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Capillary electrophoresis of urinary porphyrins with absorbance and fluorescence detection

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ABSTRACT

Urinary porphyrins are separated in a 72 cm × 50 μm I.D. fused-silica capillary by micellar electrokinetic capillary chromatography with 100 m*M* sodium dodecyl sulfate and 20 m*M* 3-(cyclohexylamino)-1-propanesulfonic acid at pH 11. Detection is accomplished by absorbance at 400 nm or fluorescence with excitation at 400 nm and emission at wavelengths above 550 nm. Substantial trace enrichment is found for porphyrins in urine samples or for porphyrin standards prepared without surfactant in the injection buffer. Limits of detection are in the 100 pmol/ml concentration range with an optimized fluorescence system. The method is shown suitable for the determination of porphyrins in clinical urine specimens. Comparisons are made between electrophoretic and chromatographic methods for the separation and detection of urinary porphyrins.

INTRODUCTION

The separation of urinary porphyrins by chromatographic methods of analysis have been thoroughly reviewed in recent years[1,2]. The chromatographic methods, in particular liquid chromatography (LC), give relatively rapid separations that are well suited for clinical determinations.

Modern high-performance capillary electrophoresis (CE) is a relatively new technique. The vast majority of published work has dealt with separations, instrumentation and detection[3–5]. There has been little work reported dealing with the handling of biological fluids in CE, particularly with regard to matrix effects.

Urinary porphyrins appear to be ideal compounds for separation by electrophoresis. These compounds have between two and eight carboxylic acid groups and in alkaline buffers should be so negatively charged. Electrophoretic migration of the ionized porphyrins will be towards the positive electrode. Since electroendoosmotic flow will direct the bulk flow towards the negative electrode[6], a counter-migration technique must be employed. Both free solution capillary electrophoresis (FSCE) and

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micellar electrokinetic capillary chromatography (MECC) were studied to determine methodology appropriate for this application.

FSCE separations are performed with a homogeneous buffer medium. The separation is based on the combination of molecular charge and size[7].

MECC employs surfactants in the run buffer. Above the critical micelle concentration (CMC) hydrophobic aggregation of surfactant molecules produce micelles which migrate countercurrent to the electroosmotic flow. The micelles provide a heterogeneous and hydrophobic "pseudo-phase" which can impart reversed-phase properties to the separation[8,9]. Separations of phenols[8], phenylthiohydantoin amino acids[10], *o*-phthalaldehyde amino acids[11] and nucleosides[12] have been reported.

In the present study, the potential for CE to measure porphyrins in a biologically relevant matrix is investigated. Detection is optimized and comparisons are made with LC. If sufficiently selective, sensitive, and reproducible, the instrumental simplicity of automated CE might be advantageous in the clinical setting.

EXPERIMENTAL

Apparatus

An automated CE instrument [Model 270A; Applied Biosystems (ABI), San Jose, CA, U.S.A.] was used, unmodified for MECC separations with absorption detection employing tungsten and deuterium lamps. A 72 cm × 50 μm I.D. capillary (ABI part No. 0602-0014) was used for all separations except for the work described in Fig. 2 where a 55 cm capillary was employed. Vacuum injection at a preset vacuum of 127 mmHg was used for the MECC work. Both electrokinetic and vacuum injection were used for FSCE.

The following modifications were performed to permit the use of fluorescence detection. The "flow-cell assembly" was removed and machined to permit the insertion of two optical fibers oriented at right angles to the separation capillary. The fibers were routed to a filter assembly. Emitted light was measured with a Hammamatsu R1527 photomultiplier tube (PMT) located as close to the filter as possible. The PMT leads were routed outside of the instrument to the photometer of an LC fluorometer (Model 980; ABI, Ramsey, NJ, U.S.A.). The fluorescence wavelengths were selected with a 550 nm longwave filter (Corion, Holliston, MA, U.S.A.).

To adapt the instrument to accept a xenon arc lamp, the following modifications were made. The lamp cradle at the rear of the monochromator was removed and replaced with a lamp mount assembly (ABI part No. 1400-0159). A 75-W xenon arc lamp (ABI part No. 2450-0193) was fitted onto the lamp hub assembly. An external 150-W power supply with an automatic starter (Model LPS 200X, Photon Technologies) was set at 13 V, 5.6 A to run the lamp.

Data were collected on either a strip chart recorder (Kipp and Zonen) or integrator (Model 4290, Spectra-Physics, San Jose, CA, U.S.A.).

Chemicals

A chromatographic marker kit containing a mixture of mesoporphyrin, coproporphyrin, pentacarboxyl porphyrin, hexacarboxyl porphyrin, heptacarboxyl porphyrin and uroporphyrin, 10 nmol each was purchased from Porphyrin Products (part No. CMK-1A; Logan, UT, U.S.A.). The individual porphyrins, as well as

uroporphyrin III and coproporphyrin III were also purchased in pure form from the same source. Sodium dodecyl sulphate (SDS) was Sequanol grade from Pierce (Rockford, IL, U.S.A.). 3-Cyclohexylamino-1-propanesulfonic acid (CAPS) buffer and sodium hydroxide were from Sigma (St. Louis, MO, U.S.A.). Water was provided by an in-house reverse osmosis, ion-exchange system (Hydro, Research Triangle Park, NC, U.S.A.). LC grade methanol was from J. T. Baker (Phillipsburgh, NJ, U.S.A.).

