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Feasibility of capillary zone electrophoresis with suppression of electroendosmotic flow in completely closed systems

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SUMMARY

Instrumental aspects of capillary zone electrophoresis in closed systems are reported. The combination of fixed-volume sample introduction, utilization of double-beam UV absorbance detection and the use of fixed membranes for the separation of the electrode reservoirs from the separation capillary proved to be a reliable instrumental configuration for capillary zone electrophoresis. Dispersion of eluted zones was studied as a function of capillary length. Repeatabilities of migration times and peak areas were determined. Several examples of separations of mixtures of organic acids and nucleotides are given. The separation of diluted and ultrafiltrated serum from patients with chronic renal failure proved to be promising.

INTRODUCTION

High-performance capillary electrophoresis (HPCE) 1–4, which includes various modes of capillary electrophoresis, has grown in popularity during the last decade[5]. Although electrophoretic techniques have been applied for many decades, the importance of using capillaries of, *e.g.*, Pyrex, glass, PTFE and fused silica was readily understood as evaporation of solvent is eliminated, dispersion is minimal and the development of detectors suitable for capillary systems makes it easy for researchers to start with HPCE.

Sometimes electroosmotic flow (EOF) is suppressed and in some experiments EOF is used as a pumping mechanism, especially because chromatographers show interest in these techniques[6,7]. Therefore, stationary phases and quasi-stationary phases are used[8–10] and a link is made between chromatography and electrophoresis. As a rule of thumb, it should be remembered that "chromatographic" interaction is generally suppressed in classical electrophoretic experiments. In this paper, capillary zone electrophoresis (CZE) with suppression of electroosmotic effects, as a specific mode of high-performance capillary electrophoresis, is described.

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TABLE I

OPERATIONAL SYSTEMS USED IN CAPILLARY ZONE ELECTROPHORESIS

Parameter	*System I*	*System II*
Carrier electrolyte	β-Alanine (0.01 *M*) Acetate (pH 3.8)	MES[a] (0.01 *M*) Histidine (pH = 6.05)
Additive	0.05% MHEC[b]	0.05% MHEC
Capillary	PTFE, 0.2 mm I.D.[c]	PTFE, 0.2 mm I.D.
Driving current	50 μA	35 μA

[a] MES = 2-(N-Morpholino)ethanesulphonic acid.
[b] MHEC = Methylhydroxyethylcellulose.
[c] Various lengths.

EXPERIMENTAL

Zone electrophoresis

The equipment used is described in detail under Results and Discussion. The separation capillary (I.D. 0.2 mm, O.D. 0.35 mm) was made of PTFE (Habia, Breda, The Netherlands). Cuprophan membranes were used in the electrode compartments. The UV absorbance detector used was a Model UV-M (Pharmacia, Uppsala, Sweden). Loading of the sample "loop" was done with a 2-ml Luer-lock syringe. A modified alpha-series (Model 807R) power supply (Brandenburg, Thornton Heath, U.K.) was used in the constant-current mode. The operational systems used are given in Table I.

Data acquisition

This was done with a Model 761S data interface and Model 2600 chromatography software (Perkin-Elmer–Nelson, Cupertino, CA, U.S.A.), running on an IBM PC/XT computer. Sampling frequencies used were 10 and 20 Hz.

Chemicals and sera

With the operational systems used, the voltage was approximately 10 kV in the constant-current mode. 2-(N-morpholino)ethanesulphonic acid (MES), histidine, β-alanine, acetic acid, picric acid, sulphanilic acid, 2,4-dihydroxybenzoic acid and *p*-nitrobenzoic acid (all of analytical-reagent grade) were purchased from Merck (Darmstadt, F.R.G). and methylhydroxyethylcellulose (MHEC) from Serva (Heidelberg, F.R.G). Uraemic sera were kept frozen at −20°C until use. Serum proteins were removed prior to zone electrophoretic analysis by ultrafiltration through centrifugation in Centrifree micropartition units (Amicon, Danvers, MA, U.S.A.) at 1800 *g*. Ultrafiltered serum samples were diluted 8-fold prior to injection.

