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LIQUID CHROMATOGRAPHIC SEPARATION OF BILE ACIDS*

N. A. PARRIS

DuPont (U.K.) Limited, Instrument Products Division, Wilbury House, Wilbury Way, Hitchin, Hertfordshire SG4 0UR (Great Britain)

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SUMMARY

The recent revival in interest in the role of bile acids in biological systems has emphasised the importance of analysing individual bile acids for the presence of closely related impurities. An assessment of the value of modern, high-performance liquid chromatography for this application has shown that mixtures of underivatised bile acids may be resolved by reversed-phase chromatography. Separation of isomeric acids and those differing in the extent of hydroxyl substitution is possible by this approach. Mixtures of amino acid conjugates of bile acids may also be resolved by this method. The use of an ultraviolet absorbance detector, operating at a wavelength in the region of 210 nm, results in a detection limit in the order of 50 ng.

INTRODUCTION

For several decades, a great deal of attention has been given to studying the biological activity and therapeutic value of steroid drugs, particularly those of the corticosteroid type. During the same period, however, the structurally related bile acids have received much less attention. In more recent times, interest in the bile acids has increased considerably, principally for two reasons.

Firstly, evidence has indicated that certain transformations of bile acids may be associated with cancer of the large intestine[1]. Secondly, certain compounds possess therapeutic properties, for instance, chenodeoxycholic acid has been proposed as an *in vivo* means of solubilising gallstones[2].

The assay of bile acids may be performed by microbiological methods which tend to be somewhat time consuming and, for the methods to be specific, it is essential to use highly purified enzyme preparations[3]. An assessment of the use of high-performance liquid chromatography (HPLC) for this application has been made since the method has proved capable of quickly providing specific quantitative data in many analytical chemical applications.

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Throughout the development of HPLC there have been many applications describing highly successful separations of the thermally labile corticosteroids and oestrogens (see, *e.g.*, refs. 4 and 5). Although bile acids are structurally related to other steroids, the presence of a carboxylic functional group, together with a lack of unsaturation or aromaticity in their structure, alters the liquid chromatographic (LC) behaviour considerably.

Separation of bile acids from lipid-like materials has been reported by Eneroth and Sjövall[6] using silica gel adsorption methods with chloroform–acetic acid mixtures as mobile phases. In conventional, low-resolution systems, the approach tends to elute all of the bile acids together, *i.e.*, a type or class separation. High-performance adsorbents, *i.e.*, small-particle-diameter packings, have been used in modern LC systems to achieve resolution of individual bile acids from a mixture. Parris[7] has reported the use of Zorbax-SIL, porous silica microspheres, for this application, where the mobile phase comprised pentane–isopropanol (70:30).

In this present work, the usefulness of reversed-phase chromatography as a means of separating bile acids and related compounds is investigated. Of particular interest is the feasibility of resolving amino acid conjugates of bile acids, isomeric acids, and those which differ in the extent of hydroxyl substitution.

EXPERIMENTAL

Apparatus

The equipment used in this investigation comprised the following units, all obtained from DuPont, Instrument Products Division (Wilmington, Del., U.S.A.): Model 830 liquid chromatograph, having a maximum operating pressure of 315 bars; Model 837 spectrophotometer with a wavelength range of 195–600 nm and a flow cell volume of 8 μl; Model 845 differential refractometer with a flow cell volume of 3 μl.

Additional items of equipment included a Model 7120 universal septumless injector (Rheodyne, Berkeley, Calif., U.S.A.) and a Model 7130A two-pen strip chart recorder (Hewlett-Packard, Avondale, Pa., U.S.A.). These latter items are also attainable from DuPont. Chromatographic columns were 250 mm long with an internal diameter of 2.1 mm and were obtained from the supplier (DuPont) prepacked with Zorbax-ODS. This chromatographic packing consists of closely sized, porous silica microspheres of 5 μm diameter having octadecylsilyl groups chemically bonded to their surface.

