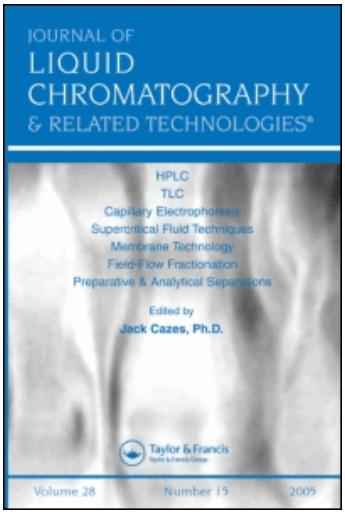

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF UROPORPHYRIN ISOMERS

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ABSTRACT

The separation of uroporphyrin I and III isomers by reversed-phase high performance liquid chromatography on ODS-Hypersil with ammonium acetate buffer - acetonitrile solvent systems is described. The effects of buffer concentration, pH, organic modifier proportion and different organic modifiers on the resolution are studied. The optimum conditions for the separation were 12-13% acetonitrile in 1M ammonium acetate buffer pH 5.10-5.20. The method also separated uroporphyrin I and III from the II isomers but the resolution of uroporphyrin III and IV isomers was not achieved.

INTRODUCTION

Uroporphyrinogen III is the universal precursor of chlorophylls, haem and vitamin B_{12}. It is formed initially by the condensation of four molecules of porphobilinogen to the unstable intermediate preuroporphyrinogen catalysed by the enzyme porphobilinogen deaminase. In the absence of this enzyme, preuroporphyrinogen is spontaneously rearranged into uroporphyrinogen I (1,2).

Much effort had been devoted to the development of separation methods for uroporphyrin I and III isomers (Figure 1), the two naturally occurring uroporphyrins. The separation is

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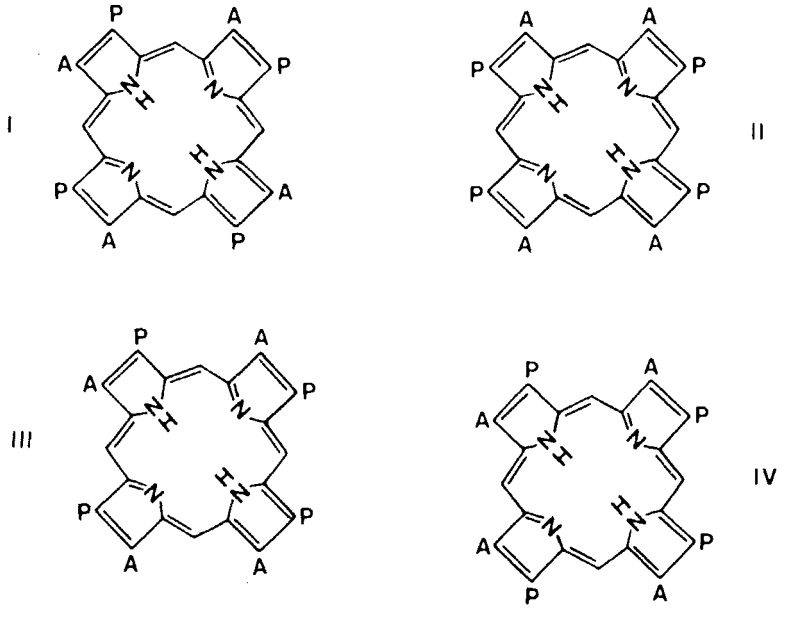

Figure 1 Structures of uroporphyrin I, II, III and IV isomers. A = CH_2COOH; P = $-CH_2-CH_2-COOH$.

important for the diagnosis of porphyrias and for developing an assay for uroporphyrinogen III cosynthetase where the quantitative separation of the I and III isomers is essential.

High performance liquid chromatography (HPLC) has been used to separate uroporphyrin I and III isomers as their octamethyl esters. Bommer et al. (3) separated the isomers after a five peak-recycle on two 30 cm silica columns in series. Nordlov et al. (4) modified the system and achieved separation without peak recycling but unfortunately the method required several hours of equilibration and a 3-hour separation. The reproducibility of such a system is doubtful. Walker et al. (5) and Jackson et al. (6) reduced the analysis time considerably but complete separation was not achieved. There has been hitherto only one paper describing the separation of uroporphyrin I and III isomers

as free acids (7) but no detailed study of their retention behaviour was presented.