RESULTS AND DISCUSSION

Instrumentation

The basic unit is shown schematically in Fig. 1. A_1 and A_2 are the electrode compartments, B is the sampling device, C the separation compartment and D the detector device.

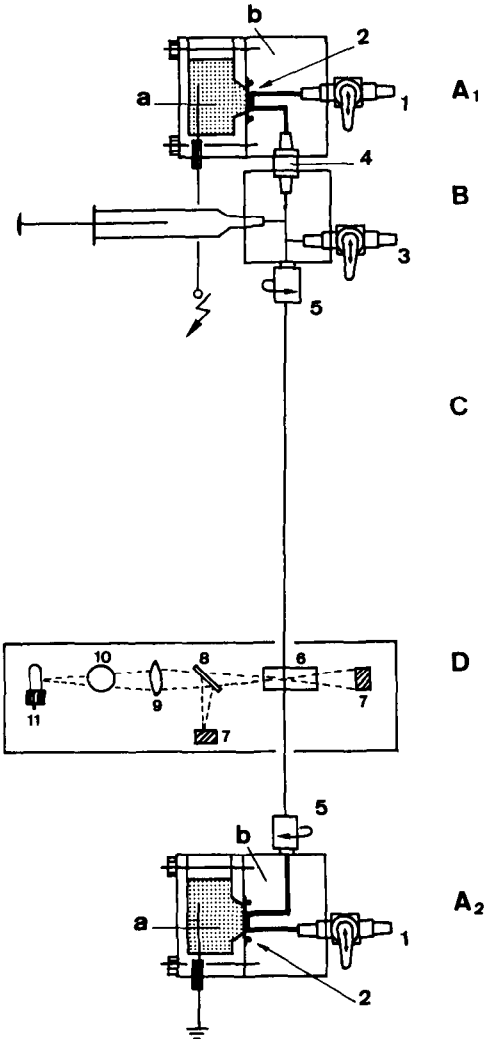

Fig. 1. Schematic diagram of electrophoretic equipment used for capillary zone electrophoresis. A_1 and A_2 = electrode blocks; B = sampling device; C = separation compartment (PTFE tubing); D = UV absorbance detector; a = electrode vessels; b = blocks used for rinsing and refilling the system; 1 = tap for rinsing and refilling the system; 2 = cuprophan semipermeable membranes; 3 = drain valve; 4 = connector of electrode block with injection system; 5 = screw connections; 6 = UV cell; 7 = photodetectors; 8 = beam splitter; 9 = lens; 10 = filter; 11 = UV source.

The electrode vessels (a) are filled with ultra pure water. The blocks (b) are provided with a channel, via which the system can be filled with electrolyte or rinsed with water via the valves 1. This channel connects the electrode vessel (a) with the sampling device and the separation compartment. Between the electrode vessels (a) and the blocks (b), flat membranes (2) are clamped by two screws and an O-ring. This means that the separation compartment is closed at both ends. If any gas is produced

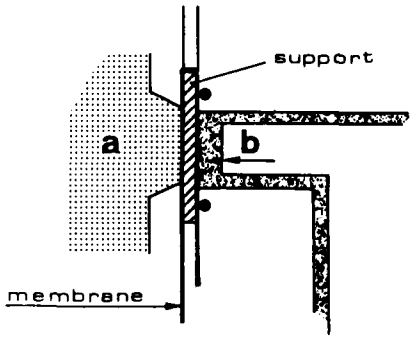

Fig. 2. Fixation of semi-permeable membrane to avoid movement during filling and rinsing. The membrane is folded around the solid support. (a) Water; (b) electrolyte.

by the electrodes, it cannot influence the analysis. Liquid applied via valves (1) passes and rinses the membranes. The volumes of the channels in the blocks (b) are large compared with the volume of the separation compartment. The potential drop in this channel, over and nearby the membrane, is small because the amount of buffer per unit length is high compared with the amount in the separation capillary. Therefore, it takes a long time before the pH jumps, which occur across the membranes owing to both the Donnan potential and electrode reactions, will enter the separation capillary and hence influence the analysis.