Buffer preparation

The MECC run buffer was prepared as follows. A 2.88-g amount of SDS plus 442 mg CAPS was dissolved in about 95 ml water. The pH was adjusted to 11.0 with 1 *M* sodium hydroxide and the volume brought up to 100 ml. The solution was filtered through a 0.2-μm filter. This gave a working concentration of SDS and CAPS of 100 and 20 m*M*, respectively. Other buffers were prepared in a similar fashion. A 15% methanolic buffer was prepared by mixing 17 ml of the above described buffer with 3 ml methanol.

Standard preparation

Standards were prepared by adding 250–1000 μl buffer to culture tubes containing 10 nmol each of the six different porphyrins and sonicating for about a minute. Further dilutions were made as required. Since porphyrins are photosensitive, they were kept in the dark as much as possible. To minimize light exposure, the autosampler illuminator within the Model 270A was disconnected and the autosampler viewing window was covered to exclude room light.

Urine samples

A 24-h pooled sample of urine from a patient suffering from porphyria cutanea tarda was used. About 0.5 ml urine was centrifuged for a few minutes prior to injection and the supernatant transferred into a 0.5-ml micro-centrifuge tube. Samples of normal urines were obtained from a volunteer and treated as noted above. To aid in peak identification, the normal urine was spiked with 300 pmol/ml of each porphyrin.

Capillary conditioning

A new capillary was flushed by vacuum for 30 min with 1 *M* sodium hydroxide followed by 10 min 0.1 *M* sodium hydroxide and then 30 min with run buffer. After that sequence, the detector-side buffer reservoir was rinsed and filled with run buffer.

Routine operation

The programming features of the Model 270A include the following sequential steps: WASH, BUFFER, MARKER, INJECTION, DETECTOR, TIME1, TIME2, TIME3 and TIME4. After the initial conditioning step described above, the WASH cycle was not used. BUFFER wash was set for 3 min to fill the capillary with the run buffer. The MARKER, which could be used for adding a neutral unretained marker or an internal standard was not used. INJECTION was set for vacuum at selected times from 1- to 20-s injections. The DETECTOR was set for 400 nm with a rise time of 0.5 s. TIME1 conditions were: voltage, 20 kV; polarity, +; temperature, 45°C; run time, 17 min. The current draw was 30 μA under these conditions with the 100 m*M* SDS, 20 m*M* CAPS, pH 11 MECC buffer. Conditions that deviate from the above are so indicated on the figure captions.

Electroendosmotic velocity measurement

The electroendosmotic velocity (V_{eo}) was measured in 3 buffers: (1) 20 m*M* CAPS, pH 11, (2) 20 m*M* CAPS, 100 m*M* SDS, pH 11 and (3) 20 m*M* CAPS, 150 m*M* SDS, pH 11. V_{eo} was measured using a neutral marker that does not partition into the micelle; 1 part methanol plus 9 parts of the respective buffer. The V_{eo} was calculated by dividing the capillary length (mm) by the migration time (s) of the methanol. The voltage was 20 kV and the capillary temperature was 45°C.

Theoretical plate calculation

Theoretical plates (N) were calculated for uroporphyrin using the formula: $N = 5.54(t_m)^2/w_{1/2}^2$ where t_m = the migration time and $w_{1/2}$ = the peak width at half height. A chart speed of 5 cm/min was used to enable accurate measurements to be made.

LC conditions

An Econosphere C_{18} column, 25 cm × 4.6 mm I.D. packed with 5-μm particles (Alltech) was used. A Spectroflow 430 gradient former and a Spectroflow 400 (Kratos, Ramsey, NJ, U.S.A.) pumping system was employed. The mobile phase was a binary gradient: solvent A: methanol–potassium phosphate, monobasic buffer, 6.9 g/l, pH 3.5 (50:50); solvent B: 100% methanol. The gradient was a ramp from 100% A to 100% B in 10 min, hold at 100% B for 10 min followed by reequilibration to initial conditions in 5 min. The detector, a Kratos FS 970 equipped with a xenon arc was set at 400 nm excitation. The wavelengths of emission were selected with a 600-nm bandpass filter, 70 nm bandwidth. The loop size was 20 μl.

RESULTS AND DISCUSSION

Free solution capillary electrophoresis

A separation employing free solution, counter migration capillary electrophoresis is shown in Fig. 1A. The elution order is consistent with the charge on each porphyrin for a counter migration mechanism. The least charged solute elutes first and the elution continues in the order of increasing negative charge. While the most negatively charged solute has the greatest electrophoretic mobility, it is directed towards the positive electrode away from the detector. Since electroosmosis results in the bulk flow being directed towards the detector, the solute with the greatest electrophoretic mobility elutes last. This elution order is exactly the opposite of reversed-phase LC (Fig. 1B[13]).