After completion of this investigation, the manufacturing process for the chromatographic packing was slightly modified. This change has resulted in columns of higher efficiency and improved permeability; thus, the columns which are currently available will tend to yield somewhat better results than are reported here.

Solvents

Mobile phases were prepared from methanol, HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain) and water, distilled and stored in glass apparatus. Phosphoric acid, analytical reagent grade (BDH, Poole, Great Britain) was used to adjust the pH of the mobile phase.

Samples

Individual samples of free bile acids and their glycine and taurine conjugates were obtained from various sources. Many appeared quite impure, a feature which sometimes hampered the interpretation of the experimental data.

Development of a reversed-phase chromatographic system

All studies were carried out using the Zorbax-ODS chromatographic column within the instruments column oven compartment, without utilising the above-ambient oven temperature controller. Under these conditions the column compartment temperature was stabilised to 25° ± 1° during the investigation. Initially pure methanol was employed as mobile phase and solutions (approximate concentration 1% w/v) of each bile acid reference compound were injected in turn, in order to establish their retention characteristics. This procedure was repeated using mobile phases containing 95, 90, 85, 80, and 75% (v/v) methanol in acidified water. In all

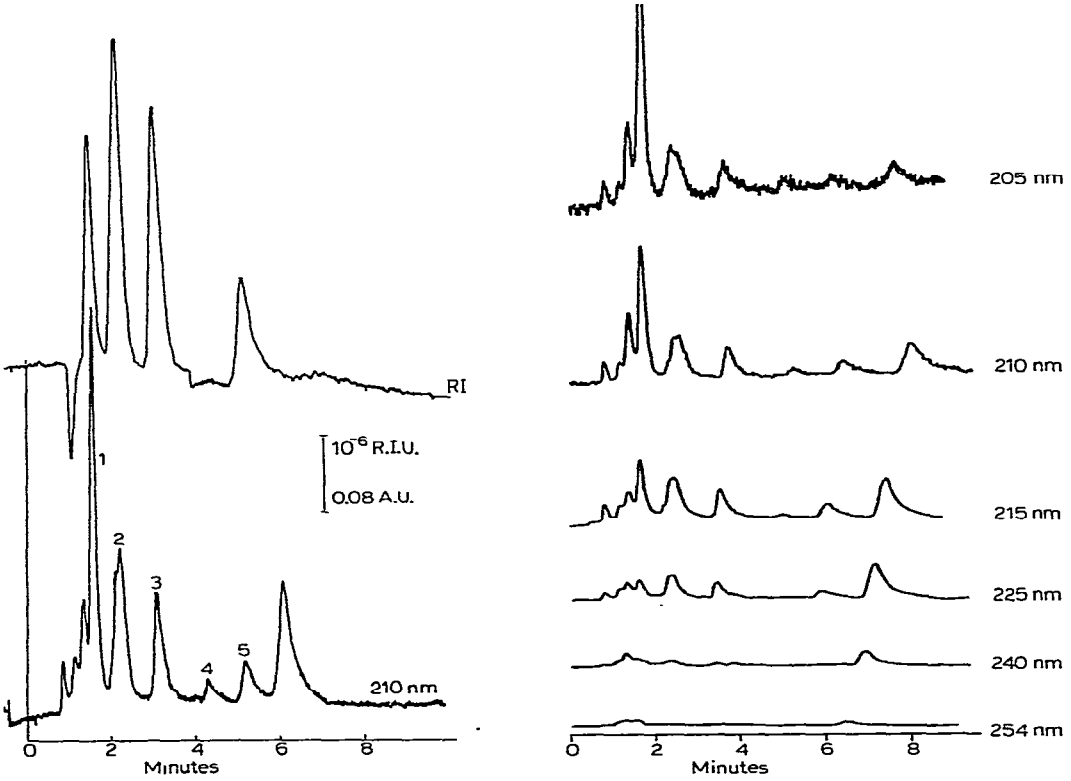

Fig. 1. Separation of six bile acids by reversed-phase chromatography. Column, stainless steel, 250 × 2.1 mm I.D.; packing, Zorbax-ODS; temperature, 25°; mobile phase, methanol–water (acidified with phosphoric acid to pH 2) (8:2); inlet pressure, 210 bars; flow-rate, 0.4 ml/min. Peaks: 1 = oxocholanic acid, 2 = cholic + chenodeoxycholic acids, 3 = hyodeoxycholic acid, 4 = deoxycholic acid, 5 = ketocholic acid. Upper trace, refractive index; lower trace, UV absorbance at 210 nm.

