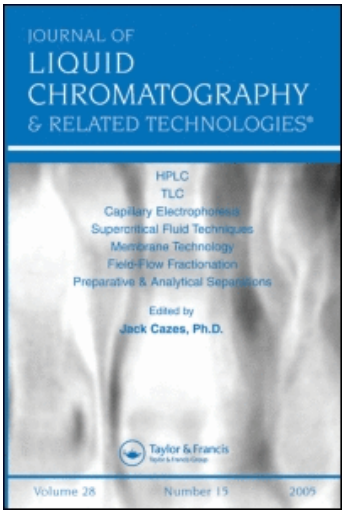

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Separation of Porphyrins Using a γ-Cyclodextrin Stationary Phase

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SEPARATION OF PORPHYRINS USING A γ-CYCLODEXTRIN STATIONARY PHASE

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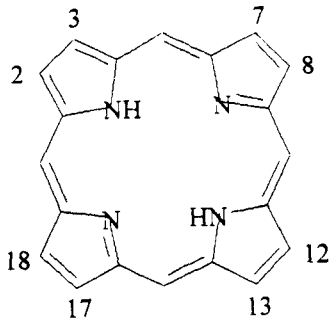
ABSTRACT

An isocratic separation of five porphyrin carboxylic acids using conventional mobile phase conditions was achieved on a commercially available γ-cyclodextrin bonded phase within 30 minutes. The effects of pH, ionic strength, organic modifier as well as the retention and separation mechanisms are discussed. The direct determination of porphyrin levels in blood serum is also discussed.

INTRODUCTION

Polycarboxylated porphyrins are intermediate metabolites of heme biosynthesis (1). Excessive production and excretion of porphyrins in biological fluids is a sign of disturbances of the heme biosynthetic pathway (2). These disorders can be inherited or caused by exposure to some toxic chemicals. Determination of relative levels of porphyrins in biological fluids, e.g. urine, stool, or blood, is essential for diagnosis of various porphyrias, lead poisoning, and iron deficiency anemia (3). In normal urine, five porphyrins may be found, containing four to eight carboxylic acid groups, namely copro-, pentacarboxyl-, hexacarboxyl-, heptacarboxyl-, and uro-porphyrin (Table 1).

Table 1. Structures of porphyrins used in this study.



Porphyrins	Side-chain substitution							
	2	3	7	8	12	13	17	18
Uroporphyrin I	A	P	A	P	A	P	A	P
Heptacarboxylporphyrin I	M	P	A	P	A	P	A	P
Hexacarboxylporphyrin I (cis)	M	P	M	P	A	P	A	P
Hexacarboxylporphyrin I (trans)	M	P	A	P	M	P	A	P
Pentacarboxylporphyrin I	M	P	M	P	M	P	A	P
Coproporphyrin I	M	P	M	P	M	P	M	P

A = $-CH_2COOH$; P = $-CH_3CH_2COOH$; M = $-CH_3$

Various chromatographic techniques have been developed to determine free porphyrin carboxylic acids. Among these methods, high-performance liquid chromatography (HPLC) is a preferred technique due to its efficiency, sensitivity, simplicity and easy quantitation (2,4).

Both normal phase and reversed-phase modes have been tried. In the normal phase mode, an aminopropyl-bonded silica phase was used for the separation of

porphyrins (5). Routinely, normal phase procedures require esterification of the porphyrin carboxylic acids before analysis. Reversed-phase systems have been tried using octadecylsilane stationary phases (2, 6-9). These reversed-phase separations almost exclusively used linear gradient elutions.

Native and derivatized cyclodextrin (CD) bonded stationary phases are best known for their ability of separating enantiomers. In addition, CD bonded stationary phases are also used for separating structural isomers (10). Many of these separations are difficult or impossible to achieve on conventional HPLC columns.

Under reversed-phase conditions, formation of an inclusion complex of the solute with the relatively nonpolar interior of the CD cavity is suggested to be an essential step to achieve separations (11). Furthermore, the hydroxyl groups lining the mouth of the CD cavity can form hydrogen bonds with polar segments of the solute molecules. The stability of an inclusion complex is a function of the size and shape of a molecule relative to a given CD (10). Separation due to inclusion complexation may not be possible if the diameter of a molecule is significantly larger than the mouth of the CD cavity. However, as long as a portion of the molecule is tightly complexed, the analytes may be effectively separated (12). Recent studies suggest that some chiral analytes may be separated by sitting atop of the CD cavity like a lid, without forming a conventional inclusion complex (13).