Two peaks are found for hexacarboxyl porphyrin that were not resolved in the above cited LC separation. The presence of two peaks is an artifact of the synthetic procedure[14]. According to the manufacturer, adjacent and opposite decarboxylation can occur during the synthesis. The adjacent isomer is about two times more likely to decarboxylate and this is represented in Fig. 1A by the peak height ratio for the two peaks of hexacarboxyl porphyrin. In the naturally occurring biological system, only one of the isomers is found. It is probable that these isomers could be separated by LC in a 1 *M* acetate buffer[1,2].

The run buffer for the Fig. 1A separation contained 10% methanol. Without the methanol, an asymmetric peak was found for coproporphyrin, presumably due to its poor solubility. The sample was dissolved in 50% methanol. Without such a high

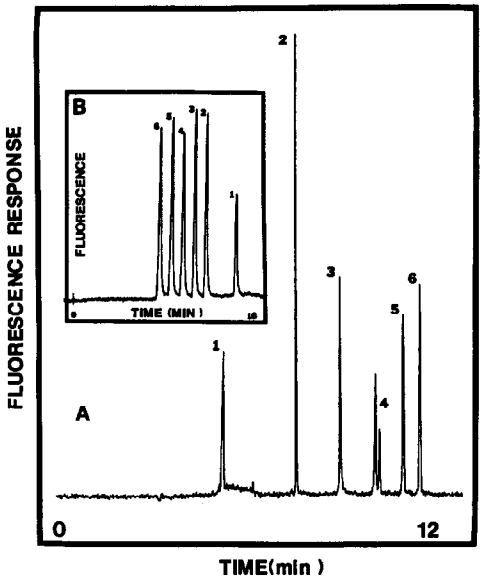

Fig. 1. (A) Free solution separation of urinary porphyrins. Buffer: 20 m*M* CAPS, pH 11 with 10% methanol. Sample: porphyrin mixture, 5 nmol/ml dissolved in methanol–20 m*M* CAPS (50:50). Injection: electrokinetic, 12 s at 10 kV. Run voltage: 30 kV. Temperature: 30°C. Detection: fluorescence, excitation wavelength 400 nm, emission wavelengths > 595 nm, with xenon arc source. Peaks: 1 = mesoporphyrin (dicarboxyl); 2 = coproporphyrin (tetracarboxyl); 3 = pentacarboxyl porphyrin; 4 = hexacarboxyl porphyrin positional isomers (two peaks); 5 = heptacarboxyl porphyrin; 6 = uroporphyrin (octacarboxyl). (B) Reversed-phase high-performance LC separation of urinary porphyrins.

concentration of methanol, mesoporphyrin is insoluble and did not appear on the electropherogram. Mesoporphyrin is not naturally occurring but was added to the test mix by the manufacturer as a typical dicarboxylic acid porphyrin.

During the course of running the system, loss of resolution and peak broadening was sometimes noticed. Regeneration of the capillary through base or acid washing did not always restore the separation. It appeared that adsorption of porphyrins was occurring at the capillary wall. Perhaps this was mediated by the marginal solubility of mesoporphyrin and coproporphyrin. Because of this problem, a more robust system was required for this separation to be useful.

MECC of urinary porphyrins

The rationale for employing MECC for this separation was not to provide for a pseudo-reversed-phase mechanism but to control wall adsorption of the analytes. Since both the porphyrins and the surfactant, SDS, are anionic at pH 11, electrostatic repulsion was expected. The elution order was expected to remain the same as in the free solution experiments. Rather than contribute to the separation mechanism, the anionic surfactant was used to bind any electrostatic or hydrophobic sites on the capillary wall that might interact with the anionic analyte.

The non-interaction hypothesis is not totally correct. As Fig. 2A and B indicates, mesoporphyrin (peak 1) shows a shift in selectivity. At 100 m*M* SDS, it elutes between

