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Note

Isotachophoresis as a preseparation technique for liquid chromatography

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High-performance liquid chromatographic (HPLC) profiles of uremic serum ultrafiltrate are rather complex [1]. For purposes of identification and characterization of the HPLC peaks, information may be obtained from chromatographic retention data, on-line and off-line HPLC—mass spectrometric analysis, off-line (Fourier) infrared analysis and to a certain extent from UV-ratio monitoring at multiple wavelengths [2]. However, it is desirable to decrease the complexity of the profiles, especially in view of the spectrometric identification techniques, where peak impurities might obscure the spectra. For this reason uremic serum ultrafiltrate was preseparated by isotachophoresis [3], the advantages of which are as follows. (1) The concentration effect of dilute samples. (2) The self-sharpening effect of zone boundaries. (3) The possibility of selecting a discrete amount of anions or cations by a proper choice of electrolyte conditions. (4) The length between leading zone and terminating zone (sample) is constant at the moment the terminator has passed the injection point. The steady-state therefore need not to be reached for sample collection. (5) Using valves for sample introduction even allows the collection of non-ionic compounds, as they remain in the valve during the isotachophoretic separation.

EXPERIMENTAL

Isotachophoresis

Separations were performed on an LKB Tachophor isotachophoretic analyzer (LKB, Bromma, Sweden) at 60 μA (stabilized current, end voltage 9 kV), in a 0.4 mm I.D. PTFE capillary instead of the original separating capillary plate. Test runs were also done in home-made equipment [3] using both UV and conductivity detection.

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Amaranth red and fluorescein were used as coloured markers in the initial experiments. Hard-cutting of the zone train migrating between amaranth red and fluorescein or terminator was done by means of a razor blade [4].

In the preparation runs, 1 μl of uremic serum ultrafiltrate was injected into the isotachophoretic analyzer. The volume collected by hard-cutting (5 μl, 4 cm) was transferred to a conical microvial (Chrompack, Middelburg, The Netherlands) and injected into the liquid chromatograph using a 10-μl syringe (Glenco, Chrompack, Middelburg, The Netherlands).

So far no hard-cutting has been performed using a PTFE valve as described by Kenndler and Kaniansky [5].

Liquid chromatography

The equipment used consisted of two Model 100 A pumps, a Model 321 controller, and a Model 160 fixed-wavelength UV detector, all from Beckman (Berkeley, CA, U.S.A.). A 1-μl aliquot of serum ultrafiltrate was diluted to 5 μl and injected into the liquid chromatograph. Further experimental conditions for isotachophoresis and liquid chromatography are given in Table I.

In this way the same absolute amounts of (anionic) solutes in the serum with and without isotachophoretic preseparation are loaded on the HPLC column. Additional experimental conditions for isotachophoresis and liquid chromatography are given in Table I.

TABLE I

OPERATIONAL SYSTEM FOR ISOTACHOPHORETIC AND LIQUID CHROMATOGRAPHIC ANALYSES

Isotachophoresis		
	Electrolyte	
	Leading	Terminating
Anion	Chloride	HEPES*
Concentration	0.025 *M*	0.025 *M*
Counter-ion	Histidine	Sodium
pH	6	9.5
Solvent	H_2O	H_2O

Liquid chromatography	
Mobile phase	100% solvent I to 100% solvent II
Gradient	Within 30 min Solvent I: 0.05 *M* ammonium formate pH 4—methanol (95:5, v/v) Solvent II: methanol
Column	25 cm × 4.6 mm, stainless steel, packed with Polygosil-60, C18, 5-μm particles**
Detection	UV at 254 nm
Flow-rate	1 ml/min

*HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, sodium salt (Sigma, St. Louis, MO, U.S.A.).

**Polygosil (Machery-Nagel & Co., Düren, F.R.G.).

Uremic serum ultrafiltrate

Uremic serum ultrafiltrate was obtained from Prof. S. Ringoir, Department of Nephrology, University Hospital of Ghent, Belgium. The filtrate became available during a sequential ultrafiltration—hemodialysis artificial kidney treatment of a uremic patient. Large molecules such as proteins are rejected by the artificial kidney membrane (molecular weight cut-off 10,000) and consequently are not present in the serum ultrafiltrate.

RESULTS

Fig. 1 shows an isotachophoretic test run of a uremic serum ultrafiltrate sample. Test runs were performed on home-made equipment using both UV and conductivity detection. From these runs zone train lengths between amaranth red as frontal coloured marker and fluorescein, useful as a terminal marker, or terminator were simply determined. Therefore, in the isotachopherogram of Fig. 1 only amaranth red is present.