In the normal phase mode, the nonpolar component of the mobile phase is thought to compete with the solute to occupy the CD cavity, thereby preventing the analyte from forming an inclusion complex. In this case, the separation is more likely due to hydrogen bonding between the solute and the hydroxyl groups at the top and bottom of the CD toroid (14).

The structural similarities of free porphyrin carboxylic acids make the isocratic separation of these compounds challenging. Recently, an isocratic separation method for free porphyrin carboxylic acids on a β-CD bonded stationary phase was reported (1, 15). The isocratic elution on the β-CD bonded stationary phase was completed in less than eight minutes (15). However, this method employed a mobile phase containing 18-crown-

6 ether, phosphate buffer, acetonitrile and pyridine. The suggested mechanism for the separation of carboxylated porphyrins was retention via adsorption rather than inclusion complexation, because the cavities of β-CDs were too small to accommodate the bulky porphyrins.

The γ-CD column is not as widely used as the β-CD column, because the γ-CD cavity is thought to be too big to tightly complex with most analytes. The current work shows that a γ-CD bonded stationary phase in conjunction with a conventional mobile phase can achieve the isocratic separations of carboxylated porphyrins within 30 minutes.

EXPERIMENTAL

Chemicals

Uroporphyrin I, heptacarboxylporphyrin I, hexacarboxylporphyrin I, pentacarboxylporphyrin I, coproporphyrin I were all purchased from Porphyrin Products, Inc.(Logan, UT). Sodium phosphate monobasic was bought from J. T. Baker Chemical Co. (Phillipsburg, NJ). Sodium phosphate dibasic was purchased from Matheson Coleman & Bell Manufacturing Chemists (Norwood, OH). HPLC grade acetonitrile and water were obtained from Fisher Scientific (Tustin, CA).

Apparatus

The HPLC system consisted of a Shimadzu LC-600 Liquid Chromatograph, a SPD-10A variable wavelength UV/vis detector, and a CR601 Chromatopac data acquisition system. The Cyclobond II Gamma column (250 x 4.6 mm i.d. stainless steel, 5 μm particle diameter) was obtained from Advanced Separation Technologies, Inc. (Whippany, NJ).

Chromatographic conditions

The separations were performed with isocratic elution. The mobile phases used were acetonitrile/phosphate buffer (Na_2HPO_4, NaH_2PO_4; *ca.* 100 mM). The isocratic elution was carried out at 18 °C at a flow rate of 0.8 mL/min. The eluates were monitored at 400 nm.

Appropriate amounts of the porphyrin samples were dissolved in methanol and stored in the dark at 4°C.

RESULTS AND DISCUSSION

Isocratic separation of five porphyrin carboxylic acids was achieved within 30 minutes using a γ-CD bonded stationary phase. The chromatographic data is summarized in Table 2.

Effect of organic modifier

Methanol was not investigated as extensively as an organic modifier as acetonitrile because preliminary results showed that selectivities and peak shapes obtained by using methanol were not as good as those obtained using acetonitrile. Therefore optimization focused on acetonitrile as an organic modifier. The effect of acetonitrile concentration on capacity factors is shown in Figure 1.

At low acetonitrile concentrations (<30%), as the volume fraction of organic modifier increases, the capacity factors tend to decrease. However, at higher acetonitrile concentrations (>40%), as the organic modifier increases, the capacity factors start to increase. The same trend exists for all five porphyrins but with apparently different "turning points". For instance, the turning point for uroporphyrin is approximately 30% acetonitrile; for coproporphyrin it is *ca.* 40% acetonitrile.

The appearance of a capacity factor minimum as a function of the composition of the organic modifier in the mobile phase suggest that there might be two retention mechanisms involved in the separations. When the mobile phase contains a low volume fraction of organic modifier, the retention mechanism is likely due to hydrophobic interactions with stationary phase. The porphyrin macrocycle is planar, highly conjugated with significant aromatic and hydrophobic character (16). The macrocycle part of the porphyrin may enter or sit on the top of the relatively nonpolar γ-CD cavity, allowing the carboxylated side chains of the porphyrins to associate with the hydroxyl groups lining the

Table 2. Chromatographic results of isocratic separation of polycarboxylated porphyrins on γ-CD bonded phase. Mobile phase: 25/75 ACN/buffer (140 mM phosphate; pH 6.9).