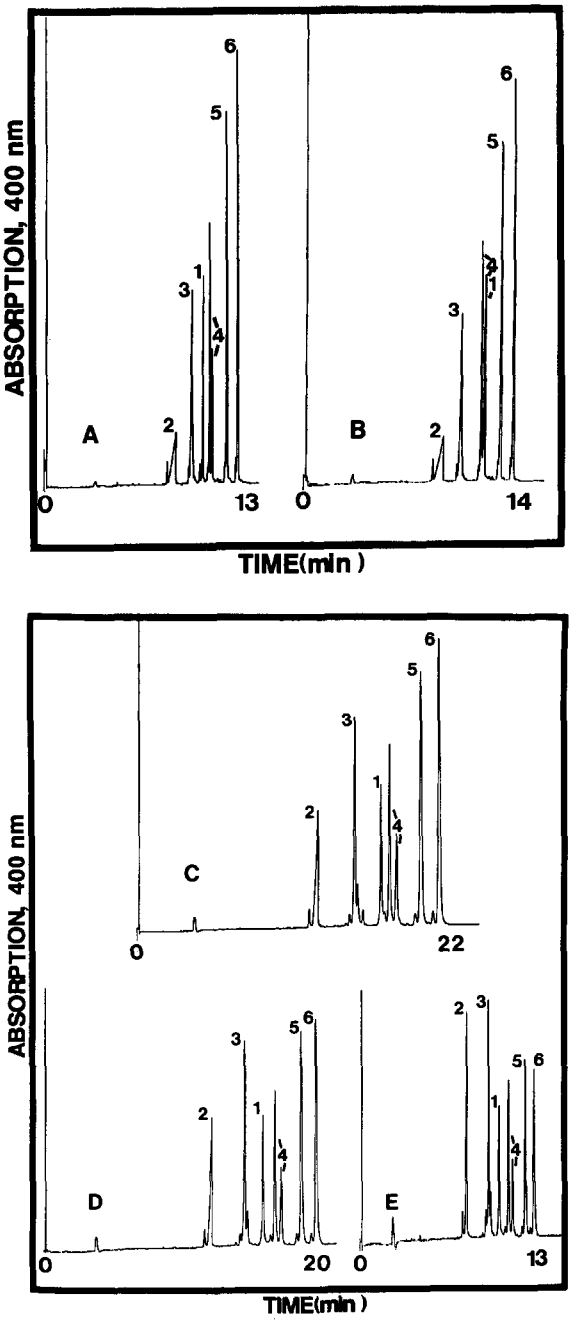

Fig. 2. MECC separation of urinary porphyrins. Sample: porphyrin mixture, 20 nmol/ml in 20 m*M* CAPS, pH 11. Injection, 3 s vacuum. Capillary: 55 cm × 50 μm I.D. Detection: absorbance, 400 nm with tungsten lamp. Sensitivity: 0.064 absorbance units full scale. (A) Run buffer: 100 m*M* SDS, 20 m*M* CAPS, pH 11; 20 kV; 45°C. (B) Run buffer: 150 m*M* SDS, 20 m*M* CAPS, pH 11; 20 kV; 45°C. (C) Run buffer: 17 ml of 100 m*M* SDS, 20 m*M* CAPS, pH 11, plus 3 ml of methanol; 20 kV; 45°C. (D) Run buffer: 17 ml of 100 m*M* SDS, 20 m*M* CAPS, pH 11, plus 3 ml of methanol; 20 kV; 55°C. (E) Run buffer: 17 ml of 100 m*M* SDS, 20 m*M* CAPS, pH 11, plus 3 ml of methanol; 30 kV; 45°C. Peaks as in Fig. 1.

the penta- and hexa-species. At 150 m*M* SDS, mesoporphyrin coelutes with the latter hexacarboxyl porphyrin isomer.

The other porphyrins did not show any changes in elution order that would be suggestive of an MECC separation mechanism. The migration times for the porphyrins are somewhat longer at the higher surfactant concentration. This is consistent with conventional MECC where longer migration times are expected as the surfactant concentration is increased. Measurement of V_{eo} gave values of 1.79 and 1.81 mm/s for 100 and 150 m*M* SDS solutions, respectively. The difference between these results is probably not significant therefore electroosmotic flow is not responsible for the differences in migration time *versus* surfactant concentration. Otsuka *et al.*[10] have defined the velocity of the micelle, $v_{mc} = V_{eo} + v_{ep}(mc)$ where $v_{ep}(mc)$ = the electrophoretic velocity of the micellar aggregate. The micellar velocity, v_{mc} is more profoundly influenced by the surfactant concentration[10]. v_{mc} decreases rapidly with increasing surfactant concentration. This is a consequence of the enhanced $v_{ep}(mc)$ of the micelle towards the positive electrode due to the reduction in viscosity from Joule heating at the higher surfactant concentration. In a similar fashion, the electrophoretic mobility of the porphyrins is expected to increase with surfactant concentration.

It is unlikely that the porphyrins, excepting mesoporphyrin are interacting hydrophobicly with the micelle. The anionic charge is well distributed in all four molecular quadrant for the porphyrins having four or more –COOH groups. Electrostatic repulsions of porphyrins from the anionic micellar aggregate is most likely responsible for the free solution mechanism of separation.

In free solution, the porphyrins elute somewhat faster than in the MECC mode. The electroosmotic flow is 2.1 mm/s at 20 kV with 20 m*M* CAPS buffer at pH 11 and the current draw is only 10 μA. Under these conditions, there is minimal Joule heating and it is likely that the electrophoretic mobility is reduced due to the higher viscosity.

The explanation for the behavior of mesoporphyrin is clear. Mesoporphyrin has both of its carboxyl groups located on adjacent indoles. For this substance, the anionic charge is highly localized. The opposite side of the molecule is free to interact hydrophobicly with SDS without experiencing electrostatic repulsion. The other porphyrins have a more uniform distribution of carboxyl groups throughout their structures. This is a further evidence for the free solution mechanism. Coproporphyrin, which contains four –COOH groups would be better solubilized if an MECC mechanism were occurring.

The surfactant–wall interaction hypothesis proposed above may indeed have some merit. Separations were always successful on both new and aged capillaries.

