Journal of Chromatography, 320 (1985) 89–97
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,278

LARGE SAMPLE VOLUME PRESEPARATION FOR TRACE ANALYSIS IN ISOTACHOPHORESIS

VLADISLAV DOLNÍK, MIRKO DEML and PETR BOČEK*
Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, CS 611 42 Brno (Czechoslovakia)

SUMMARY

An isotachophoretic device for the analysis of trace components in a sample with the efficient pre-separation and elimination of bulk components has been suggested and realized. A large volume of the sample in question is separated in a rectangular wide-bore channel packed with a suspension of a granulated polyacrylamide gel and analyzed in free electrolytes in a narrow-bore tube. Trace components, the concentrations of which are 10^{-6} mol/l, can be analyzed in 60–70 min applying *ca.* 1 ml of the sample even in the presence of bulk excess of a major component the concentration of which is by 5 orders of magnitude higher.

INTRODUCTION

Isotachophoresis has already been successfully used in various fields especially in clinical chemistry for analyzing various compounds in body fluids. However, without any sample pretreatment the analyzed substance levels of 10^{-4} mol/l are reached when analyzing *ca.* 1–10 μl of sample, so that a relatively low number of compounds can be determined by direct isotachophoretic analysis of plasma or urine.

In isotachophoresis the efforts made analyse compounds present in low concentrations has led to the use of (i) selective detectors[1,2], (ii) low concentrations of leading electrolyte[3], (iii) small detection cells[4] and (iv) large sample volumes. The last approach seems to be the most promising because of its universality and flexibility, but results in another problem: interference by bulk components. To eliminate these disturbances, the minor components are separated from bulk components by applying a high electric charge[5].

Attempts to solve this problem have been based on (i) hydrodynamic counter-flow[6], (ii) concentration cascade[7] and (iii) enhancement of separation volume (*i.e.*, use of longer narrow-bore tubing or more advantageous column coupling[8] and volume coupling[9]). Of these procedures, column coupling is the most frequently used, however, only *ca.* 30 μl of sample can be analyzed with, of course, a prolonged analysis time.

This led us to develop an isotachophoretic device for the separation of large volume samples in a wide-bore channel packed with a suspension of granulated polyacrylamide gel, followed by detection in free electrolyte in a narrow-bore tube.

0021-9673/85/$03.30

THEORETICAL

Required volume of sample

A large number of clinically significant ionogenic substances occur in plasma and urine at concentrations of 10^{-4}–10^{-6} mol/l, *i.e.*, below the usual detection limit in isotachophoresis. That is why only few compounds in body fluids can be quantitated by isotachophoresis without any sample pretreatment. As *ca.* 10^{-9} mol of a sample component is required for reproducible isotachophoretic analysis, 1 ml of a sample must be injected for reliable determination of compounds at the 10^{-6} mol/l level.

Requirements of separation charge and column hold-up

The separation charge, Q_S, and column hold-up, Q_L, required for separation of weak ions can be calculated[5] from

$$Q_S = F \cdot \frac{N_A Z_A (u_A + u_R) + N_B Z_B (u_B + u_R)}{u_A Z_A - u_B Z_B} \quad (1)$$

$$Q_L = Q_S - N_A F [1 + (u_R/u_A)] \quad (2)$$

where N_A, N_B are the amounts of ions to be separated, Z_A, Z_B their effective charges in their mixed zone, u_A, u_B, u_R are the ionic mobilities of these ions and of the counter ion and F is the Faraday constant.

The requirements for separation of 1 ml of plasma can be estimated by calculating Q_L and Q_S for a pair consisting of the bulk component and one trace component in the sample components. The separation of creatinine and sodium in a leading electrolyte of 10 mmol/l potassium acetate + acetic acid and of chloride and pyruvate in a leading electrolyte of 10 mmol/l HCl + β-alanine were selected as models for estimation of the Q_S and Q_L values required for analyses of plasma cations and anions, respectively. Data used in those calculations and the calculated values of Q_L and Q_S are summarized in Table I. For both cationic and anionic analyses, a column hold-up of *ca.* 17 C is required.

TABLE I

REQUIRED SEPARATION CHARGE AND COLUMN HOLD-UP

Data used for calculations and calculated values.

