Journal of Chromatography, 218 (1981) 647–652
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,118

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BILE PIGMENTS: SEPARATION AND CHARACTERIZATION OF THE UROBILINOIDS

ROSALIND V. A. BULL, C. K. LIM* and C. H. GRAY
Division of Clinical Chemistry, MRC Clinical Research Centre, Harrow, Middlesex HA1 3UJ (Great Britain)

SUMMARY

The detailed analysis of faecal bile pigments by high-performance liquid chromatography is described. Non-aqueous reversed-phase systems with acetonitrile–dimethyl sulphoxide or acetonitrile–dimethyl sulphoxide–methanol as the mobile phase on C_1, C_8 or C_{18}-bonded silica are used for the group separation of verdinoids, violinoids and urobilinoids. A silica column, with acetonitrile–water–tetraethylenepentamine as mobile phase, separates the laevorotatory stercobilin ($C_{33}H_{46}N_4O_6$) and half-stercobilin ($C_{33}H_{44}N_4O_6$) from the optically inactive urobilin ($C_{33}H_{42}N_4O_6$). The diastereoisomers are resolved by converting the urobilinoids into their dimethyl esters before chromatography on a silica column with *n*-heptane–methyl acetate–methanol containing 1 % of diethylamine as the solvent system.

INTRODUCTION

The urobilinoids are naturally occurring bile pigments formed from bilirubin by bacterial reduction in the intestine. The colourless "urobilinogens" formed are usually completely oxidised to the orange "urobilins" during extraction from faeces.

"Urobilins" can be classified into three groups: dipyrrolidones (stercobilin), pyrrolinone-pyrrolidone (half-stercobilin) and dipyrrolinones (urobilin). Each may exist as pairs of enantiomorphs, the 2′- and 7′-positions of the molecules being chiral centres (see Fig. 1). Half-stercobilin and urobilin can be readily oxidised to half-stercoviolin and mesobiliviolin, respectively, while stercobilin is stable; mesobiliviolin can be further oxidised to mesobiliverdin (glaucobilin) (Fig. 1).

The determination of the daily excretion of faecal bile pigments has been used as an index of the severity of haemolysis in diseases in which there is an abnormally high turn-over of haem, such as sickle cell and other haemolytic anaemias and congenital porphyria. However, due to the instability and, therefore, the complexity of the bile pigments and the lack of specific methods for their determination, they are now rarely measured.

The analysis of faecal bile pigments, especially the urobilinoids, by high-performance liquid chromatography (HPLC) has not been reported before. This paper