Coproporphyrin gave a peak that exhibited fronting. This effect was reduced but not eliminated by running at elevated temperatures. A temperature of 45°C was selected as a compromise between peak sharpness and loss of resolution due to the increase in electroosmotic flow. The improvement in peak sharpness may be due to increasing the solubility of the solute in the buffer. Adding 15% methanol to the buffer solubilizes the coproporphyrin and a sharp peak is obtained (Fig. 2C). A 55-cm capillary was used for these experiments to better manage the run time. The longer run time can also be compensated by increasing the temperature to 55°C (Fig. 2D) or the voltage to 30 kV (Fig. 2E). The use of the methanolic buffer was not pursued at this time because of expectation that even modest solvent evaporation would produce migration time drift. A non-ionic surfactant may yet prove a better choice to solubilize marginally soluble compounds.

Detection

Urinary porphyrins have major absorption maxima between 395 and 405 nm. Intense fluorescence occurs between 600 and 700 nm. The selection of the appropriate light source profoundly influences the limits of detection (LOD) found either with absorption or fluorescence detection. Table I lists the LOD values found for free solution separations with electrokinetic injection, MECC separations with vacuum injection and for comparative purposes, LC with a 20-μl loop injection.

The optimal lamp source differs depending on whether fluorescence or absorption is employed. Not surprising, the deuterium lamp was the least sensitive since a wavelength of 400 nm was used. This wavelength is in a spectral region where the deuterium lamp has little energy. For fluorescence, the xenon arc is optimal due to its substantial power for visible excitation. For absorption measurements, the tungsten lamp is superior. While less powerful than the xenon lamp, a filament based lamp is generally more stable than a gas discharge plasma.

The comparison with LC illustrates one of the limiting problems in CE, *i.e.* substantially lower concentration limits of detection (CLOD). The LC method has a CLOD 80–90 times lower than found for CE. The mass limit of detection (MLOD) for CE is superior to that found with LC. With an injection size of approximately 4 nl (1 s at 127 mmHg), the amount of material injected at the LOD is calculated as 0.1 nmol/ml · $(4 \cdot 10^{-6}$ ml) = 0.4 fmol. To contrast this loading factor with LC, the calculation gives 1 pmol/ml · 0.02 ml = 20 fmol. For CE, the MLOD is 50-fold lower than found in LC for the porphyrins. The LC flow cell had a volume of 5 μl. The on-capillary optical window for CE had a volume of less than 0.2 nl.

The advantage of CE over LC in a clinical application is ease of use and low operating costs. The LC separation requires gradient elution, expendable columns and relatively large amounts of solvents, the disposal of which is costly in light of environmental regulations.

Trace enrichment

Trace enrichment, or peak compression will always occur in chromatographic or

TABLE I

LIMITS OF DETECTION OF URINARY PORPHYRINS BY CE AND LC

Please refer to the Experimental section for the detection conditions for both CE and LC measurements. NM = Not measured.

Lamp	*Limit of detection (nmol/ml)*				
	Free solution CE[a]		*MECC*[b]		*High-performance LC*[c]*, fluorescence*
	Absorbance	*Fluorescence*	*Absorbance*	*Fluorescence*	
Deuterium	0.7–1.7	>5	NM	NM	0.01 –0.02
Tungsten	0.4–1.1	>5	0.3–1.4	NM	NM
Xenon	NM	0.08–0.2	1.1–2.5	0.1–0.4	0.001–0.002

[a] Electrokinetic injection, 6 s at 6 kV.
[b] Vacuum injection, 1 s at 127 mmHg.
[c] 20-μl loop.

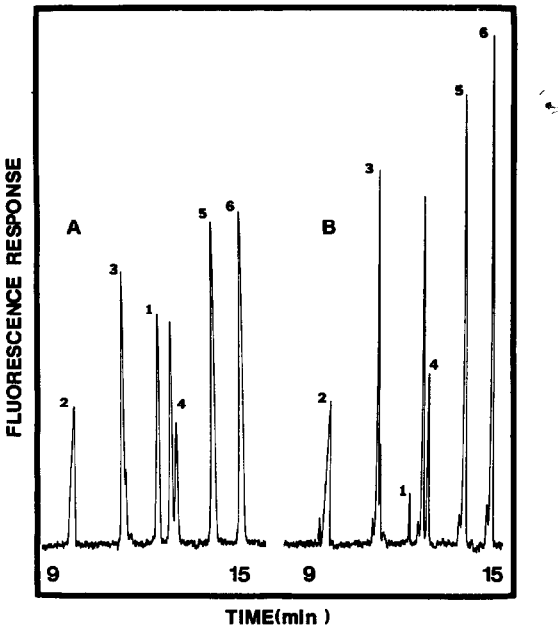

Fig. 3. Impact of injection buffer on resolution. Sample: porphyrin mixture, 5 nmol/ml in (A) run buffer (100 m*M* SDS, 20 m*M* CAPS, pH 11) and (B) 20 m*M* CAPS, pH 11. Injection: 1 s vacuum. Run voltage: 20 kV. Detection: fluorescence, excitation wavelength 400 nm, emission wavelengths > 550 nm with xenon arc. Temperature: 45°C. Peaks as in Fig. 1.

electrophoretic separations whenever the solute's velocity is greater in the injection medium than in the separation medium. In electrophoresis, this phenomenon is known as stacking.