Fig. 2. Influence of UV detector wavelength on the sensitivity of detection of bile acids. Operating conditions, as for Fig. 1.

cases the water used to prepare the mobile phase was acidified to pH 2 by the addition of phosphoric acid; this action was taken in order to suppress any dissociation of the carboxylic acid function of the bile acid samples. The inlet pressure of the chromatographic system was adjusted to maintain the mean mobile phase linear velocity close to 4 mm/sec, as determined by the appearance of the first peak on the chromatogram.

The UV absorbance and refractometric detectors were coupled in series, the former detector being nearest to the column outlet. The operating wavelength of the UV detector was varied within the range 254–205 nm so as to ascertain the response behaviour of the detector towards the compounds under examination.

RESULTS

Investigations soon showed that water–methanol mixtures could be used as mobile phases for the resolution of bile acids on a column packed with Zorbax-ODS. The most useful working range of mobile phase composition was from 80–100% methanol. Addition of an acid or an acidic buffer to the mobile phase to minimise dissociation of the carboxylic functional group was found to result in an improved peak shape. Fig. 1 shows the separation of the mixture of six bile acids, specifically:

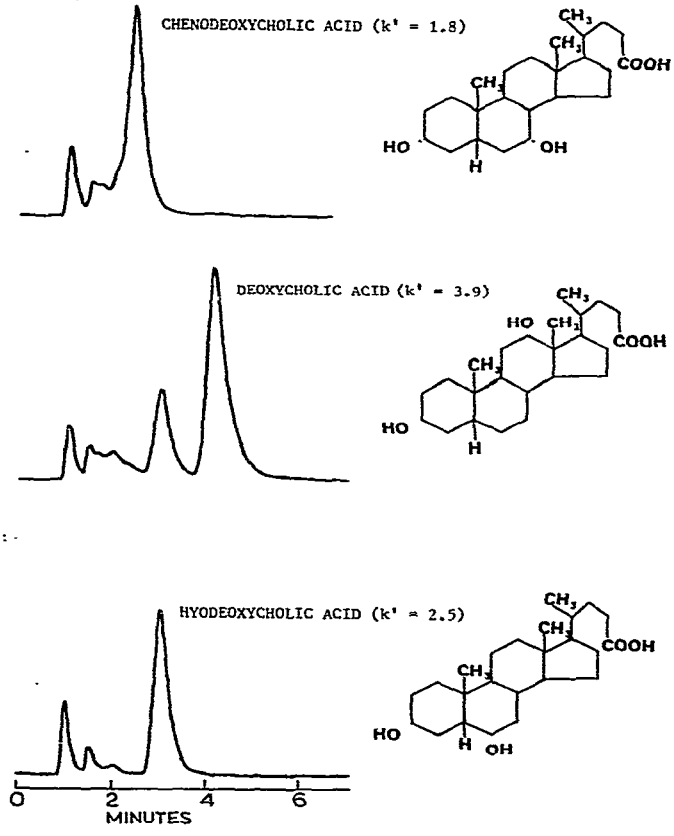

Fig. 3. LC characteristics of isomeric dihydroxy-bile acids. Operating conditions, as for Fig. 1.

oxocholanic, cholic, chenodeoxycholic, hyodeoxycholic, deoxycholic and ketocholic acids. The mobile phase contained 80% methanol and 20% water (acidified to pH 2 by addition of phosphoric acid) by volume. The separation was monitored by both refractive index and UV absorbance at a wavelength of 210 nm. The overall separation took less than 8 min to complete.