0021-9673/81/0000–0000/$02.50

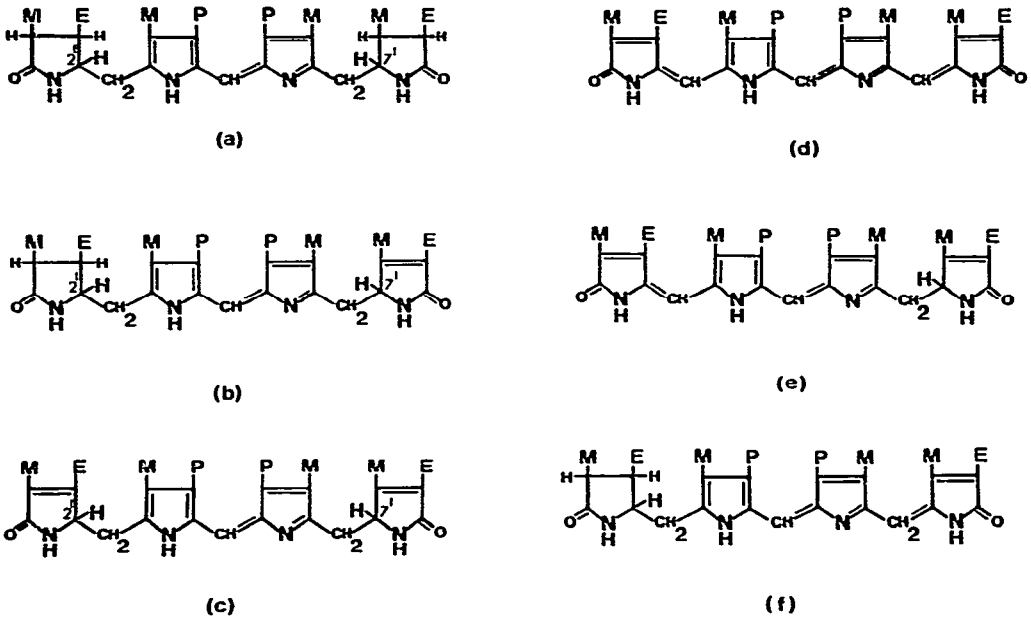

Fig. 1. Structures of bile pigments: (a), stercobilin; (b), half-stercobilin; (c), urobilin; (d), glaucobilin; (e), mesobiliviolin; and (f) half-stercoviolin. E = ethyl, M = methyl, P = propionic acid.

describes the detailed analysis of the urobilinoids by HPLC. Non-aqueous reversed-phase systems with acetonitrile–dimethyl sulphoxide (50:50) or acetonitrile–dimethyl sulphoxide–methanol (25:25:50) as solvents have been developed for the separation of the urobilinoids from the verdins and violins. The urobilinoids were separated into stercobilin, half-stercobilin and i-urobilin on a silica column (Hypersil) with acetonitrile–water–tetraethylenepentamine (85:15:0.05) as mobile phase. The diastereoisomers were resolved by converting the urobilinoids into their dimethyl esters, followed by separation on a silica column (Hypersil), with *n*-heptane–methyl acetate–methanol containing 1% of diethylamine (75:25:2) as eluent.

This efficient separation by HPLC will improve quantitation of the bile pigments, which might be important in assessing haem catabolism in clinical work.

EXPERIMENTAL

Materials

Acetonitrile (CH_3CN) was HPLC grade from Rathburn Chemicals (Walkerburn, Great Britain). Iodine was analytical grade from May and Baker (Dagenham, Great Britain). AnalaR-grade ethanol was from James Burrough (London, Great Britain). Reagent-grade anhydrous ferric chloride was from Hopkin and Williams (Chadwell Heath, Great Britain). Bilirubin was from Sigma (London). The following chemicals were from BDH (Poole, Great Britain): tetraethylenepentamine (TEPA), dimethyl sulphoxide (DMSO), *n*-heptane, methyl acetate, diethylamine (DEA), boron trifluoride in methanol (14%, w/v) and 5% palladium on charcoal were all reagent grade; glacial acetic acid, diethyl ether, hydrochloric acid, sodium acetate,

sodium hydrogen carbonate, chloroform, petroleum spirit (b.p. 60°–80°C), methanol, sodium hydroxide and mercury were all AnalaR grade.

Apparatus

Hypersil (spherical silica), SAS-Hypersil (C_1), MOS-Hypersil (C_8) and ODS-Hypersil (C_{18}), from Shandon Southern Products, Runcorn, Great Britain, were slurry packed. The column dimensions were 100 mm × 5 mm I.D., except for the Hypersil column, which was 250 × 5 mm I.D. A Pye Unicam (Cambridge, Great Britain) LC3-XP liquid chromatograph with a LC3-UV detector was used. Injection was via a loop-valve injector (Rheodyne 7125) fitted with a 20-μl loop.

Preparation and isolation of bile pigments

The urobilinoids were extracted by the method of Watson[1] from a six-day collection of faeces from a patient with haemolytic anaemia. The final extract was precipitated from petroleum spirit, but not crystallised. A portion of the product was esterified by refluxing for 10 min in methanol (3 ml) and boron trifluoride in methanol (1 ml, 14% w/v). The mixture was diluted with water after cooling and extracted into chloroform. After successive washings with sodium hydrogen carbonate solution and water, the chloroform layer was filtered and dried.