This effect was observed during the course of injecting urine directly into the instrument during an MECC separation. The resulting peaks were substantially sharper than found for standards injected in micellar media. This is illustrated in Fig. 3 where a standard porphyrin mixture was injected in 20 m*M* CAPS buffer and compared to the same mixture injected in run buffer which contained 100 m*M* SDS. Note the improvement in resolution, particularly with regard to the minor components (photodegradation products). The increase in resolution and theoretical plates, even for a short 1-s injection is substantial. The mesoporphyrin signal is much lower with a non-micellar injection solvent due to poor solubility. At lower concentrations, that solubility problem is overcome.

The impact of the injection buffer on peak compression can be substantial. When using an injection buffer with a low conductivity (high resistance) relative to the run buffer, the voltage drop differential can be substantial. For example, the 100 m*M* SDS, 20 m*M* CAPS run buffer draws 30 μA at 20 kV in a 72-cm capillary. The injection buffer, 20 m*M* CAPS draws only 10 μA under equivalent conditions. Then the resistance of these buffers is 9.2 and 27.8 MΩ, respectively. If a 5-cm zone of injection buffer is introduced, the voltage drop is 243 V/cm for the run buffer and 736 V/cm for the injection buffer. Since both V_{eo} and v_{ep} are proportional to the voltage drop and/or

the current, the impact on peak compression can be visualized in a qualitative fashion. Computation of the actual amount of peak compression is complex and beyond the scope of this paper.

A study was performed to determine the degree of trace enrichment at various injection times and the sacrifice in resolution that occurs. The electropherograms are shown in Fig. 4 with some of the figures of merit illustrated in Fig. 5.

It is possible to perform vacuum injections of up to 5 s without substantial loss in resolution. The plate count, measured by peak width at half-height declines from 373 000 to 336 000 for 1- and 5-s injections, respectively. Both peak height and area are linear within this range after which peak height, as expected begins to deviate. Peak

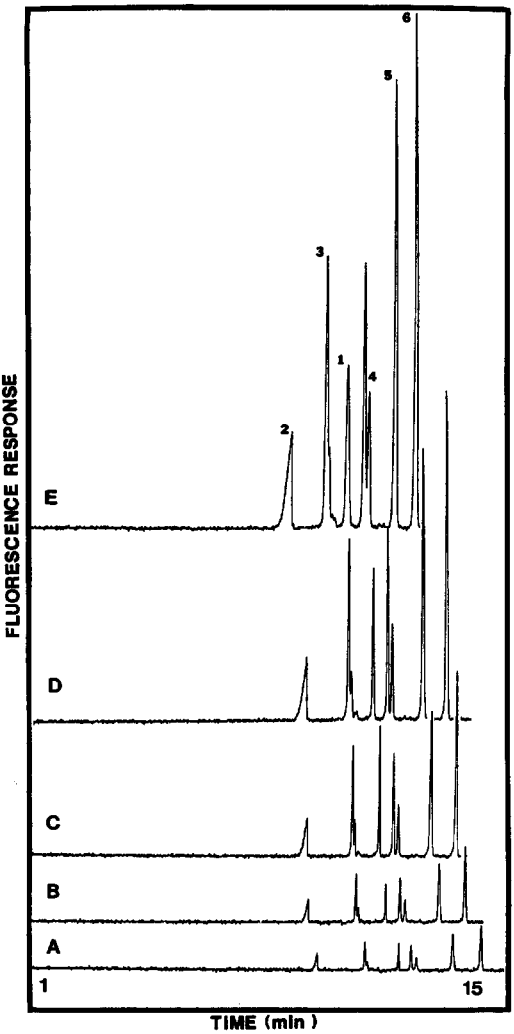

Fig. 4. Impact of injection time on resolution, migration time and response. (A) 1 s; (B) 2 s; (C) 5 s; (D) 10 s; (E) 20 s. Other conditions as in Fig. 3. Peaks as in Fig. 1.

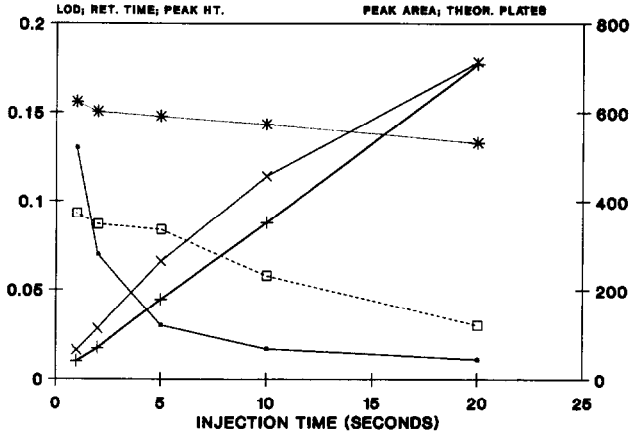

Fig.5. Impact of injection time on LOD, peak area, peak height, migration time and theoretical plate count for uroporphyrin. Key: *···* = migration time (× 0.01 min); × — × = peak height; + — + = peak area; □ --- □ = plate count; ■ — ■ = LOD (ng/ml). Values for theoretical plates should be multiplied by 10^3.

