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Use of eluent containing surfactant for the liquid chromatographic analysis of porphyrins by direct serum injection

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ABSTRACT

A gradient elution reversed-phase high-performance liquid chromatographic method was developed for the direct serum injection analysis of porphyrins based on the use of eluent containing an anionic surfactant (sodium dodecyl sulfate) at a concentration below the critical micelle concentration to elute the serum proteins at the column void volume. Separation and detection performances were tested with a mixture of porphyrin standards containing uro-, heptacarboxylic-, hexacarboxylic-, pentacarboxylic-, copro-, zinc proto- and mesoporphyrin in a model serum consisting of 50 mg/ml bovine serum albumin. Average limit of detection is 0.06 pmol with a 10-μl injection volume using fluorimetric excitation at the Soret band of porphyrins. The utility of this method for the direct serum injection analysis of porphyrins in human serum was evaluated by investigating serum samples from individuals suffering from iron-deficiency anemia and breast cancer.

INTRODUCTION

The determination of total porphyrin content as well as relative concentrations of individual porphyrin species (porphyrin profiles) in biological materials are important for the confirmation and differential diagnosis of a variety of diseases associated with impaired heme synthesis and metabolism such as inherited or acquired porphyrias [1–4]. To this end a large number of analytical techniques have been developed in recent years for the determination of porphyrin profiles in urine and feces based on thin-layer chromatographic [5,6] or high-performance liquid chromatographic (HPLC) [7–10] techniques. In fact, it has been recently demonstrated that direct injection of acidified, filtered urine into a reversed-phase column is possible for the analysis of urinary porphyrin profiles [9]. In contrast, only a few studies have been reported on the determination of porphyrin profiles in serum or plasma [11], perhaps in large part due to the experimental difficulties encountered in the extraction and subsequent separation and detection of relatively low concentrations of porphyrins in human serum or plasma.

In recent years a number of different approaches have been developed which demon-

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strated the feasibility of performing chromatographic analysis by direct serum injection, thereby eliminating the need for any sample preparation steps [12–17]. One of the most attractive and promising techniques which allows for direct serum injection to be performed in conventional chromatographic systems is micellar liquid chromatography (MLC) in which eluents containing surfactants at a concentration above the critical micelle concentration (CMC) are used to prevent protein precipitation and to minimize adsorption of serum proteins onto the stationary phase [18,19]. In this technique it is necessary to choose eluent conditions that are favorable for micelle formation and serum protein solubilization. Recently Grohs *et al.* [20] have investigated the range of surfactant and organic solvent composition in which direct serum injection is possible for the analysis of drugs using conventional reversed-phase HPLC under isocratic conditions.

Chromatographic methods based on reversed-phase HPLC and gradient elution with methanol in phosphate buffer are most popular for the rapid quantitative and qualitative analyses of urinary and fecal porphyrins [8–10]. In this paper, a similar reversed-phase HPLC method with gradient elution was developed for the direct serum injection analysis of porphyrins in model and human sera involving no extraction or derivatization step. Elution of serum proteins at the column void volume was achieved based on the use of sodium dodecyl sulfate (SDS) at a concentration (2 m*M*) below its CMC and methanol at 30% (w/w) in sodium phosphate buffer (pH 3.5). Detection was accomplished by fluorimetric excitation at the Soret band of porphyrins. The analytical merits of this method were investigated using model serum containing bovine serum albumin (BSA) and porphyrin standards, and the utility of this method for the determination of porphyrins in real samples was demonstrated for the direct serum injection analysis of human sera from patients suffering from iron-deficiency anemia and breast cancer.