Movements of the membranes will affect the reproducibilities of injection and migration times. Therefore, the wetted membranes are folded and fixed around a solid support (Fig. 2). Sample solution is introduced into the sampling device through a feeder using a Luer-type syringe. This feeder and another one, which leads to waste (valve 3), are placed perpendicular to the injection channel. For a more detailed description, see ref. 11. The separation compartment is screw-connected (5) between the sampling device (B) and the electrode compartment A_2. The volume of the sampling device is *ca.* 0.6 μl. It has been reported previously that a sampling repeatability of less than 2% (relative standard deviation, R.S.D.) can be obtained, including day-to-day variation[11].

The separation compartment is a PTFE tube (I.D. 0.2 mm, O.D. 0.35 mm). A Model UV-M UV absorbance detector (Pharmacia) was used in the zone electrophoretic experiments. This detector was originally developed for various liquid chromatographic applications. By measuring UV absorbance with use of a beam splitter and two photodetectors instead of UV absorption, as in our isotachophoresis detector[12], the signal-to-noise ratio could be increased by a factor of at least ten. An advantage of this detector is the small optical unit, which is physically separated from the electronic control unit. Hence the optical unit can be placed easily at any position along the separation capillary, which permits monitoring the separation process. A quartz flow cell designed for high-performance liquid chromatography (HPLC) (18.0684.01; Pharmacia), which focuses the UV light in the centre of the separation capillary, is used. The flow cell is a straight flow-through cuvette. The separation capillary is drawn through the flow cell, and is consequently uninterrupted. The light from the UV source passes through the quartz wall of the cell and through the PTFE wall of the separation capillary. Therefore, the actual optical path length will be *ca.* 0.2 mm.

TABLE II

REPRODUCIBILITIES OF MIGRATION TIME AND PEAK HEIGHTS OF TEST ANIONS IN CLOSED-SYSTEM ZONE ELECTROPHORESIS (n = 8)

Solute	*Migration time (sec)*		*Peak height (mm)*	
	Mean	*R.S.D. (%)*	*Mean*	*R.S.D. (%)*
Picric acid	259.2	0.75	58.34	5.4
Sulphanilic acid	287.5	0.62	138.6	2.4
2,4-Dihydroxy-benzoic acid	335.1	0.52	80.73	3.4
4-Nitrobenzoic acid	349.4	0.60	81.73	3.1

Performance of the system

For the zone-electrophoretic experiments we used operational systems I and II (see Table I). EOF was suppressed by adding MHEC to the carrier electrolyte. Assuming a zeta potential of approximately −1 mV (which is the value for HEC in PTFE tubing[13]), it can be calculated that the EOF "velocity" is only about 1% of the zone-electrophoretic velocity, and can be considered negligible. The repeatability of migration time and peak height was tested in system I using an anionic test mixture consisting of picric acid, sulphanilic acid, 2,4-dihydroxybenzoic acid and *p*-nitrobenzoic acid (all solutes of concentration 10^{-5} *M*). The results are given in Table II. The reproducibility of the migration time was *ca.* 0.6% (R.S.D., n = 8) for all components. Peak heights of the compounds in repeated injections varied between 2.4 and 5.4% (R.S.D.). These results demonstrate the possibilities of the sampling device and the usefullness of the present fixed-membrane configuration.

We determined the number of theoretical plates for the test compound sulphanilic acid at different lengths of the separation compartment. This was done by mounting a capillary of 60 cm length and moving the UV detector along the capillary. The resulting electropherograms are shown in Fig. 3. Plate numbers and plates per metre are plotted as a function of capillary length in Fig. 4. At a capillary length of *ca.* 15 cm the number of plates per metre becomes relatively independent of length (*i.e.*, 110 000 plates/m). Evidently, with shorter capillaries the contribution of instrumental factors to total band broadening becomes significant. Such factors can be related to the method of injection, *e.g.*, the shape and stability of the voltage–time curve when switching on the power supply, or diffusive wash-out of sample from "dead volumes", *e.g.*, the feeders to the injection channel. Moreover, the behaviour of the electrode compartment membranes during application of the voltage and during the analysis may be of importance. The influence of these factors may be diminished, *e.g.*, by computer control of voltage switching, choosing optimum membranes and improving the design of the sampling device and electrode blocks.