areas were linear for injection times up to 20 s. The limit of detection was improved from 130 pmol/ml to 30 pmol/ml for uroporphyrin, a 4.3-fold decrease between 1- and 5-s injections. In contrast, going from 5 to 20 s produced an LOD improvement of only 2.7 × because of the increased band broadening; the plate count was reduced to 120 000 theoretical plates.

The disadvantage of large-volume injections in non-micellar media is a shifting of migration time that is dependent on the size of the injection. With a 20-s injection, the migration time for uroporphyrin decreases by 15%. The decrease for a 5-s injection is only 5%. The decrease in migration time is due to two factors: (1) the analytes migrate faster in the free solution injection buffer due to the increased field strength as described above and (2) the injection occupies a finite space in the capillary, the length of which is dependent on the size of the injection. A 20-s injection occupies about 50 mm of capillary length. That factor alone would provide for a 10% reduction in migration time.

Matrix effects

It is desirable to inject untreated urine directly into the instrument to avoid sample handling. Unfortunately, the matrix can severely perturb the separation. This is illustrated in Fig. 6 where electropherograms of normal urine and urine spiked with 300 pmol/ml of porphyrins are shown. Injection times of 2, 3 and 5 s were used.

Based on the dramatic sharpening of the uroporphyrin peak (peak 6), it appears that urine or some urine samples may have stacking properties, at least for uroporphyrin. The uroporphyrin was positively identified by standard addition (Fig. 6B). Buffering the urine to 20 m*M* CAPS had no perceptible effect on the separation compared to unbuffered urine.

The source of this unusual peak compression has not been positively identified. As per comments at an international meeting[15], isotachophoretic focusing is a possibility. Perhaps some component or components in a urine specimen can serve as

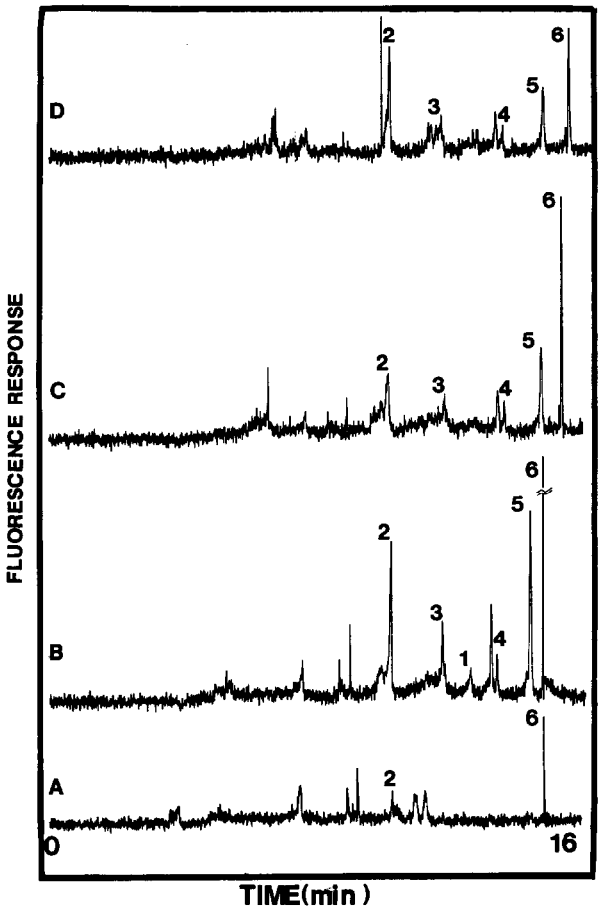

Fig. 6. Impact of injection time of a urine sample on the electropherogram. (A) Urine from a normal patient, 5-s injection; (B–D) urine spiked with 300 pmol/ml porphyrins; (B) 5-s injection; (C) 3-s injection; (D) 2-s injection. Other conditions as in Fig. 3. Peaks as in Fig. 1.

a terminating buffer, then it might be possible that an isotachophoretic zone might overlap and compress an electrophoretic peak. On a 55-cm capillary, peak compression of pentacarboxyl porphyrin was observed with the same urine specimen as employed in Fig. 6. Elevating the temperature eliminated the peak compression. This indicates that whatever the phenomenon, it can be regulated and if controllable, might be useful from the analytical perspective.

There are several important issues that can be extracted from these data: (1) migration time, peak height and peak width can be related to the injection time, the dependence of which can be influenced by the sample matrix; (2) 1–2-s injection times have only a modest impact on the electropherogram; (3) the use of such short injection times will limit detectability of the porphyrins to 100–200 pmol/ml.