EXPERIMENTAL

Reagents

SDS, BSA and normal human serum were obtained from Sigma (St. Louis, MO, USA). SDS was recrystallized from methanol before use. HPLC-grade methanol, sodium phosphate monobasic, and phosphoric acid were obtained from Fisher Scientific (Springfield, NJ, USA). Porphyrin chromatographic marker kits and zinc protoporphyrin IX standard were obtained for Porphyrin Products (Logan, UT, USA). Each marker kit contained 10 nmol each of eight-, seven-, six-, five-, four-, and two-carboxylic porphyrins. All of these porphyrins are of type I isomer, except for the two-carboxylic porphyrin which is of type IX. Purified water was obtained from a Barnstead Nanopure water system (Dubuque, IA, USA). Pathological human serum samples were obtained, with patient consent, from the Clinical Chemistry Laboratory at Our Lady of Lourdes Hospital (Binghamton, NY, USA).

Chromatographic conditions

The HPLC system consisted of a Varian 5500 liquid chromatograph (Houston, TX, USA), a Model 7125 sample injector with a 10-μl injection loop (Rheodyne, Cotati, CA, USA), and a Perkin-Elmer Model 75 absorbance detector set at 280 nm or 610-S fluorescence detector (Norwalk, CT, USA) equipped with a 10-μl flow cell. Fluorescence excitation was performed at 400 nm with a bandpass of 20 nm, and emission was collected at 620 nm with a bandpass of 20 nm. The HPLC analytical column was a 10-μm μBondapak C_{18} (300 mm × 3.9 mm I.D.) (Waters Assoc., Milford, MA, USA). This column was protected by a 3-cm refillable pellicular C_{18} guard column (Alltech, Deerfield, IL, USA). Chromatograms were recorded on a Hewlett-Packard Model 3390A integrator (Avondale, PA, USA) and on a strip-chart recorder.

HPLC-grade methanol and purified water were vacuum-filtered through a 0.45-mm filter before formulation. Aqueous mobile phase (eluent A) was prepared by first dissolving sodium phosphate monobasic in water to give a 0.05

M solution and adjusting to pH 3.5 with phosphoric acid. SDS was then added to give a 0.002 *M* solution and dissolved with gentle stirring; finally, methanol was added to give a 30% methanol mixture (w/w), and the mixture was degassed further by sonication for 3 min. Eluent B was pure methanol.

A linear gradient was applied to the system from 100% eluent A to 100% eluent B in 10 min, followed by holding at 100% eluent B for 5 min and then returned to initial conditions in 2 min and finally re-equilibration for 10 min. A total mobile phase flow-rate of 2.0 ml/min was used at all times and retention times were measured from the point of injection to the peak maxima on the chromatogram.

Sample preparation

A solution of 50 mg/ml BSA in 0.01 *M* pH 7.4 phosphate buffer was used as a model serum. Porphyrin standard solutions were prepared by dissolving the contents of a chromatographic marker kit and an appropriate amount of zinc protoporphyrin into a small volume of 1 *M* hydrochloric acid and pyridine, respectively, and diluted to 25 ml with 0.01 *M* pH 7.4 phosphate buffer. An appropriate amount of porphyrin standard solution was then spiked into the model serum. Human serum test samples were used as received.

Recovery experiments

For the preparation of porphyrin sample solutions with the presence of serum proteins, appropriate volumes of standard solutions of uroporphyrin, protoporphyrin and zinc protoporphyrin were added to normal human serum to give a final porphyrin concentration of 30 n*M* with negligible dilution of the serum. These sample solutions of porphyrins in serum were then thermally equilibrated at 37°C for at least 30 min. For the preparation of porphyrin sample solutions at the same concentration without the presence of serum proteins, equivalent volumes of porphyrin standard solutions were simply diluted with 0.01 *M* phosphate buffer (pH 7.4). To determine the average percentage recovery, the sample solutions of each porphyrin with and without the presence of serum proteins were injected directly into the HPLC system and the ratio of the areas of the porphyrin chromatographic peaks obtained from these sample matrices were calculated for five repeated runs. Chromatographic and detection conditions were identical to those described for direct serum injection analysis of porphyrin standards spiked into the model serum as shown in Fig. 1.

