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DETERMINATION OF CONJUGATED BILE ACIDS IN HUMAN BILE BY ISOTACHOPHORESIS IN A NON-AQUEOUS SOLVENT USING a.c. CONDUCTIVITY AND UV DETECTION

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SUMMARY

A method for the determination of conjugated bile acids in human bile using isotachophoresis in 95% methanol is described. The leading ion is 0.01 *M* chloride, the counter ion is hydroxylamine at its p*K** value and the terminating ion is N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES). The sample preparation consists of C_{18}-silica cartridge adsorption. Microlitre amounts of the methanol eluate are injected and analysed within 20 min in a 0.2 mm I.D. PTFE capillary. The sensitivity of the method is better than 50 ng of each of the conjugated bile acids using a.c. conductivity detection.

INTRODUCTION

Methods for the determination of bile acids have shown some improvements in the last decade. In a review in 1973[1], enzymatic methods were recommended for routine use, whereas a combination of thin-layer and gas–liquid chromatography was suggested if specific information on individual bile acids is needed. Although some improvements in enzymatic[2], thin-layer chromatographic[3] and gas chromatographic[4] methods have been reported recently, the developments in high-performance liquid chromatography (HPLC) are apparently more promising. In a more recent review[5] on the separation of bile acids by HPLC, the retention behaviour of free and conjugated bile acids in both reversed-phase and normal-phase systems was reported. For the isolation of bile acids from biological material (bile, serum, faeces), sample pre-treatment is usually time consuming (from 10 min to more than 24 h), as a group separation of free and conjugated acids is generally included.

The detection of bile acids in HPLC is mostly carried out by differential refractive index measurements[5–8]. Some mobile phases allow UV detection at wavelengths below 210 nm[8,9]. Fluorescence detection with pre-column[10] or post-column[11] derivatization has also been reported.

Another analytical technique that has developed significantly during the last decade is capillary isotachophoresis[12–14]. The equipment uses a sensitive universal

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detector compared with refractive index detection in HPLC, a.c. conductivity or potential gradient detection. Most applications are performed in aqueous operational systems, but the importance of using non-aqueous solvents was soon recognized[12,15]. One reason is that the use of the technique can be extended to ionizable compounds, sparingly soluble in water, such as long-chain fatty acids[15]. Another reason is the use of specific solvent–solute interactions to optimize certain separations. As the solubility of bile acids under aqueous isotachophoretic conditions is insufficient, a methanolic operational system is applied for the analysis of free and glyco- and tauro-conjugated bile acids in biological samples.

EXPERIMENTAL

The use of non-aqueous (*e.g.*, methanolic) systems in isotachophoresis has so far been limited by the availability of insulating material that is both resistant to these solvents and suitable for the construction of a high-resolution a.c. conductivity detector, for which Perspex is usually used[12]. The use of Teflon® has also been reported[16]. The introduction of an Araldite that meets both of these requirements was reported recently[17]. The construction of the equipment used was similar to that described earlier[12]. The separation compartment consisted of a PTFE capillary of I.D. 0.2 mm and length *ca.* 25 cm. The a.c. conductivity cell had a volume of 3 nl, corresponding to a theoretical minimum detectable amount of *ca.* $30 \cdot 10^{-12}$ equivalents. UV detection was carried out at 206 nm.

The solvent used in the isotachophoretic experiments was 95% (v/v) methanol in water. A higher methanol content is not necessary. A lower methanol content decreases the solubility of the bile acids to below the isotachophoretic steady-state concentration. The use of non-aqueous solvents in isotachophoresis is hampered by the lack of data on pK, mobility and solubility values of ions normally used in operational systems. As the leading ion chloride was used, the mobility of which is considerably lower than in water. For this particular application, however, this was irrelevant. As the counter ion we preferred buffers normally used in aqueous isotachophoresis, mainly because of their availability and relative purity. However, histidine, used preferably for neutral anionic systems, could not be used because of its limited solubility at methanol concentrations above 60%. We used either hydrox-

TABLE I

OPERATIONAL SYSTEMS FOR 95% (V/V) METHANOL IN WATER AS A SOLVENT

The counter ions were at their respective pK* values. Driving current: 25 μA in a 0.2 mm I.D. capillary

Parameter	*pH* 5.84*	*pH* 6.82*
Leading ion	Chloride	Chloride
Concentration	0.01 *M*	0.01 *M*
Counter ion	Hydroxylamine	Triethanolamine
Terminator	HEPES	BICINE*
Concentration	*ca.* 0.005 *M*	*ca.* 0.005 *M*

* BICINE = N,N-bis(2-hydroxyethyl)glycine.

ylamine or triethanolamine at their respective pK^* values. The operational systems used are listed in Table I.