Component	*Plasma concn.*[13] *(mol/l)*	$10^8 u^{14,15}$ *(m²/V · sec)*	Z^{14}	$10^8 u_R$[14] *(m²/V · sec)*	Q_S *(C)*	Q_L *(C)*
Sodium	0.14	5.19	1.0	4.24	41.5	17.0
Creatinine	10^{-4}	3.72	0.57			
Chloride	0.1	7.91	1.0	3.67	30.4	16.3
Pyruvate	10^{-4}	4.23	1.0			

Optimization of migration unit and minimization of analysis time

For the passage of a large electric charge through a separation channel in an acceptable time, a high driving current must be applied, using a high concentration leading electrolyte and/or wide-bore channel. The separated zones must be detected in a narrow-bore channel in order to maintain detection sensitivity, and that is why zones after separation in a wide-bore channel must be transferred to the detection capillary by migration through a tapered channel. However, the zones and their boundaries deteriorate during such migration and therefore an additional channel must be introduced behind each tapered channel in order to re-establish them.

The total analysis time is minimal if the sum of the times of all migration steps (*i.e.* separation, zone cross-section reduction, re-establishment of separated zones and detection) is minimal. In this case, the migration unit comprises a separation channel, a series of tapered channels and re-establishing channels and a detection capillary. The cross-sections of the individual channels, their shapes, cross-sectional ratios and the number of tapered channels can be optimized to achieve the minimum analysis time.

Selection of channel shape

To obtain short analysis times, the channel shape has to such as to enable efficient dissipation of generated Joule heat. For construction of migration channels, only two kinds of shape were considered from a technological point of view: rectangular and circular. Let us consider a circular channel with diameter r which is cooled across its whole circumference and a rectangular channel which is cooled only from side a, the cooling effect of the remaining sides $(a + 2b)$ being neglected. Both channels have equal cross-section, hold-up and thermal conductivity of the cooled wall. Let us neglect radial temperature profiles, so that the temperature at all points of the channel is the same. The minimum time, $t_{\text{min.}}$, for isotachophoretic migration through both channels is given by

$$t_{\text{min.}} = Q_{\text{L}}/\sqrt{\text{P}\kappa_{\text{T}}Sd} \tag{3}$$

where P is the maximum Joule heat which can be dissipated through unit area of the channel wall, d is the length of cooled channel perimeter, S is the cross-section of the channel and κ_{T} is the conductivity of the terminating electrolyte.

The ratio of the minimum times of migration in both channels can be expressed as:

$$\frac{t_{\text{min., circ.}}}{t_{\text{min., rect.}}} = \sqrt{\frac{d_{\text{rect.}}}{d_{\text{circ.}}}} = \sqrt{\frac{a}{2\pi r}} = \sqrt[4]{\frac{a}{4\pi b}} \tag{4}$$

In this model the times for isotachophoretic migration are the same in both types of channels, when $a/b = 4\pi$. At higher ratios the minimum time for migration is shorter in the rectangular channel. In real channels a rectangular shape is favoured because the presumptions made (neglect of radial temperature profiles and cooling effect of the remaining rectangular sides) are more advantageous for a circular shape. For these reasons a rectangular shape of separation channel has been preferred.

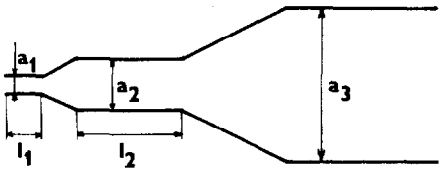

Fig. 1. Schematic arrangement of tapered and re-establishing channels. a_1, a_2 = The widths of the re-establishing channels; a_3 = the width of the separation channel; l_1, l_2 = the lengths of the re-establishing channels.

Determination of parameters of migration unit

As shown earlier the minimum analysis time is achieved when the sum of the times of the individual steps is minimal. A schematic diagram of a migration unit is shown in Fig. 1. It consists of one separation channel and two pairs of tapered channels and re-establishing channels. The ratio, K, of the inlet cross-section to the outlet cross-section is the same for all tapered channels. It is equal to the width ratio in rectangular channels and also to the length ratio of the re-establishing channels:

$$K = \frac{a_2}{a_1} = \frac{a_3}{a_2} = \frac{l_2}{l_1} \tag{5}$$

The minimum time for isotachophoretic migration through the last pair of tapered and re-establishing channels is given by

$$t_{1,\,\mathrm{min.}} = \frac{bFc_\mathrm{L}}{I_{1,\,\mathrm{max.}}} (a_1 l_1 + a_1^2 A) \left(1 + \frac{u_\mathrm{R}}{u_\mathrm{L}}\right) \tag{6}$$

where c_L is the concentration of the leading ion, u_L, u_R are the ionic mobilities of the leading ion and counter ion, b is the height of the channel and A is a parameter which depends on the ratio K and on the shape of the reduction channels (volume of tapered channel = $a^2 b\, A$).