RESULTS AND DISCUSSION

For the successful elution of serum protein (BSA) using eluents containing surfactants below the CMC, Grohs *et al.* [20] have shown that it is necessary to choose eluent conditions that are favorable for solubilization of proteins by the surfactant as well as coating of the stationary phase with the surfactant. In our experiment the initial SDS (~ 2 m*M*) and methanol (~ 30%, w/w) concentrations during which the serum albumin is being eluted from the column fall within the range of surfactant and methanol composition which permits quantitative elution of BSA as

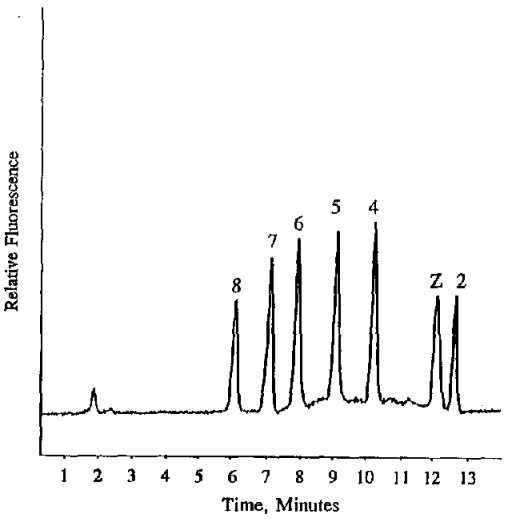

Fig. 1. Chromatogram of model serum containing 50 nmol/l of each porphyrin standard. Peaks: 8 = uroporphyrin; 7 = heptacarboxylic acid porphyrin; 6 = hexacarboxylic acid porphyrin; 5 = pentacarboxylic acid porphyrin; 4 = coproporphyrin; Z = zinc protoporphyrin; 2 = mesoporphyrin.

suggested by Grohs *et al.* [20] (SDS lower limit, 1 m*M*; methanol upper limit, 40% w/w). However, as the gradient moves toward higher methanol composition, it is possible that desorption of SDS from the stationary phase could occur, resulting in a decrease in the amount of surfactant coated onto the stationary phase and an increase in the amount of adsorbed protein at subsequent injections. A short re-equilibration time (10 min) at the initial eluent condition was applied at the end of the gradient to replenish any desorbed surfactant [21].

The influence of the gradient on the elution of BSA was evaluated by injecting a series of model serum samples and determining the peak areas of the eluted albumin using absorbance detection. It was found that the BSA peak area rose with the initial few injections and reached a plateau after the fourth injection. The coefficient of variation in BSA peak area was less than 5% for 25 injections within this plateau region. Furthermore, the effects of the gradient program on the chromatographic behavior of porphyrins were evaluated using fluorescence detection. Peak area and retention times obtained from repeated direct serum injection analysis of a mixture of porphyrin standards spiked into the model serum were found to be highly reproducible. For 25 injections, the coefficients of variation calculated from peak areas of each of the porphyrin standards as shown in Fig. 1 were found to be less than 3.0% while retention times of the porphyrin peaks did not vary by more than ± 10 s. Column lifetime was examined by observing the variation in the efficiency and resolution of the porphyrin standards and we found no significant changes of these parameters after 200 injections of the spiked model serum samples. These data are in agreement with similar results obtained by Grohs *et al.* [20] under isocratic conditions, suggesting the gradient used in this experiment had minimal effects on the reproducible elution of BSA from the reversed-phase column.

Fig. 1 shows a typical chromatogram of a mixture of porphyrin standards spiked into the model serum. It can be seen that the standard mixture which contained mesoporphyrin (a dicarboxylic analogue of protoporphyrin), zinc protoporphyrin (ZnPP), coproporphyrin, penta-, hexa- and heptacarboxylic acid porphyrins, and uroporphyrin were completely resolved within 15 min of direct serum injection. The elution order of these porphyrins are governed primarily by the number of carboxyl groups in the periphery of the porphyrin ring and is identical to those reported in the literature for the separation of porphyrin standards in aqueous solutions without the presence of serum albumin using reversed-phase HPLC [7–10].