For positive peak identification, standard addition techniques may be necessary to confirm the identity of unusual peaks when large volume injections are made.

Alternatively, sample preparation techniques such as solvent or solid-phase extraction may be employed to minimize matrix effects. An additional benefit of these techniques is the potential to improve the sensitivity of the method through off-line trace enrichment.

Precision and linearity

Peak area linearity was assessed at concentrations of 0.64, 1.4, 4 and 10 nmol/ml. The calibration curves were linear ($r^2 > 0.9994$) for all natural porphyrins and passed close to the origin. Mesoporphyrin, showed a modest loss of linearity at the higher concentrations ($r^2 = 0.9593$), presumably due to low solubility in the non-micellar injection buffer.

Peak height precision was assessed at three concentration levels. The following data excludes coproporphyrin which will be discussed later. At the 40 nmol/ml level, injecting with micellar buffer, the relative standard deviations (R.S.D.) ranged from 0.9–2.5% ($n = 5$). At 4 nmol/ml, injecting with CAPS buffer, the R.S.D. values were from 2.8–4.6% ($n = 9$). At 1 nmol/ml, injecting with CAPS buffer, the R.S.D. values were 3.6–8.5% ($n = 9$).

Coproporphyrin was not as well-behaved electrophoretically, presumably due to solubility problems. Occasionally, peak shape changes were noted. Injecting with micellar solvent may help here as an R.S.D. of 1.9% was calculated at the 40 nmol/ml concentration level. At 1 and 4 nmol/ml, the R.S.D. values were 18 and 14%, respectively. When coproporphyrin must be determined, the previously described methanolic buffer should give superior results.

Migration time precision was not calculated owing to a reproducible bias due to variations in the electroosmotic flow. Run-to-run variation measured for each of the 7 peaks was 1.2–2.5 s/run. This corresponds to a relative migration drift of 0.2–0.3%. Replenishment of the buffers after several runs restored the migration times to the initial values. The cause of the drift is probably depletion of one or more of the buffer components.

Patient sample

An electropherogram of a urine specimen from a patient suffering from porphyria cutanea tarda is shown in Fig. 7A. Splitting of the peaks in the uro- and hepta-areas was noted. Spiking experiments with pure porphyrins indicated that the second peak of each doublet was the correct signal. Suspecting separation of uro(I) and uro(III) isomers a mixture of the two was injected and coelution was obtained. This method will not discriminate between uro(I) and uro(III), nor copro(I) and copro(III).

The urine sample was an aliquot from a 24-h collection. Although it was stored frozen, the sample was a few months old. Since porphyrins are known to degrade photochemically, a standard mixture was exposed to fluorescent room light for an hour. The ensuing electropherogram is shown in Fig. 7B. Photodegradation of all of the porphyrins were noted. In particular, the splitting pattern for uro- and hepta- very closely matched that found for the standard.

The highly elevated uro- and heptaporphyrins found in the patient sample is consistent with porphyria cutanea tarda.

These results suggest that CE may be useful in the clinical setting. While less concentration sensitive than LC, the instrumental simplicity, minimal sample usage,

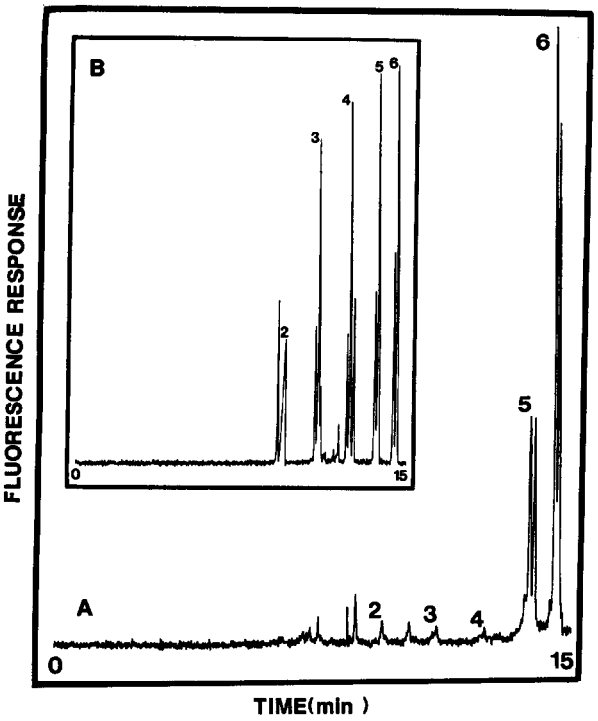

Fig. 7. (A) Electropherogram of a urine sample from a patient with porphyria cutanea tarda. Sample preparation: centrifugation for 1 min. Injection: 2 s vacuum. Other conditions as in Fig. 3. (B) Electropherogram of a partially decomposed porphyrin standard. Initial sample concentration, 5 nmol/ml. Conditions as in Fig. 3. Peaks as in Fig. 1.

low reagent consumption and freedom from organic solvents should be attractive to clinical chemists. If the general detection problem in CE can be solved, this technique will have unequivocal advantages over LC for this application.

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