Mesobiliverdin (glaucobilin) was prepared from mesobilirubin, synthesised from bilirubin by the method of Fischer and Haberland[2]. Mesobilirubin (50 mg) was dissolved in 10 ml of DMSO and quickly added, with continuous stirring, to a solution of 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (41 mg) in DMSO (10 ml). After 5 min, 0.5% acetic acid (60 ml) was added to precipitate the mesobiliverdin formed. After centrifugation, the precipitate was washed once with 0.5% acetic acid then repeatedly with water until the supernatant liquid was clear. The final product was dried in a vacuum desiccator.

Mesobiliviolin was prepared by oxidation of mesobilirubinogen[3] obtained by reduction of bilirubin[4].

i-Urobilin was prepared from bilirubin as described by Watson[4].

High-performance liquid chromatography

All HPLC solvents were made up by volume per cent. The free urobilinoids extracted from faeces were separated on a Hypersil column by elution with CH_3CN–H_2O–TEPA (85:15:0.05) at a flow-rate of 1 ml/min; the samples were dissolved in chloroform for injection, and detection was at 450 nm.

The urobilinoid dimethyl esters were resolved on a Hypersil column with *n*-heptane–methyl acetate–methanol containing 1% of DEA (75:25:2) as mobile phase; other HPLC conditions were as described above.

SAS-Hypersil, MOS-Hypersil and ODS-Hypersil were used for the separation of glaucobilin, mesobiliviolin and i-urobilin, with CH_3CN–DMSO (50:50) and CH_3CN–DMSO–CH_3OH (25:25:50) as mobile phases. The flow-rate was 1 ml/min; the samples were injected in DMSO. The detector was set at 340 nm for the detection of glaucobilin and mesobiliviolin and at 450 nm for the detection of urobilin. Other verdinoids (biliverdin), violinoids (half-stercoviolin) and urobilinoids (stercobilin and half-stercobilin) were similarly chromatographed.

RESULTS AND DISCUSSION

The separation of mesobiliverdin, mesobiliviolin and i-urobilin on SAS-Hypersil with CH_3CN–DMSO (50:50) and with CH_3CN–DMSO–CH_3OH (25:25:50) as eluents is shown in Fig. 2. The presence of DMSO is essential for resolution; the pigments were not eluted by CH_3CN, CH_3OH or a mixture of CH_3CN–CH_3OH suggesting that DMSO may be preferentially extracted into the stationary phase, modifying the column selectivity. The chain length of the bonded phases had little influence on the capacity ratios (k') of the bile pigments, and similar results were obtained using SAS-Hypersil (C_1), MOS-Hypersil (C_8) and ODS-Hypersil (C_{18}), probably because DMSO was extracted similarly into the stationary phases, resulting in columns with virtually identical selectivities. To achieve reproducible results with non-queous reversed-phase systems, the column needs to be throughly equilibrated for at least 2 h with the mobile phase.

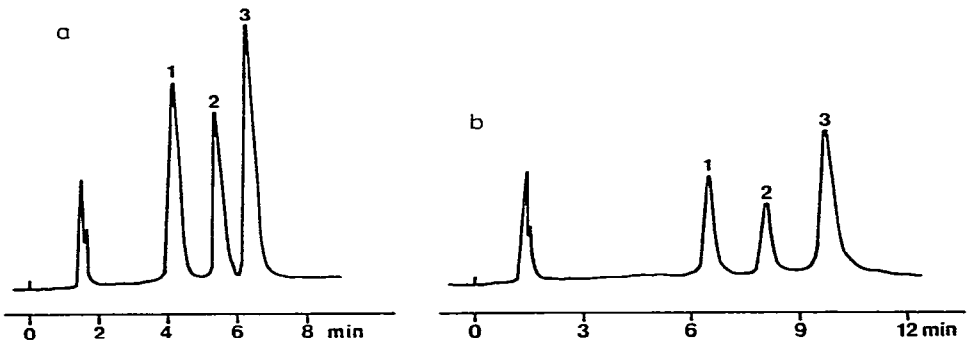

Fig. 2. Separation of bile pigments by non-aqueous reversed-phase HPLC with (a) CH_3CN–DMSO (50:50) and (b) CH_3CN–DMSO–CH_3OH (25:25:50) as mobile phase. Column: SAS-Hypersil; flow-rate, 1 ml/min; detection, 340 nm for glaucobilin (1) and mesobiliviolin (2), and 450 nm for urobilin (3).