The small peak which appeared at the void volume of the column (~ 2 min after injection) as shown in Fig. 1 corresponds to the elution of BSA from the column. This was confirmed by injecting porphyrin-containing and non-porphyrin-containing model serum samples into the system and comparing peak area at the column void volume. To assess the effects of binding of porphyrins by proteins, recovery of two metal-free porphyrins with large differences in hydrophobicity (uroporphyrin and protoporphyrin having eight and two carboxyl groups, respectively) and a metalloporphyrin (ZnPP) from normal human serum were determined. At a concentration of 30 n*M* each of uroporphyrin, protoporphyrin, and ZnPP, the average recovery for five replicate injections was found to be 92.2, 96, and 95.3%, respectively, and the corresponding coefficients of variation were 5.5, 3.2, and 4.3%, respectively. These recovery data are comparable to those reported for techniques employing various organic solvents systems for the extraction of protein-bound porphyrins prior to HPLC analysis [22,23].

The limits of detection (LOD) calculated from the calibration plots based on a signal-to-noise ration of 3 was ~ 0.05 pmol for coproporphyrin, penta-, hexa-, and heptacarboxylic acid porphyrins, and was ~ 0.08 pmol for uroporphyrin, ZnPP, and mesoporphyrin. The integrated area showed a linear relationship with amounts of up to 10 pmol for each porphyrin. The average relative LOD that can be achieved using fluorimetric excitation at the Soret band (~ 400 nm) and a 10-μl injection volume for the porphyrins sepa-

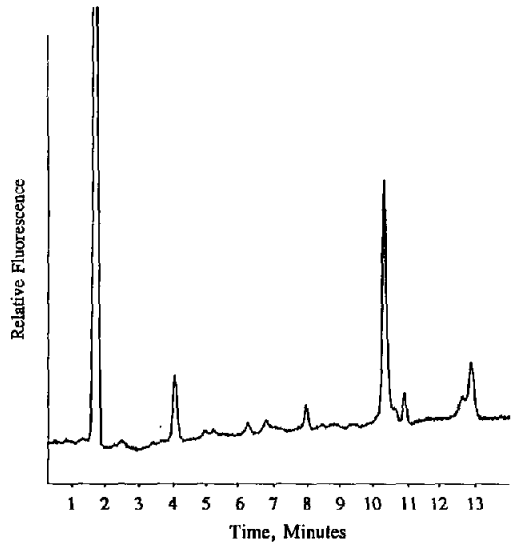

Fig. 2. Chromatogram of serum from an individual with iron-deficiency anemia.

rated in a chromatogram as shown in Fig. 1 is ~ 0.6 nmol per 100 ml, which should be sufficient for the confirmation and differential diagnosis of mild to acute cases of a number of inherited or acquired porphyrias [11]. Improvement in LOD could be achieved by employing a visible line of an argon-ion laser for fluorimetric excitation. Significant enhancement in detectability and selectivity has already been demonstrated in our research group using visible laser fluorimetry for the determination of total porphyrin contents as well as relative concentrations of individual porphyrin components present in biological fluids [24,25].

It is well known that excess amounts of ZnPP can be found in the red blood cells of those with lead poisoning or iron-deficiency anemia [22,23,26]. However, very little is known about the porphyrin levels or profiles in serum or plasma from these individuals. In Fig. 2, a chromatogram obtained from a serum sample of a patient with iron-deficiency anemia is presented. Separation was carried out using chromatographic conditions identical to those described for direct serum injection analysis of porphyrin standards spiked into the model serum as shown in Fig. 1. It can be seen in Fig. 2 that a peak that saturated the detector appears at ~ 2 min after injection, most likely due to the elution of serum proteins and non-retained fluorescent species at the column void volume. The principal peak located at ~ 10.5 min, which correlates with the retention time of coproporphyrin standard spiked into either model or human serum, was found to occur in four cases out of the seven subjects investigated. Further evidence supporting the identity of the principal peak as coproporphyrin come from the excitation and emission spectral data obtained by stopping the flow at ~ 10.5 min and scanning the peak, which showed the characteristic spectral features of coproporphyrin, having major excitation and emission maxima centered at 400 and 620 nm, respectively [4]. By comparison with peak areas obtained from the direct serum injection of a series of coproporphyrin standards, the area of the principal peak as shown in Fig. 2 corresponds to a concentration of ~ 4 nmol per 100 ml, which is at least two orders of magnitude higher than coproporphyrin concentrations usually found in normal human serum [11].

