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IMPROVED SEPARATION AND DETECTION OF FREE PORPHYRINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Porphyrins were separated using ion-pair reversed-phase high-performance liquid chromatography. The eluents were aqueous potassium phosphate buffer and tetrabutylammonium phosphate in methanol. The influences of pH value and ionic strength of the phosphate buffer and molarity of the ion-pair reagent in methanol were investigated to improve separation and detection. A linear response curve was obtained from 0.38 to 7.64 pmol for coproporphyrin I. The detection limits were determined to be 0.12 pmol for coproporphyrin I and 0.22 pmol for uroporphyrin I.

INTRODUCTION

Determination of porphyrins from human materials is essential for diagnosis of porphyrias. These diseases are caused by disturbances of the haem biosynthesis, which are provoked by inborn or acquired defects of the corresponding enzymes.

During investigation of porphyrias different analytical principles have been used for porphyrin quantification, but in recent years high-performance liquid chromatography (HPLC) has become the favoured technique because it allows rapid and quantitative determination of the interesting porphyrins from human materials. Most HPLC procedures include some extraction and esterification steps prior to HPLC analysis and detection by photometry[1,2]. Recently, however, several methods have been published by our group and others that describe the separation of free porphyrin carboxylic acids on reversed-phase columns followed by fluorescence detection[3–14].

Some authors use acidified samples and chromatograph them with different buffers[3–6]. Recently, coating techniques employing different substances were described for normal-phase[7] or reversed-phase columns[8]. Determinations of free porphyrin carboxylic acids from urine, faeces and blood were investigated by our group and others using the ion-pair reagent tetrabutylammonium phosphate dissolved in organic solvents and phosphate buffers as eluents[9–14].

Up to now, we have been using a commercially available solution of tetrabutylammonium phosphate and therefore the influence of parameters of the eluents, such as pH value, molarity and ionic strength, could not be studied. Without knowl-

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edge of the influence of these parameters a final optimization of the chromatographic process is impossible to perform. Therefore, we investigated the effects of varying the pH value, molarity and ionic strength of aqueous and organic eluents on the separation performance and detectability of different free porphyrin carboxylic acids.

EXPERIMENTAL

Apparatus

Two different HPLC systems were used. One system consisted of two Model 8500 syringe pumps (Varian, Palo Alto, CA, U.S.A.) attached to a solvent programmer, a Rheodyne 7105 injector (Rheodyne, Berkley, CA, U.S.A.), a filter fluorimeter (Fluorichrom, Varian) and an SP 4100 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). The excitation filter was an 400-nm interference filter and for detection a 570-nm cut-off filter was used. The column was a LiChroCART RP 18 (7 μm, 25 cm × 4 mm I.D.) (Merck, Darmstadt, F.R.G.) which was protected by a guard cartridge LiChroCART RP 18 (4 mm × 4 mm I.D.) (Merck). The precolumn was attached directly to the top of the analytical column and both columns were held by the pneumatic Auto-fix-system (Merck). The other liquid chromatographic system, a Vista 54 (Varian) consisting of a HPLC Model 5600 and a Data System CDS 401, was mostly used for gradient elutions. An autosampler Wisp 710B (Waters, Milford, MA, U.S.A.) was attached to the Vista 54. The column was a μBondapak C_{18} (10 μm, 30 cm × 4 mm I.D.) (Waters). The fluorescence of the column eluate was measured with a Model 3000 spectrofluorimeter (Perkin-Elmer, Norwalk, CT, U.S.A.). The excitation wavelength was set at 400 nm (slit width, 10 nm) and the emitted fluorescence was detected at 630 nm with a slit of 20 nm.

Reagents

Spectrograde methanol was purchased from Merck and Baker (Phillipsburg, NJ, U.S.A.). Water was de-ionized and then processed with a Milli-Q-filtration system (Millipore, Bedford, MA, U.S.A.) to a conductivity of 16 $M\Omega^{-1}$. The ion-pair reagent tetrabutylammonium phosphate was purchased from Waters or prepared from tetrabutylammonium hydroxide (Fluka, Buchs, Switzerland) by addition of an appropriate amount of phosphoric acid (Merck). Phosphate buffers of different pH values were prepared from potassium dihydrogen phosphate and dipotassium hydrogen phosphate (Merck). Coproporphyrin I standards were obtained from Sigma (St. Louis, MO, U.S.A.). The other porphyrins applied as coproporphyrin III, uroporphyrin I and the synthetic mixture containing 8- to 2-carboxylic porphyrins were purchased from Porphyrin Products (Logan, UT, U.S.A.).