The separation of these ionizable bile pigments by reversed-phase chromatography without the need for buffer solutions or ion-pairing reagents is attributed to the ability of DMSO to associate strongly with both the –NH and –COOH groups of the bile-pigment molecules rendering them "neutral". This explains the reversal of the elution order from that expected for reversed-phase chromatography, because mesobiliverdin is the least and i-urobilin the most polar molecule. Such a molecular association between solutes and DMSO would lead to changes in the polarity of the former.

Although non-aqueous reversed-phase systems do not separate biliverdin from mesobiliverdin, half-stercoviolin from mesobiliviolin and stercobilin from half-stercobilin and i-urobilin, the group separation is useful for the estimation of total faecal bile pigments and for the preparative isolation of bile pigments in haem turn-over studies using labelled precursors.

Stercobilin, half-stercobilin and i-urobilin were completely resolved (Fig. 3) on a silica column (Hypersil) with CH_3CN–H_2O–TEPA (85:15:0.05) as eluent. Verdinoids and violinoids were also separated in this system, but their separation form urobilinoids within a reasonable time required gradient elution. Similar systems developed for the separation of oligosaccharides have been shown to operate by the

normal-phase mechanism[5,6], and the elution order of verdins, violins and "urobilins" was that expected for normal-phase chromatography. However, although amino-group bonded silica columns are satisfactory for the separation of oligosaccharides[7–10], urobilinoids were not separated on aminopropylsilica (APS-Hypersil). Normal-phase ion-pair chromatography cannot be excluded, because the bile pigments can form ion-pairs with TEPA.

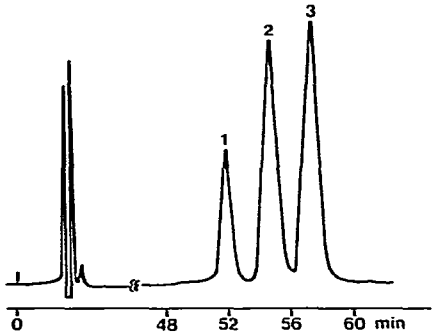

Fig. 3. Separation of stercobilin (1), half-stercobilin (2) and urobilin (3). Column, Hypersil; mobile phase, CH_3CN–H_2O–TEPA (85:15:0.05); flow-rate, 1 ml/min; detection, 450 nm.

The compounds were identified by their characteristic chemical reactions with ferric chloride–hydrochloric acid as described by Watson *et al.*[11]. Stercobilin is stable to this reagent; half-stercobilin is largely oxidised to the stable half-stercoviolin and i-urobilin is converted via mesobiliviolin into mesobiliverdin (glaucobilin).

The urobilinoids can exist as (*RR,SS*) and (*RS,SR*) diastereoisomers, since the inner α-carbon atoms (2′ and 7′) of the end ring are chiral centres (Fig. 1)[12]. They must be converted into their dimethyl esters in order to resolve the diastereoisomers (Fig. 4). Adsorption chromatography on Hypersil with *n*-heptane–methyl acetate–methanol containing 1 % of DEA (75:25:2) as mobile phase was used for the separation, a small amount of DEA in the mobile phase being essential to eliminate poor resolution due to peak tailing.

