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Short communication

Analysis of bile acids and their conjugates using high-pH anion-exchange chromatography with pulsed amperometric detection

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Abstract

Bile acids and their conjugated forms may be separated by anion-exchange chromatography in alkaline media (0.9 *M* sodium acetate, 0.1 *M* sodium hydroxide, 15% v/v acetonitrile) on a CarboPac PA-100 column. The effluent was monitored at high sensitivity, with detection limits of less than 10 μM, using a pulsed amperometric detector. Free bile acids and their glyco- and tauro-conjugated forms were separated and detected within 40 min under isocratic conditions.

1. Introduction

The analysis of bile acids and salts in faeces gives important information concerning their metabolism and reabsorption in the intestines. Several GC methods have been described [1–3] involving pre-column derivatisation. In recent years, reversed-phase high-performance chromatographic (HPLC) methods have become more popular and a number of HPLC techniques have been described for the separation and detection of bile acids. These methods used reversed-phase (RP-HPLC) columns which have been shown to separate the bile acids in ca. one hour [4–9]. Part of the difficulties arising with these methods concerns the method of detection. Unconjugated bile acids, which form the majority of the bile acids occurring in faeces, show only low ultraviolet absorption in the far UV (ca. 195–210 nm) and this makes the detection method insensitive and extremely prone to interferences. Although conjugated bile acids show a higher sensitivity, use of the far UV still causes problems due to non-specific interferences. In addition the elution gradient causes large baseline shifts [5]. Unconjugated bile acids are difficult to separate under conditions used for the separation of conjugated bile acids, and hence fractionation prior to chromatography is recommended [9]. The use of post-column detection [6] or pre-column derivatisation [7,8] result in a more complex analytical process. Both methods have negative features. Pre-column derivatisation reduces the inherent differences between the bile acids, introduces interfering products from the derivatisation reaction and may not be suitable for taurine conjugates which consequently have to be analyzed separately [8]. Post-

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column detection using an immobilised-enzyme reactor needs great care and regular regeneration of the enzymic activity.

The present work shows that bile acids may be separated by high-pH anion-exchange chromatography and detected using a pulsed amperometric detector (HPAEC-PAD), conditions often used for carbohydrate analysis. This method is capable of detecting the bile acids at a sensitivity comparable to that obtained for the carbohydrates.

2. Experimental

2.1. Chemicals

Bile acids and their conjugates were obtained from Sigma (Poole, Dorset, UK) and HPLC grade solvents and sodium acetate from Fisons (Loughborough, Leics., UK). Mixed standard solutions of the bile acids were made up in 0.8 *M* NaOH, 8% (v/v) acetonitrile.

2.2. Liquid chromatography

HPLC was performed on a Dionex DX-300 HPLC system fitted with a PAD-2 pulsed amperometric detector with a gold working electrode and a 250 × 4 mm I.D. Dionex CarboPac PA-100 column (bead diameter 8.5 μm) protected by CarboPac PA-100 guard column. A 25-μl aliquot of analyte was injected onto the column.

Standard solutions of the bile acids were chromatographed at room temperature, under a number of isocratic and gradient conditions until optimum isocratic conditions were achieved (0.9 *M* sodium acetate, 0.1 *M* sodium hydroxide, 15% (v/v) acetonitrile, flow-rate 0.8 ml min^{-1}). The PAD conditions chosen were $V_1 = +0.05$ V, $t_1 = 480$ ms, $V_2 = +0.60$ V, $t_2 = 120$ ms, $V_3 = -0.60$ V, $t_3 = 60$ ms. Changing the PAD conditions did not significantly increase the sensitivity of detection. For bile acid concentrations of ca. 300 μM (i.e. about 8 nmol injected) the PAD range is 1K nA. These conditions are similar to those generally chosen for carbohydrate analysis. is in agreement with the cyclic voltammetric behaviour of cholic acid which was independently found to be similar to that for glucose.

3. Results and discussion

The bile acids and their conjugates were separated under isocratic conditions (Fig. 1). The CarboPac PA-100 column is a highly efficient anion-exchange column with a pronounced hydrophobic character which is marketed primarily for carbohydrate analysis.

The bile acids are separated both by their anionic character and their hydrophobicity; the more hydrophillic ones eluting earlier and the more anionic taurine derivatives eluting later. The number of alcohol groups, and hence the

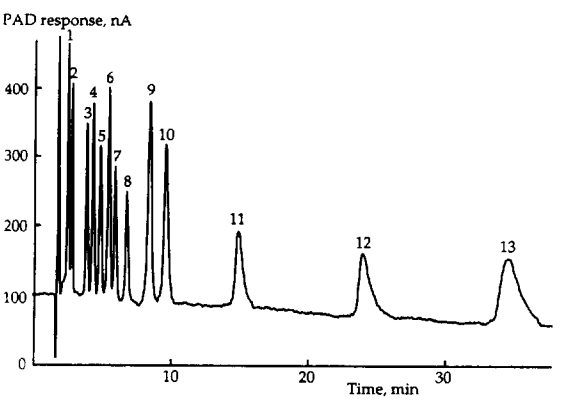

Fig. 1. HPAEC-PAD of the bile acids under isocratic conditions, 0.9 *M* sodium acetate, 0.1 *M* sodium hydroxide, 15% (v/v) acetonitrile, flow-rate 0.8 ml min^{-1}. Peaks: 1 = glycocholic acid (4.9 nmol); 2 = cholic acid (5.0 nmol); 3 = taurocholic acid (4.8 nmol); 4 = glycodeoxycholic acid (5.0 nmol); 5 = glycochenodeoxycholic acid (4.9 nmol); 6 = deoxycholic acid (5.0 nmol); 7 = ursodeoxycholic acid (7.4 nmol); 8 = chenodeoxycholic acid (4.8 nmol); 9 = taurodeoxycholic acid (9.6 nmol); 10 = taurochenodeoxycholic acid (10.4 nmol); 11 = glycolithocholic acid (5.5 nmol); 12 = lithocholic acid (7.4 nmol); 13 = taurolithocholic acid (13.6 nmol).