Method

Porphyrin standard solutions were prepared by dissolving appropriate amounts of the porphyrins in 50 mmol/l solutions of tetrabutylammonium phosphate in methanol–water (80:20). Small portions of these standard solutions were stored at −20°C until use.

Urine samples containing porphyrins either prepared by addition of porphyrin standards to urine or originating from porphyric patients were filtered through a 0.2-μm membrane filter (Gelman, Ann Arbor, MI, U.S.A.) to protect the column from particulate matter.

Chromatographic conditions

Ion-pair reversed-phase HPLC separations were performed with isocratic or multilinear gradient elution. The mobile phase consisted of aqueous potassium phosphate buffers and tetrabutylammonium phosphate in methanol. The pH value and ionic strength of phosphate buffers were varied between 5 and 8 and 5 and 30 mmol/l phosphate, respectively. The amounts of ion-pair reagent added to methanol ranged from 2.5 to 10 mmol/l. The gradient programme for separation of a synthetic porphyrin mixture on a μBondapak C_{18} column began at 28% methanol in water and was increased to 33% methanol in water within 0.5 min, then within 1.5 min to 39%, within a further 15 min to 54%, to 62% within another 3 min and after 0.1 min to a final methanol concentration of 90%, which was maintained for 3 min. Between two analyses the column was reconditioned for 10 min with the starting mixture.

For both columns the flow-rate was set at 1 ml/min. The sample volumes injected varied from 5 to 25 μl.

RESULTS AND DISCUSSION

Separation performance

pH value. It is well known that the pH value of an eluent can strongly influence the capacity ratios (k') of porphyrin carboxylic acids[3,8]. Adjusting the pH value of the aqueous mobile phase between 5 and 8 resulted in a significant decrease of capacity ratios for coproporphyrin I under isocratic conditions (Fig. 1). In the range between pH 7 and 8 the variation of k' values is much smaller than between lower pH values, therefore no drastic change of the retention behaviour of coproporphyrin occurs in the former range. In these experiments the concentrations of phosphate buffer and ion-pair reagent were constant at 10 mmol/l and 5 mmol/l, respectively.

By applying a multilinear gradient elution with the same mobile phase components as described above, a synthetic mixture of free porphyrin carboxylic acids from uro- to coproporphyrin was separated (Fig. 2). No complete separation of all porphyrins could be achieved with pH values below 6.5. A comparable decrease of capacity ratios is shown for the individual porphyrins as coproporphyrin I in the

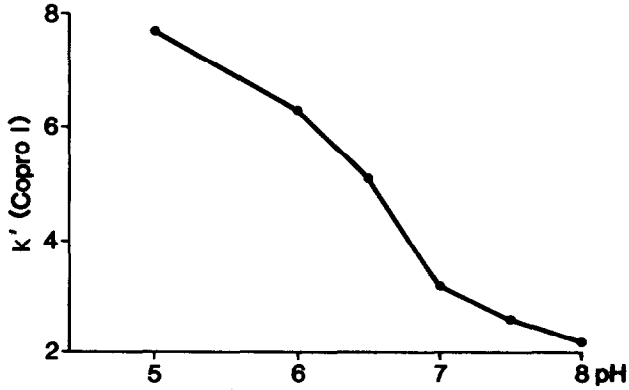

Fig. 1. Capacity ratios (k') of coproporphyrin I for different pH values of the aqueous phosphate buffer (isocratic mode). Eluent, 5 mmol/l tetrabutylammonium phosphate in methanol–10 mmol/l potassium phosphate buffer (70:30, v/v); flow-rate, 1 ml/min; column, LiChroCART RP 18 with guard cartridge.