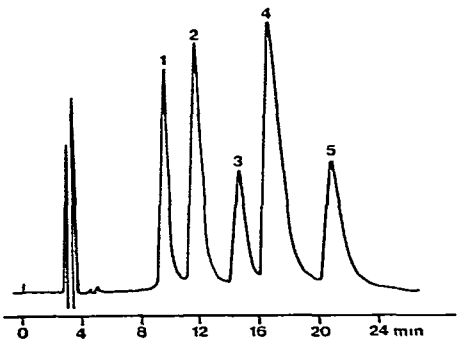

Fig. 4. Separation of dimethyl esters of (*SS*)-stercobilin (1), (*RR,SS*)-half-stercobilin (2), (*RR,SS*)-urobilin (3), (*RS,SR*)-half-stercobilin (4), and (*RS,SR*)-urobilin (5). Column, Hypersil; mobile phase, *n*-heptane–methyl acetate–methanol containing 1 % of DEA (75:25:2); detection, 450 nm.

Naturally occurring stercobilin is in the *SS* configuration[12]. Thus, only one peak corresponding to (*SS*)-stercobilin was observed. Both half-stercobilin and i-urobilin were resolved into two peaks corresponding to the *RR,SS* and *RS,SR* forms. The peaks were identified by chromatography of the dimethyl esters prepared from pure stercobilin, half-stercobilin and i-urobilin isolated by HPLC. The *RR,SS* isomers are eluted before the *RS,SR* isomers[13,14].

The analysis of faecal specimens by HPLC has shown that the urobilinoids are mixtures of stercobilin, half-stercobilin and i-urobilin in various proportions. Stercobilin and i-urobilin are formed from stercobilinogen and i-urobilinogen, respectively. The origin of half-stercobilin, however, is uncertain, because it could be formed artifactually by rearrangement from stercobilinogen and i-urobilinogen[13].

The resolution of the urobilinoids into diastereoisomers allows the isolation of the bile pigments suitable for detailed characterization by physico-chemical methods such as mass spectrometry, nuclear magnetic resonance spectrometry and circular dichroism.

ACKNOWLEDGEMENT

R. V. A. Bull was supported by a grant from the Medical Research Council to C.H.G.

REFERENCES

1 C. J. Watson, *J. Biol. Chem.*, 105 (1934) 469.
2 H. Fischer and H. W. Haberland, *Hoppe-Seyler's Z. Physiol. Chem.*, 232 (1935) 236.
3 C. H. Gray, A. Kulczycka and D. C. Nicholson, *J. Chem. Soc.*, (1961) 2276.
4 C. J. Watson, *J. Biol. Chem.*, 200 (1953) 691.
5 K. Aitzetmüller, *J. Chromatogr.*, 156 (1978) 354.
6 B. B. Wheals and P. C. White, *J. Chromatogr.*, 176 (1979) 421.
7 J. C. Linden and C. L. Lawhead, *J. Chromatogr.*, 105 (1975) 125.
8 R. B. Maegher S.J. and A. Furst, *J. Chromatogr.*, 117 (1976) 211.
9 R. Schwarzenbach, *J. Chromatogr.*, 117 (1976) 206.
10 F. M. Rabel, A. G. Caputo and E. T. Butts, *J. Chromatogr.*, 126 (1976) 731.
11 C. J. Watson, J. W. Hall, III and M. Weimer, *Biochem. Med.*, 2 (1969) 461.
12 H. Brockmann, Jr., G. Knoblock, H. Plieninger, K. Ehl, J. Ruppert, A. Moscowitz and C. J. Watson, *Proc. Nat. Acad. Sci., U.S.*, 68 (1971) 2141.
13 M. S. Stoll, C. K. Lim and C. H. Gray, in P. D. Berk and N. I. Berlin (Editors), *Chemistry and Physiology of Bile Pigments*, U.S. Department of Health, Education and Welfare, 1977, p. 483.
14 C. H. Gray, C. K. Lim, J. M. Rideout, P. O. Skacel and M. S. Stoll, in A. M. Lawson, C. K. Lim and W. Richmond (Editors), *Current Developments in the Clinical Applications of HPLC, GC and MS*, Academic Press, London, New York, Toronto, Sydney, San Francisco, 1980, p. 75.