Fig. 2 illustrates the repetitive analysis of bile acids where only the detection wavelength was changed and demonstrates the enhanced sensitivity of using UV detection at wavelengths approaching 200 nm. At 254 nm little, if any, response to a bile acid is obtained. The sensitivity of detection improves progressively as the wavelength of the detector is reduced. At 205 nm, however, the noise level also increases significantly; this wavelength corresponds to the UV cut-off of the mobile phase.

Three examples of the resolution obtainable from the type of LC system described are presented to indicate the power of the technique at solving difficult separation problems.

Firstly, in Fig. 3, the chromatograms from the three dihydroxy-bile acids are shown. These acids, *viz.*, deoxy-, hyodeoxy-, and chenodeoxycholic acids, show quite different retention characteristics and also the presence of impurities. The analysis conditions for this mixture were the same as those given in Fig. 1.

The second example is the resolution of bile acids containing a different number of hydroxy groups. Taurine-conjugated bile acids, namely taurocholic, taurolithocholic and taurodeoxycholic acids, can similarly be resolved; chromatograms of these compounds are shown in Fig. 4. It is interesting to observe that the order of elution corresponds to the decreasing number of hydroxyl groups.

Finally, Fig. 5 indicates the problems that can be encountered in this area of work. Here the LC behaviour of "pure" tauro- and glycocholic acids is presented.

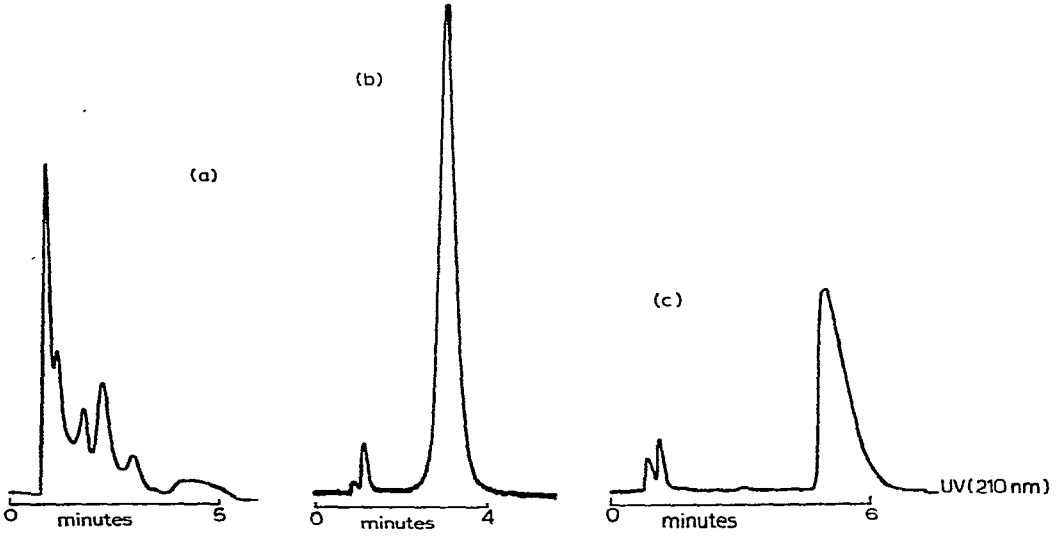

Fig. 4. LC characteristics of taurine-conjugated bile acids. (a) Taurocholic acid, (b) taurodeoxycholic acid, and (c) taurolithocholic acid. Operating conditions, as for Fig. 1.

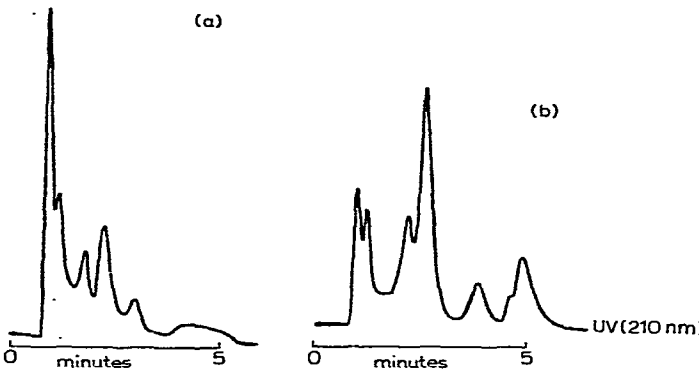

Fig. 5. LC characteristics of (a) taurocholic and (b) glycocholic acids. Operating conditions, as for Fig. 1.