In order to estimate the smallest detectable amount of the test mixture in this equipment and electrolyte system, various dilutions of this mixture were injected. Fig. 5 shows an electropherogram of 0.6 μl injected from a 1 μmol/l solution of the test components. Each peak represents 0.6 pmol. This means that for sulphanilic acid *ca.*

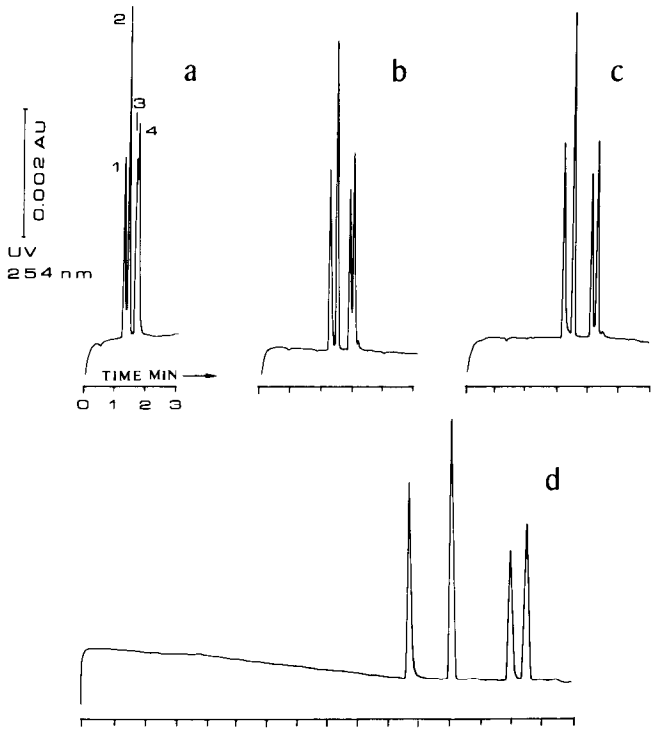

Fig. 3. Zone electropherograms of a test mixture of acids (electrolyte system I, Table I). The UV detector was placed at different positions along a separation capillary of 60 cm length. (a) 8 cm; (b) 13 cm; (c) 18 cm; (d) 58 cm. Peaks: 1 = picric acid; 2 = sulphanilic acid; 3 = 2,4-dihydroxybenzoic acid; 4 = *p*-nitrobenzoic acid.

10 pg (0.1 μmol/l), present in the original injection volume, can be detected. However, when diffusion coefficients of the test solutes are smaller, *i.e.*, with larger molecules, the plate number is expected to increase[14,15] and consequently lower detection limits can be obtained, assuming these solutes have UV absorption in the range selected. By using a capillary that is more transparent for UV light, the signal-to-noise ratio will increase, and consequently lower detection limits are possible.

A calibration line of peak area *versus* amount injected was obtained for aqueous solutions of hippuric acid. Constant volumes (0.6 μl) of solutions with different concentrations were injected. The calibration line is shown in Fig. 6. The following linear model was fitted to the data: area = (19.738 · amount) − 27.2. The correlation coefficient was $r = 0.998$ ($P < 0.0001$). This demonstrates a very good fit of peak area *versus* concentration. It should be noted, however, that these results cannot be extrapolated to the quantification of specific solutes in complex samples, *e.g.*, blood serum.

In Fig. 7 a zone electropherogram is shown of the test mixture. On the left the concentrations of the components are all $2 \cdot 10^{-5}$ *M*. On the right the concentration of sulphanilic acid is 100 times that of the other solutes. The migration times of components 3 and 4 changed by *ca.* 3%. The high concentration difference of sulphanilic acid causes a sharpening of the zone boundaries of the ionic solutes migrating with a lower effective mobility.