The pH measurements were carried out with a Type 135 digital pH meter (Corning, Halstead, Essex, Great Britain), calibrated with aqueous standard buffer solutions (pH 6.0 and 8.0) (Merck, Darmstadt, G.F.R.). The pH values of the methanolic systems in Table I were not corrected for the methanol content and are therefore denoted with an asterisk. The pK^* values of the counter ions were determined by pH* measurement of a solution of the buffer chloride salt, half of which was subjected to anion exchange. As the terminator for methanolic systems lithocholic acid has been used[12], but we prefer to use weak ions such as 2-morpholinoethanesulphonic acid (MES), N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) and tris(hydroxymethyl)methylaminopropane sulphonic acid (TAPS). As in aqueous isotachophoresis, additives to the leading electrolyte may be needed to decrease the effect of electroosmosis[12], although these disturbances are expected to be of less importance in methanolic systems[18].

The sample pre-treatment procedure used in the present investigation consisted of C-18 silica cartridge adsorption (Sep-Pak; Waters Assoc., Milford, MA, U.S.A.) as described by other workers[9] for an HPLC method. The procedure was slightly modified. A volume of 20 μl of human bile was diluted in 4 ml of 0.5 M phosphate buffer (pH 7.0) and applied to the cartridge, which was then washed with 15 ml of water and eluted with 3 ml of methanol. The average recovery of bile acids added to human bile was 91%. Microlitre amounts of the eluate or a standard solution of bile acid conjugates in methanol were injected directly. The time of analysis was 15–20 min at 25 μA in a PTFE capillary of I.D. 0.2 mm. This longer than usual analysis time was caused by the relatively low transport number (< 0.5) of the leading ion in the solvent used.

RESULTS AND DISCUSSION

One of the most important parameters used to achieve separation in isotacho-

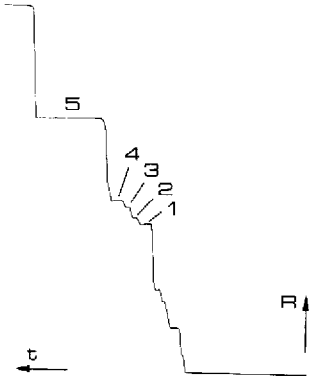

Fig. 1. Isotachophoretic separation of five bile acids in a standard mixture. The operational conditions are summarized in Table I. The pH* of the leading electrolyte was 6.82. 1 = 0.2 nmol taurochenodeoxycholic acid; 2 = 0.2 nmol taurocholic acid; 3 = 0.1 nmol glycochenodeoxycholic acid; 4 = 0.3 nmol glycocholic acid; 5 = 3.0 nmol cholic acid. R = Increasing resistance, t = increasing time.

phoresis, besides the difference in absolute mobility, is the difference in the pK values of the compounds to be separated. pK* values for bile acids in methanol are not known, but pK values in water have been mentioned in the literature[5]: for free bile acids pK = 5–6, for glycine conjugates *ca.* 4 and for taurine conjugates *ca.* 2 was reported.

Fig. 1 shows the isotachophoretic separation at pH* 6.82 of five bile acids in a standard mixture. From the fact that the non-conjugated cholic acid migrates with a considerably lower effective mobility than the conjugated bile acids, it is concluded that the pK* value of cholic acid shows a positive shift with respect to its value in

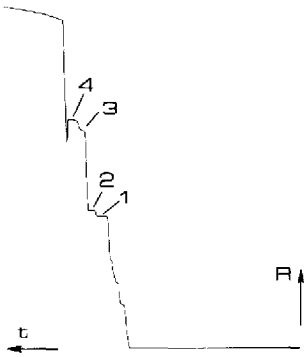

Fig. 2. Isotachophoretic separation of tauro- and glyco-conjugated acids according to their respective pK* values at pH* 5.84. The operational conditions are summarized in Table I. Zone numbers as in Fig. 1.

water. The same applies to the glyco-conjugated bile acids, as is illustrated in Fig. 2. Group separation on glyco- and tauro-conjugates is achieved at pH* 5.84 so that the pK* values of the glyco-conjugates can be calculated to be greater than 5. The pK* of the tauro-conjugates remains below 5, as the effective mobility does not change in the interval $5.84 < \text{pH}^* < 6.82$.