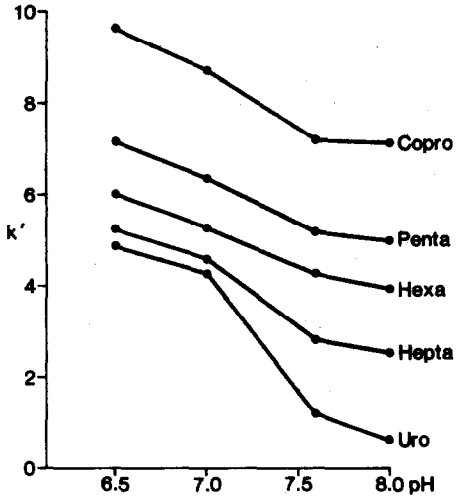

Fig. 2. Change in retention of a synthetic mixture of free porphyrins (uroporphyrin to coproporphyrin) with pH value of aqueous phosphate buffer (gradient mode). Eluent, 5 mmol/l tetrabutylammonium phosphate in methanol–10 mmol/l potassium phosphate buffer; gradient, see chromatographic conditions; flow-rate, 1 ml/min; column, μBondapak C_{18}.

isocratic elution. A large decrease of the retention time of uroporphyrin is observed in the pH range between 7 and 7.5 and nearly no retention at pH 8. The differences between the capacity ratios increase with increasing pH. Therefore separation at pH 7.5 seems to be the optimum, where uroporphyrin is still retained.

Ion-pair reagent. Effects on retention behaviour of the porphyrins could be expected as a consequence of variation of the tetrabutylammonium phosphate concentration in the methanolic eluent. Changing the concentration of the ion-pair reagent from 2.5 to 10 mmol/l resulted in a slight increase of the capacity ratio of coproporphyrin (Fig. 3). Uroporphyrin was not retained under these conditions at

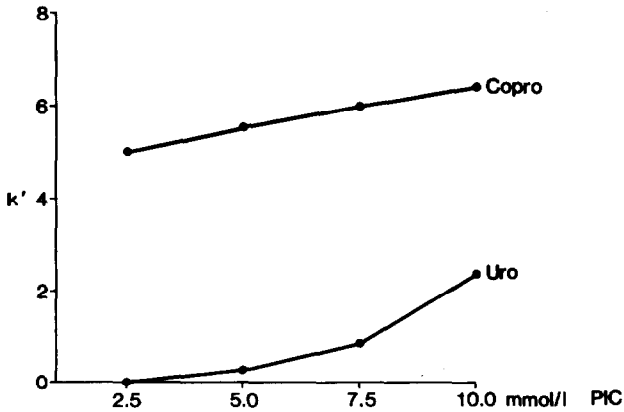

Fig. 3. Capacity ratios (k') of coproporphyrin and uroporphyrin for different concentrations of tetrabutylammonium phosphate (PIC) in methanol (gradient mode). Eluent, tetrabutylammonium phosphate in methanol–10 mmol/l potassium phosphate buffer (pH 7.5). Gradient: 5 min, 30% methanol; 5 min, 30–50% methanol; 50% methanol isocratic. Flow-rate, 1 ml/min; column, μBondapak C_{18}.

the lowest concentration of 2.5 mmol/l. At higher concentrations the capacity ratio of uroporphyrin increased more rapidly than that of coproporphyrin. Therefore a decreasing difference of retention times was obtained for increasing amounts of ion-pair reagent. At 5 mmol/l of tetrabutylammonium phosphate in methanol, uroporphyrin was retained on the column and the greatest resolution between these porphyrins was observed.

Phosphate buffer. Variation of the ionic strength of the aqueous phosphate buffer between 5 and 30 mmol/l phosphate resulted in no measurable change in the retention time of coproporphyrin under isocratic conditions.

Detectability

pH value. The absorbance and fluorescence properties of porphyrins depend on their actual charge distribution. Increased fluorescence intensity for coproporphyrin and uroporphyrin could be observed using isocratic elution with aqueous eluents at a pH between 5 and 7, whereas from pH 7 to 8 the detector response was nearly constant (Fig. 4). These investigations were performed on a spectrofluorimeter. With a filter fluorimeter similar results were obtained. The increase of fluorescence intensity is caused by higher and smaller absorption bands at high pH values, whereas at pH 5 a relatively low absorption band with two maxima is observed. To obtain a good selectivity with fluorescence detection it is necessary to use only a small wavelength range of *ca.* 10 nm with both fluorimeters. Best detectability for the porphyrins occurs at a pH of 7.5.

Phosphate buffer. A decrease of the detector response was observed for coproporphyrin when the concentration of phosphate buffer was increased from 5 to 30 mmol/l. The separations were run at pH 7.5 in isocratic mode (55% methanol). This effect might be caused by a weak absorption of the phosphate buffer at *ca.* 400 nm.