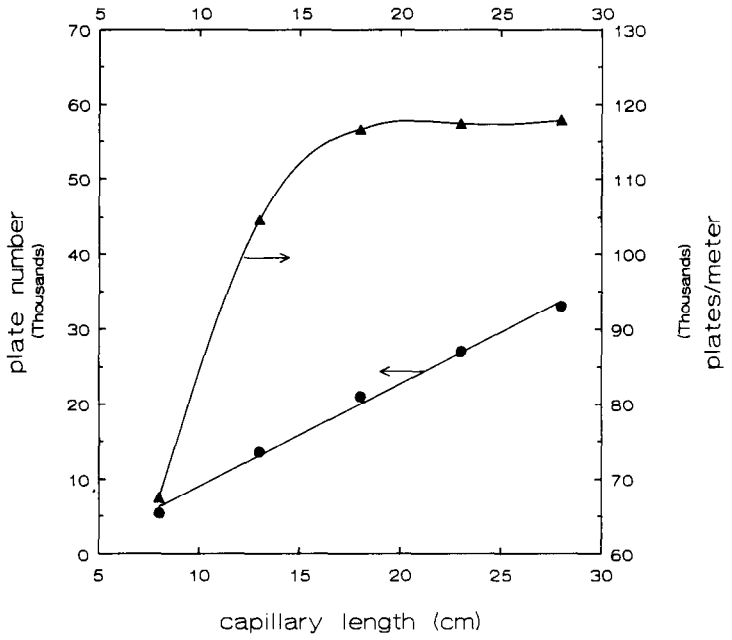

Fig. 4. Plots of number of theoretical plates and plates per metre as a function of length of separation compartment. Test compound, sulphanilic acid.

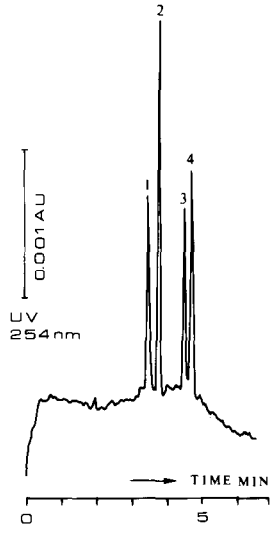

Fig. 5. Zone electropherogram of a standard test mixture at low concentration (electrolyte system I, Table I). Peaks as in Fig. 3. Each peak represents 0.6 pmol injected.

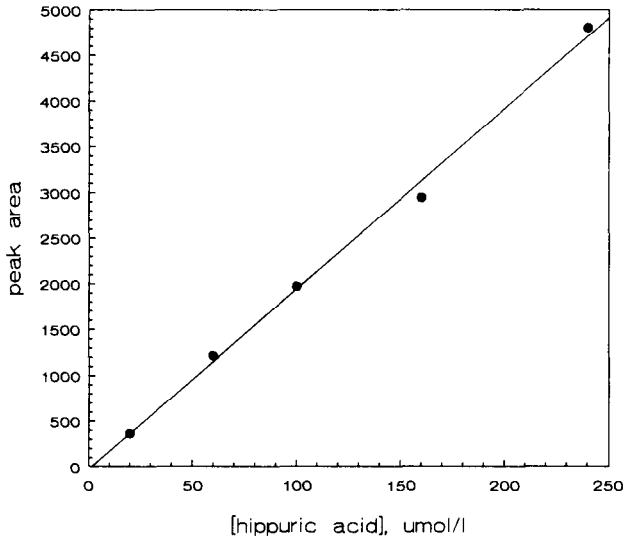

Fig. 6. Calibration line of hippuric acid concentration in aqueous solution *versus* peak area in CZE analysis. For statistics see text.