Porphyrins	k'	α
Uroporphyrin I	0.521	1.960
Heptacarboxylporphyrin I	1.021	1.427
Hexacarboxylporphyrin I (1)	1.457	1.339
Hexacarboxylporphyrin I (2)	1.951	1.240
Pentacarboxylporphyrin I	2.615	1.336
Coproporphyrin I	3.493	

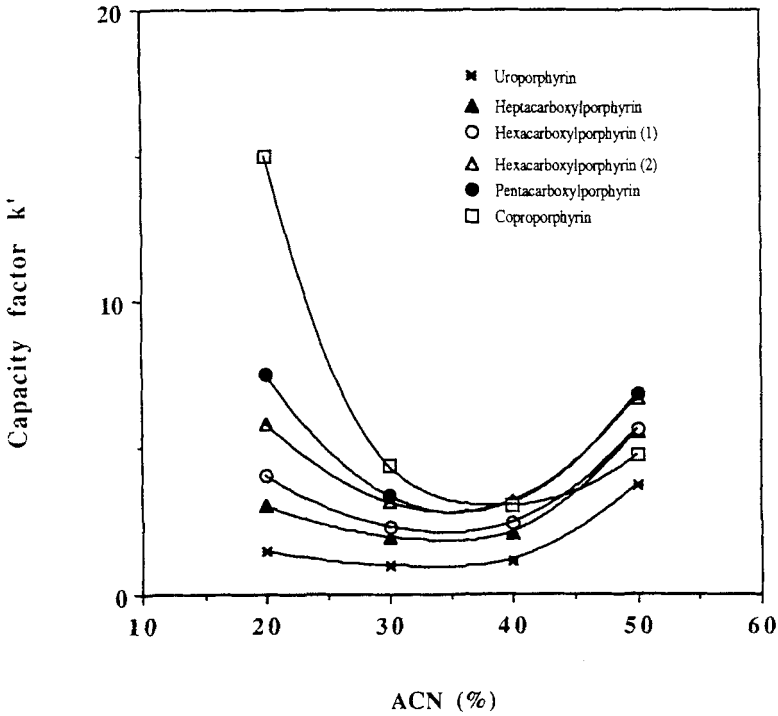


Figure 1. Effect of acetonitrile concentration on capacity factors of polycarboxylated porphyrins. Mobile phase: ACN/buffer (140 mM phosphate; pH 6.7).

mouth of the CD cavity. As the organic modifier concentration increases, retention decreases which is typical of a reversed phase mode of retention. In this region of mobile phase composition, the porphyrins elute according to polarity, with the most polar porphyrin, uroporphyrin, eluting first, and followed by heptacarboxylporphyrin, hexacarboxylporphyrin, pentacarboxylporphyrin, with the least polar porphyrin, coproporphyrin, eluting last. This elution order at low organic modifier concentration is also consistent with a reversed phase mode of retention.

The increase in retention when the concentration of acetonitrile increases beyond the "turning point" may be due to interaction between the porphyrin carboxylated side chains and CD hydroxyl groups. The least polar porphyrin, coproporphyrin, was eluted before hepta-, hexa- and penta-carboxylporphyrin.

Effect of ionic strength

The effect of ionic strength on retention was studied and the results are shown in Figure 2. As can be seen from the figure, when the concentration of phosphate buffer increases, the capacity factors of all five polycarboxylated porphyrins decrease. In addition, it was found that better peak shapes and resolution were obtained at high buffer concentration. However, the concentration of phosphate buffer was limited to *ca.* 140 mM, because acetonitrile miscibility decreases as phosphate buffer increases.

Effect of pH

Given the presence of carboxylic acid (-COOH) side chains, pyrrole (=NH) groups, and pyrrolenine (≡N) groups, it is not too surprising that the elution of porphyrin carboxylic acids is pH sensitive (Figure 3). The retention of all five porphyrins increase when pH decreases. As pH decreases, porphyrins tend to be protonated, therefore the solubility in the mobile phase decreases. Complete elution of the five porphyrins within 60 minutes requires a pH of 6.5 or higher.

Other considerations

Optimum conditions for the separation was found to be 25/75 (140 mM) of acetonitrile/phosphate buffer (Na_2HPO_4, NaH_2PO_4). The pH of the aqueous portion was 6.9. A typical chromatogram is shown in Figure 4.