Recently the presence of porphyrin-like spectral features in the fluorescence spectra of non-chromatographed serum samples obtained from cancer patients was reported [27]. Using similar procedures, we also observed these same fluorescence features in serum samples obtained from cancer patients (~ 70% in lung and breast cancers) [28]. However, when we analyzed these same samples using this direct serum injection technique, we were unable to find a significant number of samples from cancer patients which showed distinctively different porphyrin profiles when compared to those obtained from the controls. It appears that the existence of porphyrin-like emission from most of the non-chromatographed serum samples from cancer patients may be due to some type of matrix effect, leading to an increase in fluorescent quantum yields of some of the serum porphyrins.

Fig. 3 shows a chromatogram of serum sample obtained from a patient suffering from breast cancer. The principal peak located at ~ 12 min

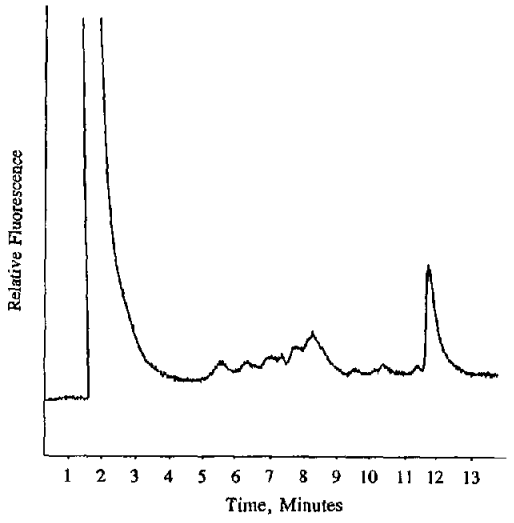

Fig. 3. Chromatogram of serum from an individual with breast cancer.

after injection correlates with the retention time of ZnPP standard spiked into either model or human serum with excitation and emission spectra resembling those of chromatographed ZnPP standard spiked into model or human serum, showing a major excitation and emission band centered at ~ 415 and 595 nm, respectively [4]. However, this peak occurred in only five cases out of the fifty subjects investigated, and to the best of our knowledge, the occurrence of ZnPP in human serum or plasma has not been reported. More studies are necessary to gain further insight into the identity of this peak in future experiments.

As in the case of model serum, about 200 human serum samples were analyzed with no apparent degradation to the chromatographic system using the same chromatographic system and eluent conditions, provided that the guard column was changed after each 50 injections. The chromatographic performance of the system was evaluated by injecting a standard porphyrin mixture spiked into model serum after every fifth injection of the human serum. The coefficients of variation calculated from peak areas of the porphyrin standards over the injection of 25 human serum samples within a single day were found to be less than 5%. Similar reproducibility in peak areas for the porphyrin standards were observed for about 200 injections of human serum samples into the same column over a period of several days. However, variations in column efficiency and resolution became noticeable after 200 injections.

In summary, this work demonstrated that eluent containing SDS at concentrations below the CMC and methanol at relatively high proportion in a phosphate buffer can be used for the gradient elution separation of a mixture of hydrophobic and hydrophilic porphyrins present in serum without the use of any sample preparation step. This method should complement those already developed for the rapid determination of porphyrin profiles in urine and feces using conventional reversed-phase HPLC, thus providing clinical and biomedical researchers with enhanced analytical capabilities. These capabilities may allow for improvements in the confirmation and differential diagnosis of various forms of porphyrias, in the correlation of porphyrin profiles with the occurrence of certain types of cancer, and in the understanding of the biochemical processes through which elevated levels of porphyrins are produced in a variety of diseases associated with impaired heme synthesis and metabolism.

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