranging from 20 to 50°C and with acetonitrile contents in the mobile phase ranging from 50 to 42.5%. To obtain a good separation between these acids, it is necessary to maintain

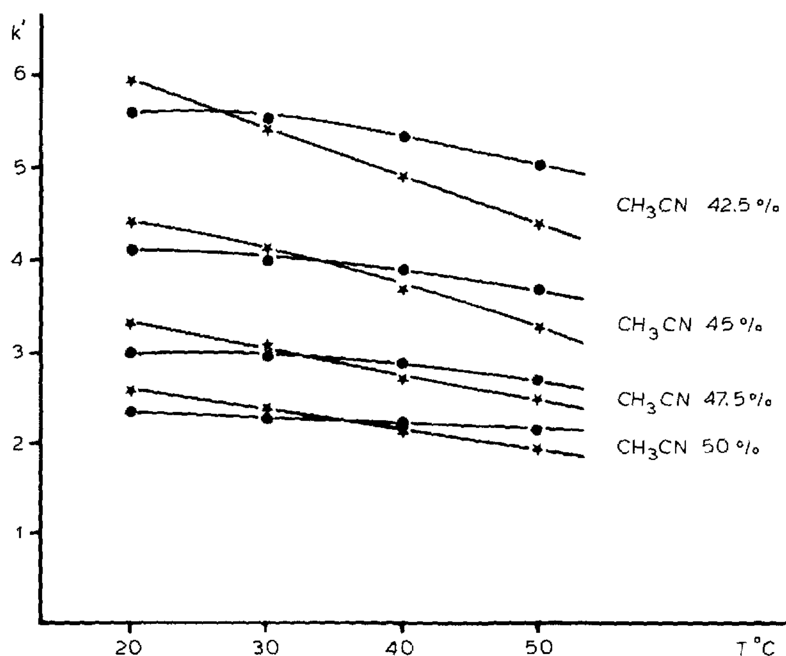

Fig. 4. k' values of CDCA (●) and 3,7-dioxocholanic acid (★) *versus* temperature and acetonitrile content in the mobile phase.

the column at room temperature (20°C) or at 50°C. The critical temperature of this separation is about 30–35°C. At these temperatures these two acids have the same retention time and cannot be separated by varying the acetonitrile content of the mobile phase.

ACKNOWLEDGEMENTS

We thank Mr. C. Cavallaro, Mr. R. Magaraggia and Mr. C. Velgi for their technical assistance.

REFERENCES

1 J. Goto, M. Hasegawa, H. Kato and T. Nambara, *Clin. Chim Acta*, 87 (1978) 141.
2 G. A. Bloch and J. B. Watkins, *J. Lipid Res.*, 19 (1978) 510.
3 R. Shaw and W. H. Elliott, *Lipids*, 13 (1978) 971.
4 M. S. Sian and A. J. Harding Rains, *Clin. Chim. Acta*, 98 (1979) 243.
5 F. Nakayama and M. Nakagaki, *J. Chromatogr.*, 183 (1980) 287.
6 K. Maruyama, H. Tanimura and Y. Hikasa, *Clin. Chim. Acta*, 100 (1980) 47.
7 A. T. Ruben and G. P. van Berge-Heregouwen, *Clin. Chim. Acta*, 119 (1982) 41.
8 K. Shimada, M. Hasegawa, J. Goto and T. Nambara, *J. Chromatogr.*, 152 (1978) 431.
9 R. Shaw, J. A. Smith and W. H. Elliott, *Anal. Biochem.*, 86 (1978) 450.
10 R. Shaw, M. Rivetna and W. H. Elliott, *J. Chromatogr.*, 202 (1980) 347.
11 J. Goto, H. Kato, Y. Saruta and T. Nambara, *J. Liquid Chromatogr.*, 3 (1980) 991.
12 S. Terabe, K. Yamamoto and T. Ando, *J. Chromatogr.*, 239 (1982) 515.
13 A. D. Reid and P. R. Baker, *J. Chromatogr.*, 247 (1982) 149.