Applications

Nucleotides. In order to illustrate the difference in selectivity between the closed system with CZE (and no EOF) and that with CZE with EOF, a mixture of the nucleotides ATP, ADP, UMP, GMP, and AMP was analysed in system I (see Table

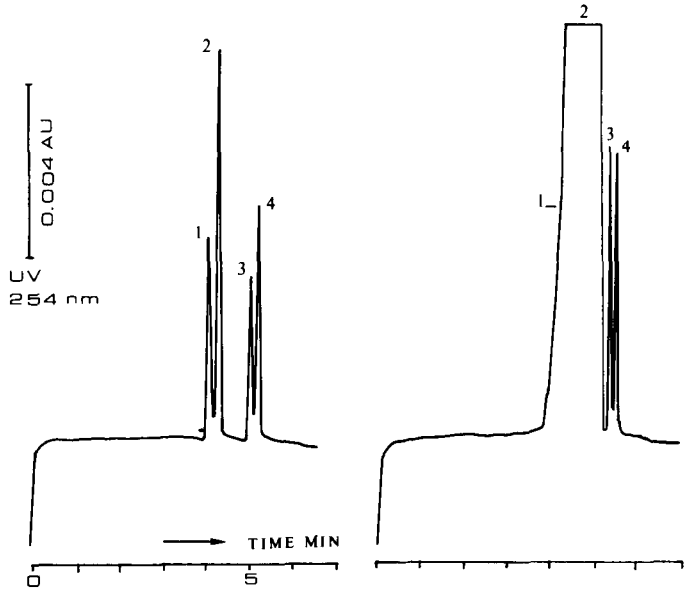

Fig. 7. Zone electropherograms of the test mixture, as shown in Fig. 3 (electrolyte system I, Table I). The concentration of sulphanilic acid is 100 times that of the other solutes. Peaks as in Fig. 3.

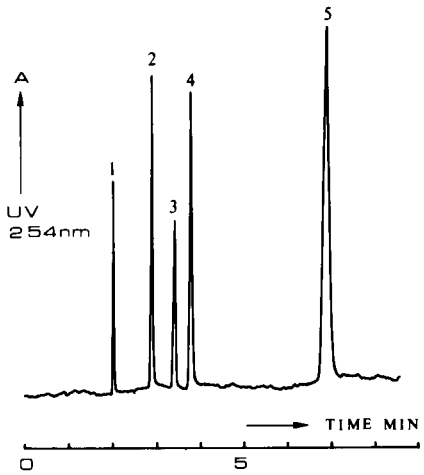

Fig. 8. Zone electropherogram of nucleotides analysed in electrolyte system I (Table I). Peaks: 1 = ATP; 2 = ADP; 3 = UMP;4 = GMP; 5 = AMP. Length of separation capillary, 16 cm.

I). The concentrations injected were $2 \cdot 10^{-5}$ *M* for all solutes. The results are shown in Fig. 8. The elution order in this zone-electrophoretic experiment is opposite to that reported by Tsuda *et al.*[16], which is explained by the fact that the latter workers used EOF in addition to differences in electrophoretic mobility[17].

Anions in uraemic sera. Blood levels of many substances are raised in patients with chronic renal failure. They include nitrogenous waste products such as urea, creatinine, uric acid and methylguanidine, organic (aromatic) acids, polyols, aluminium, the hypothetical "middle molecules", parathormone, β_2-microglobulin and many others[18,19]. We studied the kinetic behaviour and distribution between patients of a range of accumulated solutes by HPLC[20,21]. This HPLC profiling technique was designed to monitor UV-absorbing and fluorescent solutes from different solute classes. Both anionic and cationic substances were analysed. Especially the organic (aromatic) acids may play an important role in the etiology of the uraemic manifestations. Therefore, it is of importance to determine the aromatic acids in uraemic sera. This can be achieved by sample pretreatment prior to HPLC, *e.g.*, solid-phase extraction or isotachophoretic preseparation of anions[22]. However, capillary zone electrophoresis seems to be promising for the one-step analysis of these solutes in uraemic sera.