The separation of the porphyrins as free acids is advantageous as the complicated and tedious extraction and derivatisation steps are avoided. We report here a rapid, highly effective and reproducible reversed-phase HPLC system for the separation of uroporphyrin I and III isomers as their free octacarboxylic acids. The retention behaviour of the isomers on ODS-Hypersil (5 μm spherical silica chemically bonded with octadecyl silyl groups) with buffered ammonium acetate-acetonitrile as the mobile phase is studied in detail. The separation of uroporphyrin II and IV isomers (Figure 1) is also described.

The practical applications of the method are demonstrated by the separation of the isomers in the urine of a patient with congenital porphyria and in the incubation mixture used in the determination of uroporphyrinogen III cosynthetase.

EXPERIMENTAL

Materials and Reagents

Porphobilinogen, uroporphyrin I and uroporphyrin III octamethyl ester were obtained from Sigma London Ltd. (Poole, U.K.). A statistical mixture of uroporphyrin I, II, III and IV was prepared by heating porphobilinogen in HCl (8). Ammonium acetate, glacial acetic acid and tetrahydrofuran were AnalaR grade from BDH Chem. Ltd. (Poole, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn Chem. Ltd. (Walkerburn, U.K.).

HPLC Apparatus

A Pye Unicam (Cambridge, U.K.) LC3-XP solvent delivery system was used with a Perkin-Elmer (Beaconsfield, U.K.) LS-3

fluorescence detector. The excitation and emission wavelengths were 406 nm and 619 nm respectively. A Rheodyne 7125 injector fitted with a 100 μl sample loop was used for injection.

HPLC Column and Mobile Phases

A 25 cm x 5 mm ODS-Hypersil (Shandon Southern Products, Runcorn, U.K.) reversed-phase column was used for the analysis. The mobile phases were acetonitrile (12-15%) in 0.25, 0.5, 0.75, 1.0 and 1.5M ammonium acetate buffer (pH 4.0-7.0). The pH was adjusted with acetic acid. Other eluents such as methanol-1M ammonium acetate pH 5.15 (16.6 : 83.4 $^{v}/v$), tetrahydrofuran-1M ammonium acetate buffer pH 5.15 (10.2 : 89.8 $^{v}/v$) and tetrahydrofuran-methanol-ammonium acetate buffer pH 5.15 (5.1 : 8.3 : 8.6 by vol.) were also used. The flow rate was 1 ml/min throughout.

RESULTS AND DISCUSSION

The Effect of Buffer Concentration on Retention and Resolution

The molar concentration of ammonium acetate buffer in the mobile phase significantly affected the retention and resolution of uroporphyrin I and III isomers. The variation of the capacity ratio (k') with the buffer concentration is shown in Figure 2. The optimum buffer concentration was 1M. At below 0.5M, excessive retention and peak broadening resulted while at above 1.5M, rapid elution with loss of resolution was observed. This observation suggested that ammonium acetate competes effectively with the solutes for extraction onto the stationary phase and is perhaps also a good masking agent for the residual silanol groups on the silica of the reversed-phase packing. It is therefore important that a high concentration of ammonium acetate solution (0.75-1M) is used to ensure good resolution and a short separation time.

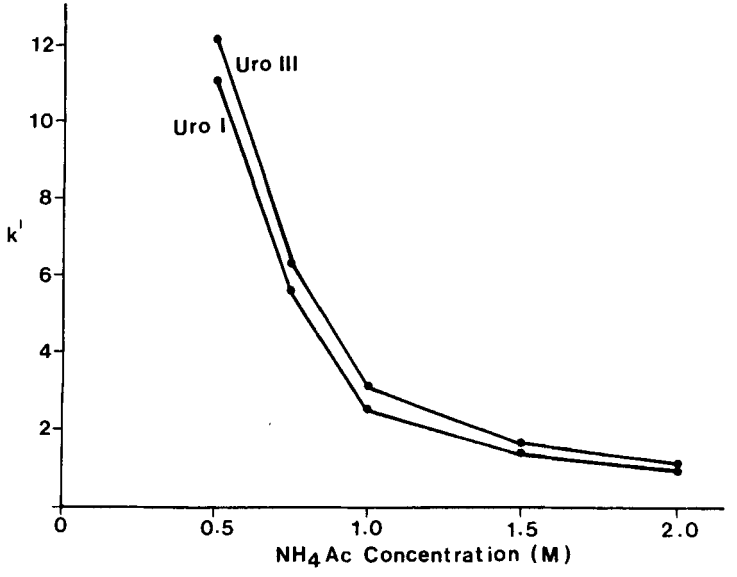

Figure 2 Variation of the capacity ratios (k') of uroporphyrin I and III with the molar concentration of ammonium acetate buffer in the eluent. The mobile phase was maintained at pH 5.15 and contained 13% acetonitrile.