hydrophillic/hydrophobic character, determines the order of elution of the bile acids and their derivatives in a manner similar to that achieved by RP-HPLC; the trihydroxylated cholic acid elutes first followed by the dihydroxylated, deoxycholic, ursodeoxycholic and chenodeoxycholic acids and finally the monohydroxylated lithocholic acid (Table 1). In contrast to RP-HPLC the glycine conjugates, being more hydrophillic, elute before the free bile acids whereas the taurine conjugates, being more anionic, elute later. The mobile phase contains a high salt concentration which helps to release the anions from the chromatographic matrix but which increases the hydrophobic attractions. Acetonitrile is added to the mobile phase to weaken these hydrophobic attractive forces and does not interfere with the PAD.

The relative responses of the bile acids to the PAD parallels their hydroxyl content, in a manner similar to that found in carbohydrate analysis–the more hydroxyl groups the higher the response. The conjugated bile acids give slightly lower molar responses (Table 1), possibly due to the increased difficulty of structural alignment, for electrochemical oxidation, on the gold electrodes.

The composition of the mobile phase ensures that most possible interferences do not bind to the column; bound molecules generally being both hydrophobic and anionic. As analysis of bile acids from natural sources such as faeces by GC or HPLC involves solvent [2,3] and Sep-Pak C_{18} [5,7] extraction, the occurrence of possible interferences is further reduced. In addition, the use of PAD has advantages with respect to the specificity of detection. Only compounds which are electrochemically oxidised under the conditions used are detected. As they possess α-linked hydroxyl groups which lie on the same, more hydrophillic, side of the molecule to the anionic carboxylate group, bile acids can interact with the positively charged gold electrode (Fig. 2). The size of the PAD response depends only slightly on the mobile phase used. Carbohydrates do not interfere with this chromatographic procedure as they elute before the bile acids and

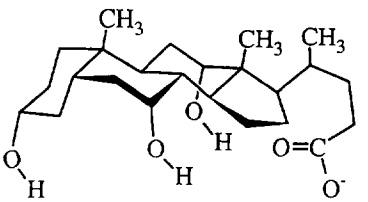

Fig. 2. The structure of cholic acid showing how the carboxylic acid and hydroxyl groups may lie on the same side of the molecule, opposite a hydrophobic surface.

Table 1
Relative responses and retention times of bile acids

Bile acid	Retention time (min)	Relative response[a]
Glycocholic acid	2.76	0.81
Cholic acid	2.98	1.00
Taurocholic acid	4.04	0.65
Glycodeoxycholic acid	4.37	0.70
Glycochenodeoxycholic acid	4.69	0.54
Deoxycholic acid	5.38	0.66
Ursodeoxycholic acid	5.87	0.46
Chenodeoxycholic acid	6.70	0.81
Taurodeoxycholic acid	8.84	0.52
Taurochenodeoxycholic acid	9.70	0.76
Glycolithocholic acid	15.05	0.46
Lithocholic acid	24.02	0.54
Taurolithocholic acid	34.90	0.41

[a] Molar response relative to cholic acid
Conditions as Fig. 1.

would be removed, in any case, by the prior clean-up process.

In conclusion, bile acids and their conjugated forms may be detected using PAD at concentrations close to those found for carbohydrate analysis, allowing for the number of hydroxyl groups present. They may be separated by anion-exchange chromatography in alkaline media, under isocratic conditions, on a CarboPac PA-100 column prior to detection. This method improves on current RP-HPLC methods as it has high sensitivity without pre-column derivatisation or post-column reaction, and does not require separate treatment of free bile acids and their conjugated forms.

4. References

[1] T. Ilda, T. Itoh, K. Hagiwara, F.C. Chang, J. Goto and T. Nambara, *Lipids*, 24 (1989) 1053.
[2] J. Ejderhamn, G. Hedenborg and B. Strandvik, *Scand. J. Lab. Invest.* 52 (1992) 697.
[3] K.D.R. Setchell, A.M. Lawson, N. Tanida and J. Sjövall, *J. Lipid Res.*, 24 (1983) 1085.
[4] S. Lam and A. Karmen, in C.K. Lim (Editor), *HPLC of Small Molecules: A Practical Approach*, IRL Press, Oxford, UK, 1986, pp. 103–116.
[5] S. Scalia, *J. Chromatogr.*, 431 (1988) 259.
[6] R.H. Zhao, B.Y. Li, N. Chen, Y.K. Zhang, Z.Y. Wang and P.C. Lu, *Biomed. Chromatogr.*, 7 (1993) 139.
[7] G. Wang, N.H. Stacey and J. Earl, *Biomed. Chromatogr.*, 4 (1990) 136.
[8] T.R. Driscoll, H.H. Hamdan, G. Wang, P.F.A. Wright and N.H. Stacey, *B. J. Ind. Med.*, 49 (1992) 700.
[9] T. Nambara and J. Goto, in K.D.R. Setchell, D. Kritchevsky and P.P. Nair (Editors), *The Bile Acids, Chemistry, Physiology and Metabolism*, Plenum Press, New York, NY, 1988, pp. 43–64.