Ultrafiltered serum from an uraemic patients was analysed using operational system II (Table I). The elution pattern shown in Fig. 9 was obtained. Duplicate profiles form two separate injections are shown. As can be seen, they are highly reproducible. Peak identification was effected by co-elution of the respective compounds with the serum samples, and by comparison with the earlier described HPLC profiles in which these compounds were also detected by UV absorbance at 254 nm. Repeatabilities of peak height, peak area and migration time of uric acid in this complex mixture are listed in Table III. The determination of hippuric acid in uraemic sera by capillary zone electrophoresis will be compared with HPLC in another publication[23]. In the electropherograms it can be seen that some early eluting peaks are very sharp. The number of theoretical plates, as defined by Giddings[14], was calculat-

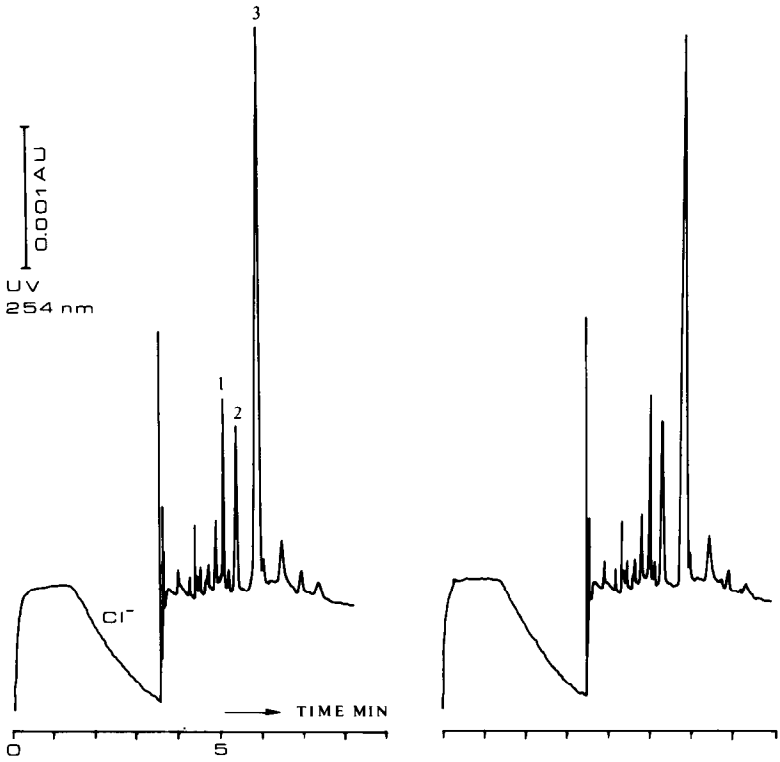

Fig. 9. Duplicate analyses, demonstrating repeatability, of zone electrophoretic separation of UV-absorbing anions in ultrafiltered uraemic sera (electrolyte system II, Table I). Peaks: (tentative): 1 = hippuric acid; 2 = *p*-hydroxyhippuric acid; 3 = uric acid. Length of separation capillary, 25 cm.

ed for these peaks and amounted over 10^6 (see also Fig. 8). This phenomenon will be described elsewhere, because it is rather complex, but Kohlrausch' law[24] must be obeyed. This results in a sharpening up after a large concentration "disturbance". The large negative peak observed in the electropherograms originates from chloride present in the serum samples (at approximately 110 mmol/l in the undiluted sample).

TABLE III

REPRODUCIBILITY OF MIGRATION TIME, PEAK AREA AND PEAK HEIGHT OF URIC ACID IN ULTRAFILTRATED URAEMIC SERUM, AFTER REPEATED INJECTION (n = 5)

Parameter	*Mean*	*R.S.D. (%)*
Migration time (s)	403	0.73
Peak area (μVs)	44 741	6.5
Peak height (μV)	8294	4.5

CONCLUSION

It has been shown that zone electrophoresis in PTFE capillaries (I.D. 0.2 mm) with the described instrumental set-up is reliable with respect to migration time and peak area for several samples. Suppression of electroosmotic effects could be achieved by separating the electrode compartments from the separation compartment, and by adding a surface-active agent to the carrier electrolyte. Any hydrodynamic movement of the background electrolyte was avoided with the closed system. It was shown that the distance between injection compartment and detector should be at least 15 cm in order to avoid instrumental factors of dispersion. Experiments have shown that injections of solutes both in distilled water and in salt gradients gave a sharpening up of the zone boundaries. Plate numbers as large as 10^6 could be achieved.

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