The Effect of pH on Retention and Resolution

The retention and resolution of the porphyrins was greatly influenced by the pH of the ammonium acetate buffer used. Increasing the pH decreased the k' values (Figure 3) with loss of resolution. Figure 3 clearly shows that the usable pH range is narrow, to achieve rapid and effective separation the pH should be carefully adjusted to 5.0-5.2.

Acetonitrile Concentration Effect

The effect of acetonitrile concentration on the k' values of the porphyrin isomers (Figure 4) is that expected for reversed-phase chromatography. The k' values decreased with

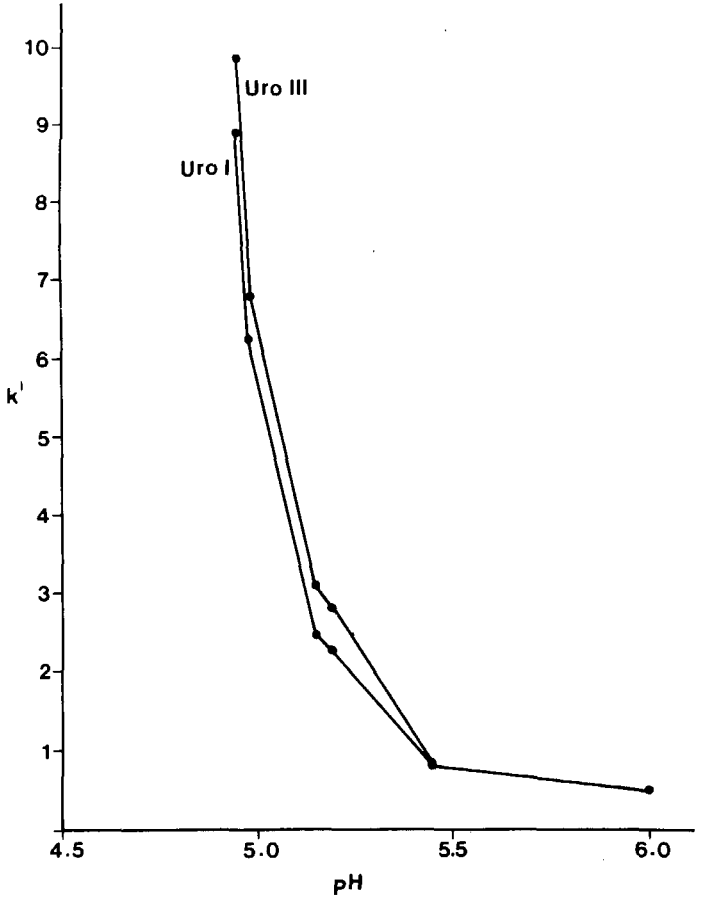

Figure 3 The effect of pH on the capacity ratios (k') of uroporphyrin I and III. The eluent was 13% acetonitrile in 1M ammonium acetate buffer.

increasing acetonitrile content in the mobile phase. The optimum concentration of acetonitrile was found to be 12-13%.

Organic Modifier Specificity

The solvent system described here is organic modifier specific. Replacing 13% acetonitrile with methanol or

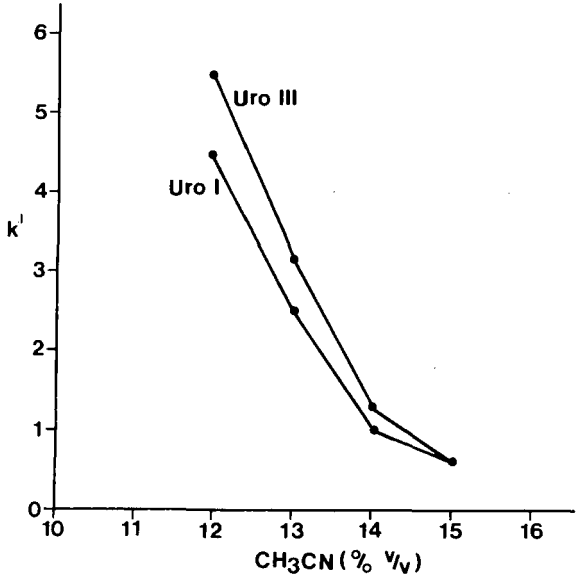

Figure 4 Relationship between capacity ratios (k') of uroporphyrin I and III and acetonitrile content in the mobile phase. The eluent was maintained at 1M ammonium acetate buffer pH 5.15.