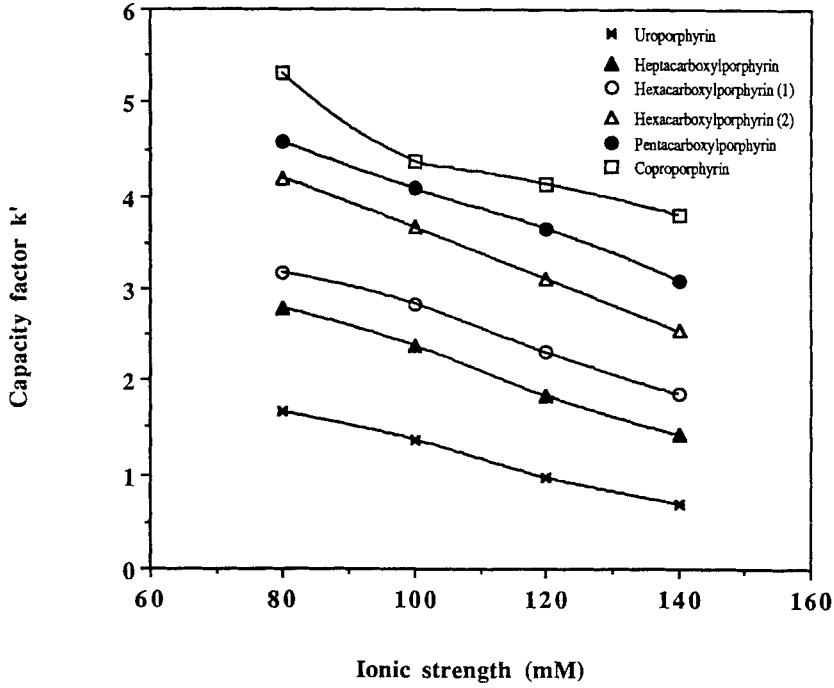


Figure 2. Effect of ionic strength on the retention of polycarboxylated porphyrins. Mobile phase: 30/70 ACN/buffer (phosphate; pH 6.7).

It is interesting to note the presence of an additional peak upon injection of standard hexacarboxylporphyrin I (Figure 5). Further testing by the manufacturer precluded the presence of an impurity. Various measures were taken to ensure that the additional peak was not an artifact of the experiment protocol. The retention times and the peak areas of these two peaks were found to be very reproducible even from various lots of hexacarboxylporphyrin from the same manufacturer.

Hexacarboxylporphyrin I is manufactured by decarboxylation of uroporphyrin I. There are two potential isomers, cis-and trans-hexacarboxylporphyrin (Figure 6) resulting from the decarboxylation process. One is formed by decarboxylation of two acetic acid

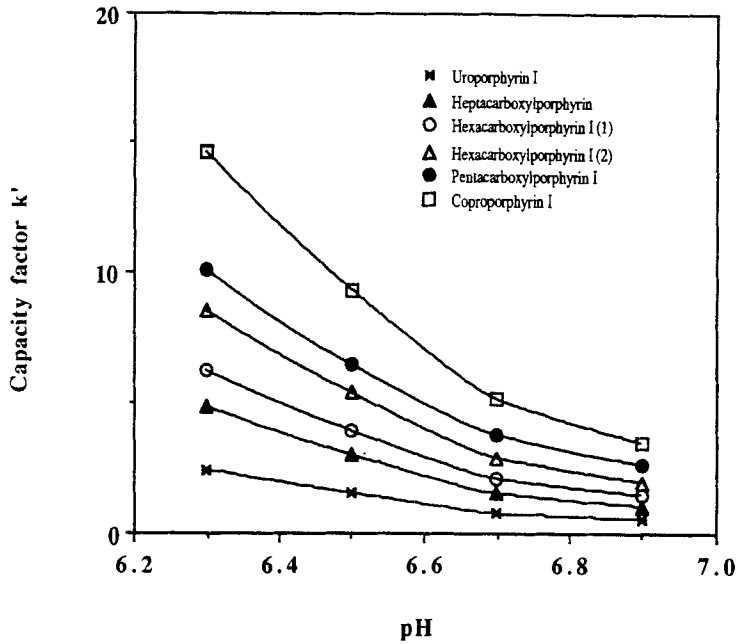


Figure 3. Effect of mobile phase pH on the retention of polycarboxylated porphyrins. Mobile phase: 25/75 ACN/buffer (140mM phosphate).

groups on two adjacent pyrroles, and the other by the decarboxylation of two acetic acid groups on two opposite pyrroles (17). Chu & Chu also observed two isomers as their ester derivatives using paper chromatography(18). For each of the remaining four porphyrins studied, only a single isomer results from decarboxylation. From statistical estimation, the ratio of cis to trans isomers produced in the decarboxylation of uroporphyrin is approximately 2:1. Assuming that the extinction coefficients of the two isomers are similar, the larger area of the second peak suggests that this peak may correspond to the cis isomer. Further confirmation of this interpretation is still under investigation. To the best of our knowledge, this may well be the first report of a chromatographic separation of the two native hexacarboxylporphyrin I isomers.