Linearity

A linear relationship between peak area and injected amount of coproporphyrin I over the range 0.38–7.64 pmol was obtained (Fig. 5). The coefficient of

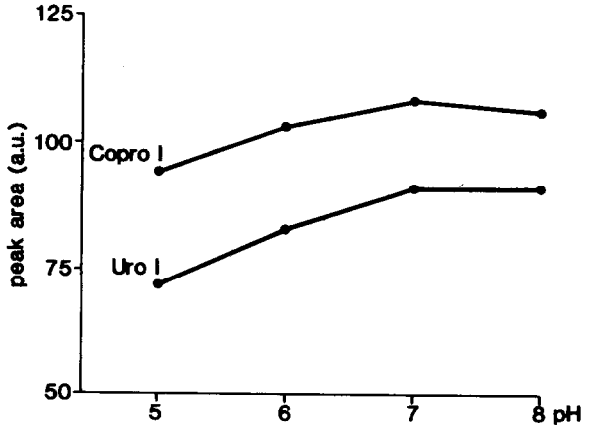

Fig. 4. Change of peak areas of coproporphyrin and uroporphyrin I with pH (isocratic mode). Eluent, 5 mmol/l tetrabutylammonium phosphate in methanol–10 mmol/l potassium phosphate buffer (70:30, v/v); flow-rate, 1 ml/min; column, μBondapak C_{18}.

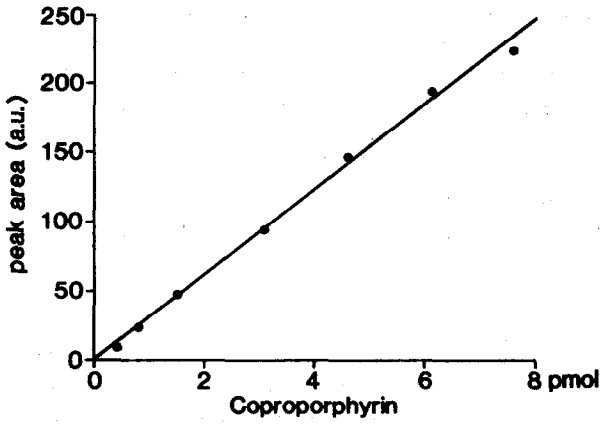

Fig. 5. Relationship between the amount of coproporphyrin injected and the area of the detected fluorescence peak (isocratic mode). Eluent, 5 mmol/l tetrabutylammonium phosphate in methanol–10 mmol/l potassium phosphate buffer, pH 7.6 (65:35, v/v); flow-rate, 1 ml/min; column, LiChroCART RP 18 with guard cartridge.

correlation was 0.997. These determinations were run under isocratic conditions [5 mmol/l tetrabutylammonium phosphate in methanol, 10 mmol/l phosphate buffer (pH 7.5), 65:35 (v/v)]. The detection limits for uroporphyrin and coproporphyrin I were 0.22 and 0.12 pmol, respectively.

Clinical applications

The pathological status of enzymes of haem biosynthesis in porphyric patients leads normally to an increased production of some porphyrins. Therefore, porphyrin profiles determined from materials from those patients differ significantly from those obtained from normal humans.

The first objective in performing porphyrin analysis is to confirm a tentative diagnosis of porphyria. The material to be analysed first is urine, but only if porphyrins are excreted by the patient in increased amounts[11]. This total porphyrin content is determined by semiquantitative methods using ion-exchange column chromatography and/or second derivative spectroscopy. The diagnosis of some porphyrias, such as porphyria variegata, requires the determination of porphyrins from faeces, but this is very rare[12]. If the patient shows clinical symptoms and signs for erythropoietic porphyrias or lead intoxication it is necessary to determine porphyrins from red blood cells[13]. The diagnosis of a porphyria is founded mainly on the porphyrin profile and not so much on the absolute amounts of the porphyrins produced.

The surveillance of porphyric patients is a second reason for performing porphyrin analysis as a clinical chemical routine. The material to be used for these porphyrin determinations depends on the particular porphyria.

CONCLUSION

The variation of elution parameters influences both chromatographic performance and the detectability of free porphyrin carboxylic acids. Thus we could optimize the pH value (7.5) and the ionic strength (10 mmol/l) of the phosphate buffer, and

the molarity (5 mmol/l) of the ion-pair reagent in methanol. This enabled us to improve the quantitative determination of porphyrins from human materials.

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