TABLE 1

Capacity ratio (k') of uroporphyrin I and III isomers in mobile phases of equal polarity. Solvent A is 1M ammonium acetate-acetic acid pH 5.15. Column is ODS-Hypersil (2.5 cm x 5 mm).

Mobile phase B in A	k'	
B	URO I	URO III
13% CH_3CN	2.50	3.14
16.6 CH_3OH	>20	>20
10.2% THF	0.43	0.43
8.3% CH_3OH + 5.1% THF	2.30	2.64

tetrahydrofuran to give equal mobile phase polarity resulted in no elution and no retention respectively (Table 1).

A ternary mobile phase system of tetrahydrofuran-methanol-1M ammonium acetate buffer pH 5.15 (5.1 : 8.3 : 86.6 by vol.) which is equivalent to 13% acetonitrile in 1M ammonium acetate buffer pH 5.15 in terms of mobile phase polarity, did separate the isomers but the resolution was not as good.

The Optimum Solvent System for the Separation of Uroporphyrin I and III Isomers

From the results obtained it becomes obvious that for the fast and effective separation of the isomers on a 25 cm x 5 mm I.D. ODS-Hypersil column the recommended mobile phase is 13% acetonitrile in 1M ammonium acetate buffer at pH 5.15. A typical separation is shown in Figure 5.

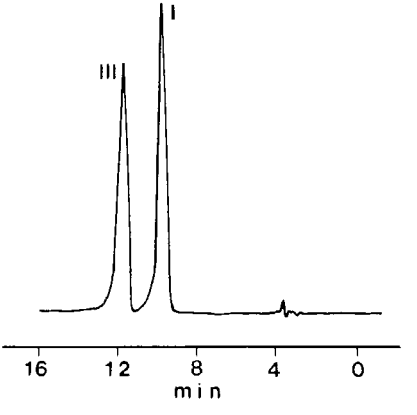

Figure 5 Separation of uroporphyrin I and III isomers. Column, ODS-Hypersil (25 cm x 5 mm); mobile phase 13% acetonitrile in 1M ammonium acetate buffer pH 5.15; flow rate, 1 ml/min., detector, fluorescence excitation 406 nm, emission 619 nm.

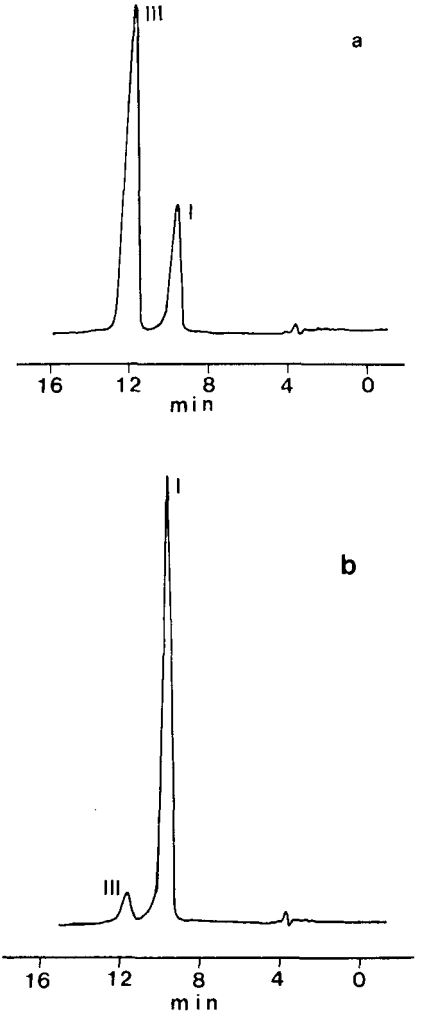

Figure 6 Separation of uroporphyrin I and III isomers in (a) incubation mixture for the determination of uroporphyrinogen III cosynthetase, (b) congenital porphyric urine. HPLC conditions as in Figure 5.