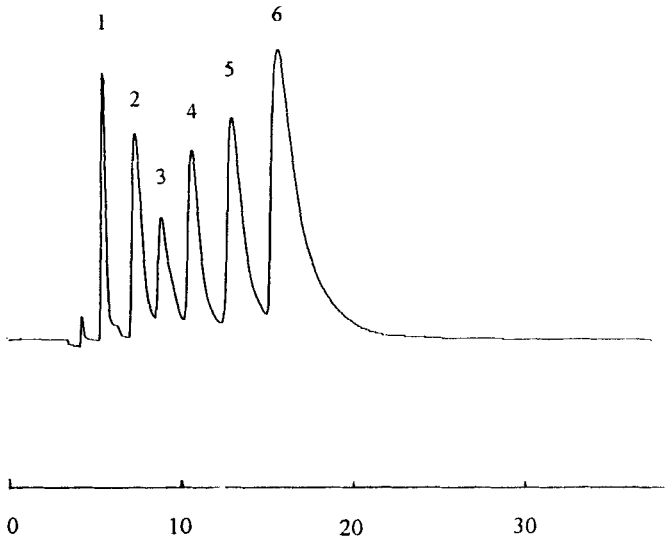


Figure 4. Chromatogram of optimized isocratic separation of polycarboxylated porphyrins on a 25 cm x 4.6 mm I.D. column. Mobile phase: 25/75 ACN/buffer (140 mM phosphate; pH 6.9). Flow rate 0.8 mL/min. 1. Uroporphyrin; 2. Heptacarboxylporphyrin; 3. Hexacarboxylporphyrin (1?); 4. Hexacarboxylporphyrin (2?); 5. Pentacarboxylporphyrin; 6. Coproporphyrin.

Biological samples

For clinical purposes, the determination of porphyrin is carried out in biological samples, such as urine and blood. There have been several reports of the direct injection of urine samples (6-9). However, direct injection of serum sample for the analysis of porphyrins without prior extraction procedures has not been reported. Recently, Stalcup & Williams (19) successfully applied direct enantiomeric resolution of chiral drugs in human serum using a commercially available β-CD column. They attributed the feasibility

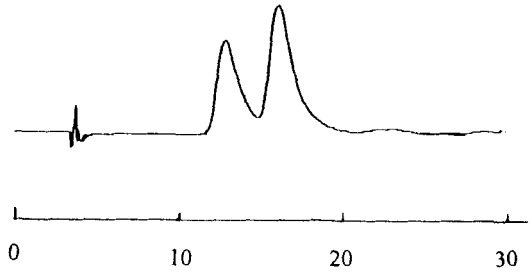


Figure 5 Chromatogram of hexacarboxylporphyrin I standard. Mobile phase: 30/70 ACN/buffer (100 mM phosphate; pH 6.7). Flow rate 0.8 mL/min.

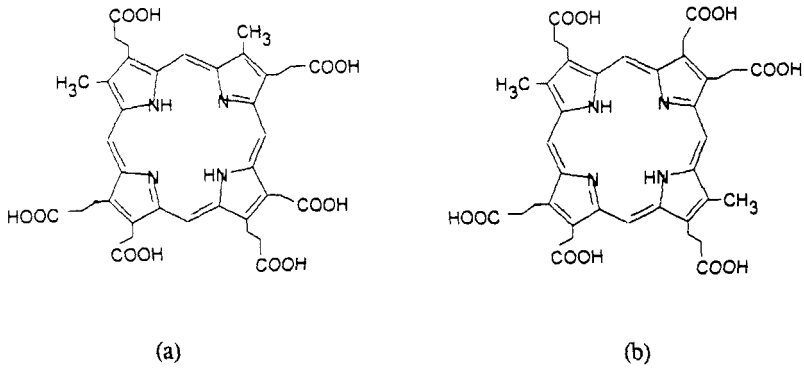


Figure 6. Structures of hexacarboxylporphyrin I isomers. a) cis-isomer b) trans-isomer.

of direct injection of serum onto the CD column to the high aqueous content of mobile phase and the diol character of the stationary phase. The high aqueous mobile phase composition for the separation of the porphyrins suggested that it might also be possible for direct injection of human serum on the γ-CD column which is synthesized analogously to the β-CD column.