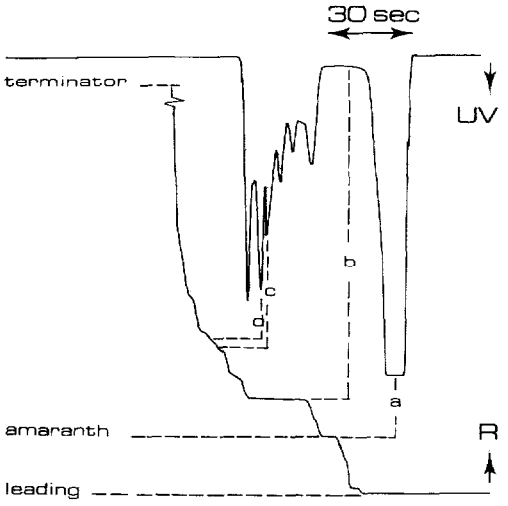

Fig. 1. Anionic separation of uremic serum ultrafiltrate (0.5 μl injected) by isotachophoresis (test run). UV absorption and conductivity detector traces are shown (R = resistance). a = Amaranth red; b = phosphate; c = hippurate; d = urate. Conditions are given in Table I.

Hard-cutting was done on the 0.4 mm I.D. capillary in the LKB apparatus, between amaranth red and the terminator, of which the position of the zone boundary was calculated from the test runs performed. Fluorescein was not used here to prevent interference in the HPLC analysis.

In Fig. 2 the HPLC profiles of uremic ultrafiltrate with and without anionic preseparation are compared. After anionic preseparation a number of peaks in the HPLC profiles have disappeared. These are either cationic or neutral constituents.

From the chromatographic retention data tentative peak assignments have been made for some major peaks, as given in the figure legend.

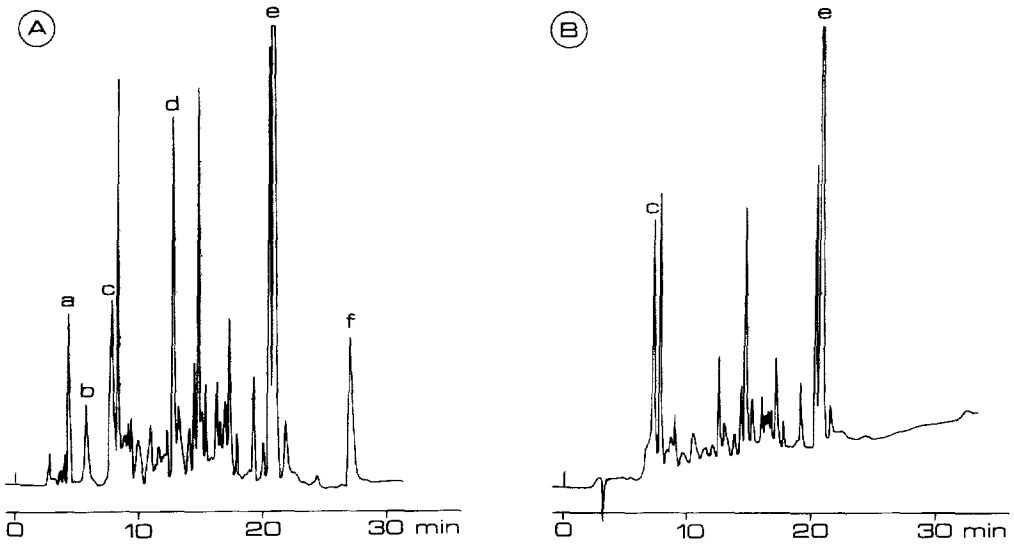

Fig. 2. HPLC profiles of uremic serum ultrafiltrate without (A) and with (B) anionic preseparation by isotachophoresis. Conditions are given in Table I. Tentative assignments: a = creatinine; b = uracil; c = uric acid; d = xanthine; e = hippuric acid; f = caffeine. These peaks have been identified by spiking with appropriate standards.

DISCUSSION

From the experiments it can be concluded that the combination of isotachophoresis and liquid chromatography can give valuable information about the identity or character of solutes in a complex diverse matrix such as biological fluids.

In this study proteins were not present in the samples, but they can be readily separated from the anionic or cationic low molecular weight solutes in the sample in the same isotachophoretic (pre)separation run [6], by choosing suitable operational conditions.

Combination of the selectivities of isotachophoresis and HPLC makes a powerful combination. In isotachophoresis a choice is made between anionic and cationic preseparation. Variation of the pH of the leading electrolyte influences the mobility of the different species, as they have different pK values. In HPLC selectivity can be influenced by the nature of both the mobile phase and the stationary phase in a most flexible way.

Direct transfer of the aqueous samples from isotachophoresis to HPLC imposes the use of reversed-phase liquid chromatography. However, isotachophoresis in non-aqueous media, which is at present being developed [7], will be compatible with normal-phase liquid chromatography as well. With some technical developments that are available or will be available in the near future [5, 8] it might be possible to select more discrete regions of the migrating zone train. These regions in capillary isotachophoresis necessarily represent small sample volumes (< 1 μl). Combination with microbore liquid-chromatography columns (e.g. 1 mm I.D.) therefore seems promising. As the chromatographic

dilution in the microbore columns (1 mm I.D.) is much less than in wide-bore columns (4.6 mm I.D.), the former will have a higher mass sensitivity by a factor of 20. This will be an advantage in those cases where only small sample volumes are available, because in wide-bore columns larger sample volumes can be injected.

The on-line coupling of the techniques of isotachophoresis and microbore liquid chromatography and microbore liquid chromatography and mass spectrometry are at present under investigation.

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