Applications of the Separation

The most important application is in the development of a rapid and specific assay for the enzyme uroporphyrinogen III cosynthetase. Our preliminary results clearly demonstrate this possibility and are shown in Figure 6(a), the separation of uroporphyrin III from the I isomer in the incubation mixture used for the determination of uroporphyrinogen III cosynthetase in haemolysed red blood cells. Figure 6(b) shows another practical application, the identification of uroporphyrin I in the urine of a patient with congenital porphyria. The separation and identification of the isomers is important for the diagnosis of this condition in order to differentiate it from other forms of porphyria.

The Separation of Uroporphyrin I, II, and III + IV Isomers

A mixture of four uroporphyrin isomers prepared by heating porphobilinogen in acid was resolved into 3 peaks as shown in Figure 7. The system could not separate uroporphyrin III from the IV isomers but uroporphyrin II was easily resolved. The separation of the I and III isomers from the II and IV isomers is unimportant, however, as the II and IV isomers are not naturally occurring compounds and therefore no attempt was made to improve the separation. The proportion of the four isomers (12.9% I, 12.0% II, 75.1% III + IV) correlates well with the statistical proportion of 12.5% I, 12.5% II, 50% III and 25% IV isomer (8).

Reproducibility of the Mobile Phase System

One of the major problems with the separation of uroporphyrin isomers as octamethyl esters by adsorption chromatography on silica is poor reproducibility. This is often due to the various degree of hydration and purity of the organic

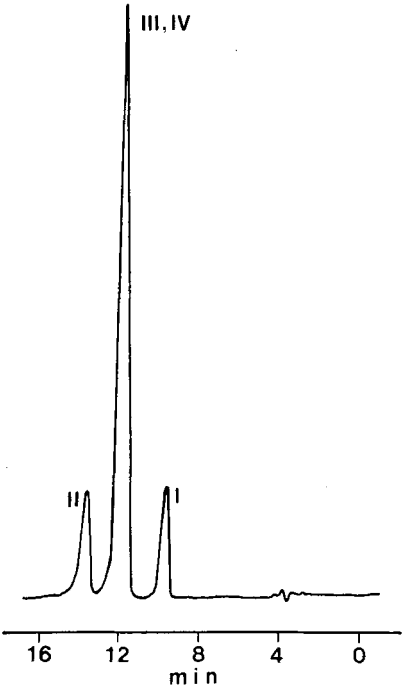

Figure 7 Separation of uroporphyrin isomers prepared by heating porphobilinogen in acid.

solvents used. Reversed-phase chromatography with buffer-controlled mobile phases is much less likely to suffer from such a variation. The reproducibility of the present system has been thoroughly tested. Several hundred analyses have been performed on the same column and on different columns packed with the same stationary phase. The results are highly reproducible. Any deterioration of column performance can often be cured by replacing the top 2 mm of the column packing.

CONCLUSION

The factors affecting the separation of uroporphyrin I and III isomers by reversed-phase chromatography have been studied

in detail. It is concluded that rapid and effective separation of the isomers required a mobile phase of 12-13% acetonitrile in 0.75-1.25M ammonium acetate buffer pH 5.0-5.2.

REFERENCES

1. Burton, G., Nördlov, H., Hosozawa, S., Matsumoto, H., Jordan, P.M., Fagerness, P.E., Pryde, L.M. and Scott, A.I., J. Amer. Chem. Soc., 101, 3114, 1979.

2. Battersby, A.R., Fookes, C.J.R., Matcham, G.W. and McDonald, E., Nature (London), 285, 17, 1980.

3. Boomer, J.C., Burnham, B.F., Coulson, R.E. and Dolphin, D., Anal. Biochem., 95, 444, 1977.

4. Nördlov, H., Jordan, P.M., Burton, G. and Scott A.I., J. Chromatogr., 190, 221, 1980.

5. Walker, I.C., Gilbert, M.T. and Stubbs, K., J. Chromatogr., 202, 491, 1980.

6. Jackson, A.H., Rao, K.R.N. and Smith, G., Biochem. J., 203, 515, 1982.

7. Wayne, A.W., Straight, R.C., Wales, E.E. and Englert, E., J. High Resolut. Chromatogr. Chromatogr. Commun., 2, 621, 1979.

8. Mauzerall, D., J. Amer. Chem. Soc., 82, 2601, 1960.