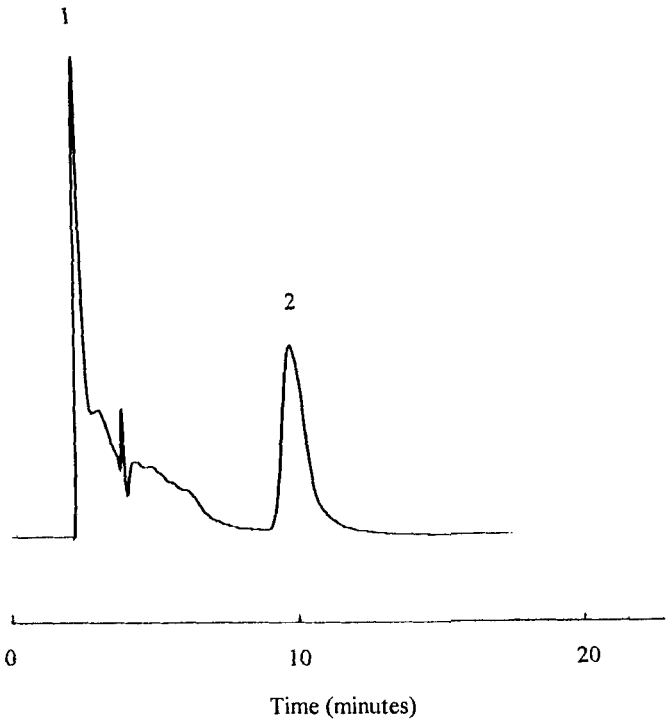


Figure 7a. Chromatogram of direct injection of serum. Mobile phase: 25/75 ACN/buffer (140 mM phosphate; pH 6.7). 1. Protein; 2. Unknown.

Human serum samples were obtained in the same way as previously reported (19). Uroporphyrin and coproporphyrin were spiked into the serum. The chromatographic results (Figure 7 a, b) showed that the detection of uroporphyrin and coproporphyrin were not affected by serum interferences. Thus, direct injection of serum onto the γ-CD column has the potential to facilitate quantitation of porphyrin levels in human blood.

CONCLUSIONS

Separations of porphyrin carboxylic acids on the γ-CD column takes advantage of the large cavity of the γ-CD. The isocratic separation of five porphyrin carboxylic acids

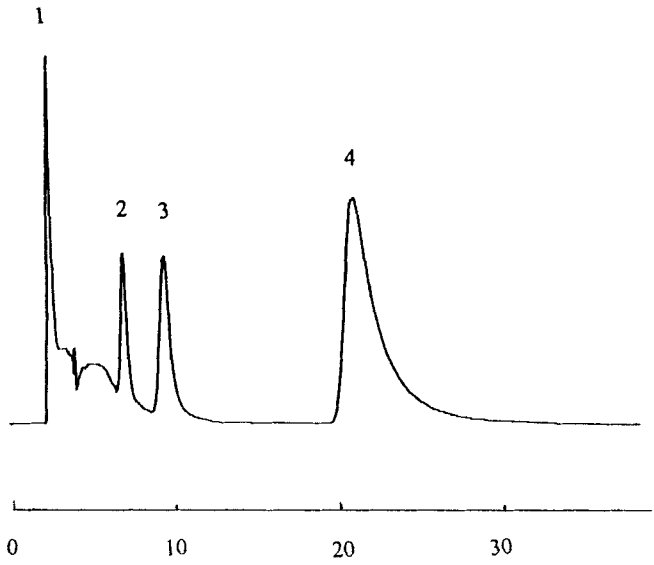


Figure 7b. Chromatogram of direct injection of serum sample spiked with uro- and copro-porphyrin (400 nm). Mobile phase: 25/75 ACN/buffer (140 mM phosphate; pH 6.7). 1. Protein; 2. Uroporphyrin; 3. Unknown; 4. Coproporphyrin.

has been achieved. The experimental conditions used may facilitate the clinical investigation of porphyrin related diseases.

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