These observations agree with reversed-phase liquid chromatographic data[5,6] where at pH* 4.7 the separation of the tauro- from the glyco-conjugates is mainly

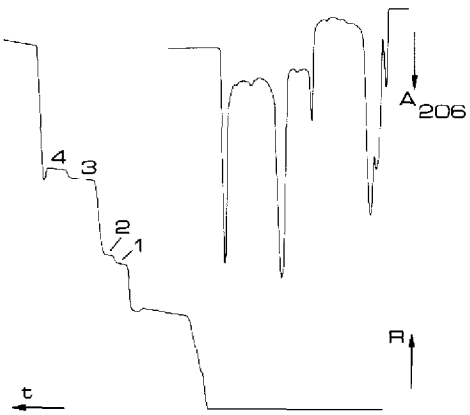

Fig. 3. Analysis of a sample of human bile of a patient. Zone numbers as in Fig. 1. The concentrations of the individual bile acid conjugates were in the range 10–40 nmol/μl. A = Increasing UV absorption.

ascribed to pK^* differences. In reversed-phase chromatography there is also a significant effect of the number and/or orientation of the hydroxyl groups on retention behaviour[6] in 65 % methanol. In isotachophoresis in 95 % methanol these effects were less pronounced, but sufficient to achieve complete separation of conjugated cholic and chenodeoxycholic acids, as illustrated in Fig. 2.

Fig. 3 shows the analysis of a sample of human bile of a patient (a suspected bile stone former). Taurochenodeoxycholic, taurocholic, glycochenodeoxycholic and glycocholic acids were determined. Because of the group separation at this pH*, the clinically significant glyco-tauro ratio is easily seen.

We are currently investigating the possibilities of adapting the method for the determination of bile acids at serum levels. One of the advantages of using a more hydrophobic solvent in isotachophoresis is that a number of sample pre-treatment procedures used in chromatography can be applied to remove unwanted polar substances such as sodium chloride.

This study has shown that the use of non-aqueous isotachophoresis is very promising. For the determination of bile acid conjugates it compares favourably with liquid chromatography in terms of sensitivity and analysis time.

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REFERENCES

1 M. T. Ravi Subbiah, *Ann. Clin. Lab. Sci.*, 3 (1973) 362.
2 H. Kimura, N. Suzuki, T. Sato, J. Goto and T. Nambara, *Jap. J. Clin. Chem.*, 8 (1979) 126.
3 G. Szepesi, K. Dudás, A. Pap, Z. Végh, E. Mincsovics and T. Tyihák, *J. Chromatogr.*, 237 (1982) 137.
4 G. Lepage, C. C. Roy and A. M. Weber, *J. Lipid Res.*, 22 (1981) 705.
5 W. H. Elliott and R. Shaw, *Chromatogr. Sci.*, 16 (1981) 1–40.
6 C. A. Bloch and J. B. Watkins, *J. Lipid Res.*, 19 (1978) 510.
7 J. Goto, M. Hasegawa, H. Kato and T. Nambara, *Clin. Chim. Acta*, 87 (1978) 141.
8 T. Nambara, J. Goto, M. Hasegawa and H. Kato, in G. L. Hawk (Editor), *Biological and Biomedical Applications of Liquid Chromatography II*, Marcel Dekker, New York, 1978.
9 A. Th. Ruben and G. P. van Berge-Henegouwen, *Clin. Chim. Acta*, 119 (1982) 41.
10 S. Kamada, M. Maeda and A. Tsuji, *J. Chromatogr.*, 272 (1983) 29–41.
11 S. Baba, R. Uenoyama, K. Suminoe, F. Takeda, S. Hašegawa and Y. Kameno, *Kobe J. Med. Sci.*, 26 (1980) 89.
12 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis —Theory, Instrumentation and Applications* (*J. Chromatography Library*, Vol. 6), Elsevier, Amsterdam, 1976.
13 A. Baldesten and S.-G. Hjalmarsson, *CRC Crit. Rev. Anal. Chem.*, 11 (1981) 261–352.
14 Z. Deyl (Editor), *Electrophoresis* (*J. Chromatography Library*, Vols. 18A and B), Elsevier, Amsterdam, 1982.
15 J. L. Beckers, *Thesis*, Eindhoven University of Technology, Eindhoven, 1976.
16 D. Kaniansky, *Thesis*, Komensky University, Bratislava (1982).
17 Th. P. E. M. Verheggen and F. M. Everaerts, *J. Chromatogr.*, 249 (1982) 221–230.
18 J. C. Reijenga, G. V. A. Aben, Th. P. E. M. Verheggen and F. M. Everaerts, *J. Chromatogr.*, 260 (1983) 241–254.