CONCLUSIONS

In other areas of chemical application, reversed-phase chromatography has become very important for the separation of compounds having an appreciable lipophilic character. The potential application of this method to bile acids was investigated since the compounds are freely soluble in methanol but are essentially insoluble in water, a characteristic which is typical of many substances that have been successfully analysed by reversed-phase chromatography. The results presented in this paper confirm the usefulness of the reversed-phase method for the separation of bile acids. The presence of dilute phosphoric acid in the mobile phase did not appear to reduce the performance of the chromatographic column during the investigation, which took place over a period of several weeks. Acidification of the mobile phase, however, did improve the shape of the eluting peaks by reducing tailing, presumably by suppressing the ionisation of the carboxylic acid functional group present in the acids. In the past, the use of UV absorbance as a means of detection has considerably simplified many LC applications since, in general, such detectors are very stable and easy to use. It is quite probable that much of the successful LC applications in the pharmaceutical field had been a result of their good UV absorbance characteristics. The use of UV detection for essentially saturated molecules requires that detectors operate at wavelengths about or below 200 nm. The data shown in Fig. 2 clearly indicate the advantage of operating at wavelengths in the region of 205–215 nm, relative to 254 nm, the absorption wavelength used in many commercial detectors. The wavelength region below 215 nm, however, imposes a considerable restriction on the choice of solvents used to prepare the mobile phase. In this current investigation absorption of UV light by methanol prevented operation at wavelengths below 205 nm. Acetonitrile has been used as an organic modifier in place of methanol in many reversed-phase studies and when highly purified, will transmit UV light to about 190 nm. However, in the present study, the solubility of the bile acids in this latter solvent was much inferior to that in methanol.

The use of two detection systems for monitoring the analysis of bile acids has led to some interesting observations. In the first instance, it was anticipated that the UV detection would offer a considerably high sensitivity, particularly in the case of

acids containing other UV-absorbing chromophores, *e.g.*, olefinic bonds. This anticipation has proved fully justified in the case of bile acids which are conjugated with amino acids such as glycine and taurine, as well as in the case of unsaturated bile acids. The detection limit of a completely saturated compound appears to vary considerably from compound to compound. A critical opinion on this point cannot be given since most of the substances investigated proved to be quite impure. A general feature of the chromatogram obtained from only one bile acid sample was a single peak, in addition to the solvent front, recorded from the refractive index detector; conversely, the UV detector often indicated the presence of many peaks. It was assumed that the single peak from the refractive index detector output was, in fact, the major component and in turn was the species in question. The assignments of peak identity in all chromatograms presented here have been based on this assumption.

In conclusion, it has been found that reversed-phase LC is sufficiently selective to enable individual and isomeric bile acids and amino acid conjugates of the same to be resolved within a few minutes without derivatisation. Both refractive index and UV absorbance (at wavelengths in the region 205–215 nm) provide effective methods of detection, the latter detection method offering greatest opportunity to observe impurities in the sample.

REFERENCES

1 M. J. Hill, *Amer. J. Clin. Nutr.*, 27 (1974) 1475.
2 P. J. Barnes, *Amer. Lab.*, *May* (1976) 67.
3 R. Engert and M. D. Turner, *Anal. Biochem.*, 51 (1973) 399.
4 N. A. Parris, *Instrumental Liquid Chromatography*, Elsevier, Amsterdam, 1976, p. 161.
5 J. F. K. Huber and C. A. M. Meijers, *Anal. Chem.*, 44 (1972) 111.
6 P. Eneroth and J. Sjövall, *Methods Enzymol.*, 15 (1969) 237.
7 N. A. Parris, *Lab. Equip. Dig.*, Nov. (1974) 77.