The minimum time for isotachophoretic migration in the ith pair of channels can be expressed as:

$$t_{\mathrm{i,\,min.}} = K^{(i-1)} t_{1,\,\mathrm{min.}} \tag{7}$$

The minimum time for isotachophoretic migration through a migration unit consisting of a separation channel and n pairs of tapered and re-establishing channels is given by:

$$t_{\mathrm{total,\,min.}} = \frac{1}{I_{1,\,\mathrm{max.}}} \left[\frac{Q_\mathrm{S}}{K^n} + (a_1 l_1 + a_1^2 A) \left(1 + \frac{u_\mathrm{R}}{u_\mathrm{L}}\right) bFc_\mathrm{L} \cdot \sum_{i=1}^{n} K^{(i-1)}\right] \tag{8}$$

The required length of the last re-establishing channel can be calculated from

$$l_1 = \frac{Q_{\mathrm{mix.}} u_\mathrm{L} u_\mathrm{D}}{a_1 b c_\mathrm{L} F (u_\mathrm{L} + u_\mathrm{R})(u_\mathrm{D} - u_\mathrm{E})} \tag{9}$$

where D, E are strong electrolyte ions which form the largest mixed zone during their migration through the tapered channel, $Q_{mix.}$ is the charge of this mixed zone and u_D, u_E are the ionic mobilities of ions D, E. $Q_{mix.}$ and its dependence on the ratio K can be obtained by recording the detector signal at the end of tapered channels having different K values.

Combining eqns. 8 and 9 we obtain:

$$t_{total,\ min.} = \frac{1}{I_{1,\ max.}} \left\{ \frac{Q_S}{K^n} + \sum_{i=1}^{n} K^{(i-1)} \left[Q_{mix.} \frac{u_D}{u_D - u_E} + a_1^2 b c_L F A \left(1 + \frac{u_R}{u_L} \right) \right] \right\} \quad (10)$$

The equation can be used directly for optimization of the migration unit. Based on preliminary experiments phosphate and lactate have been selected as the model pair of ions D, E. The dependence of their $Q_{mix.}$ on the ratio K has been determined by measuring the electric charge required for passage of the mixed zone through the boundary between the tapered and re-establishing channels using tapered channels having different values of the ratio K. For our purposes, a simple plot of log $Q_{mix.}$ *vs.* K was sufficient (Fig. 2), and can be expressed by:

$$Q = 0.00485 \cdot 10^{0.024K} - 0.00513$$

Using the minimum dimensions of a_1 and b, the minimum analysis time has been found for a migration unit with three tapered channels and a width ratio $K = 4$. However, in real experiments, disconnections and connections of the power supply take time and therefore only one tapered channel with a width ratio $K = 20$ has been used in a compartment of the rectangular bore.

Hydrophilic gels have been suggested for stabilization of migrating zones in separation channels.

Requirements for electrolyte purity

The limiting concentration of impurity in the leading electrolyte, $c_{imp.,\ lim.}$, at

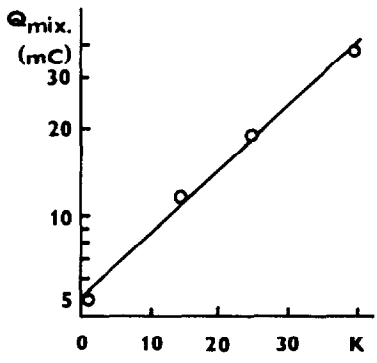

Fig. 2. Dependence of mixed zone charge, $Q_{mix.}$, on the ratio K. For explanation see text.

which the impurity is just detected can be calculated from eqn. 11 obtained by modifying eqn. 1

$$c_{\text{imp., lim.}} = \frac{c_L Z_L(u_L + u_R)}{\frac{Q_S(Z_L u_L - Z_{\text{imp.}} u_{\text{imp.}})}{FN_{\text{imp.}}} - Z_{\text{imp.}}(u_{\text{imp.}} + u_R)} \tag{11}$$

where the subscript L refers to the leading ion and imp. to impurity, $N_{\text{imp.}}$ is the amount of impurity corresponding to its detection limit (10^{-10} mol taken in calculations) and the other symbols have the same meanings as in eqn. 1.

The calculated limiting concentrations of impurities in the leading electrolytes (a) 10 mmol/l HCl + β-alanine, pH 3.3 and (b) 10 mmol/l potassium acetate + acetic acid, pH 4.7 are shown in Fig. 3 for some real examples. Obviously, the concentration of impurities in the leading electrolyte should be kept under 3–10 nmol/l.

The relative admissible content of impurities in chemicals used for the preparation of leading electrolytes can also be determined in this manner.

The described procedure can also be applied to the determination of purity requirements in terminating electrolytes. However, in this case it is necessary to take account of the volume of the terminating electrolyte and of the difference between the pH of the terminating electrolyte and of the adjusted zone of the terminating ion.

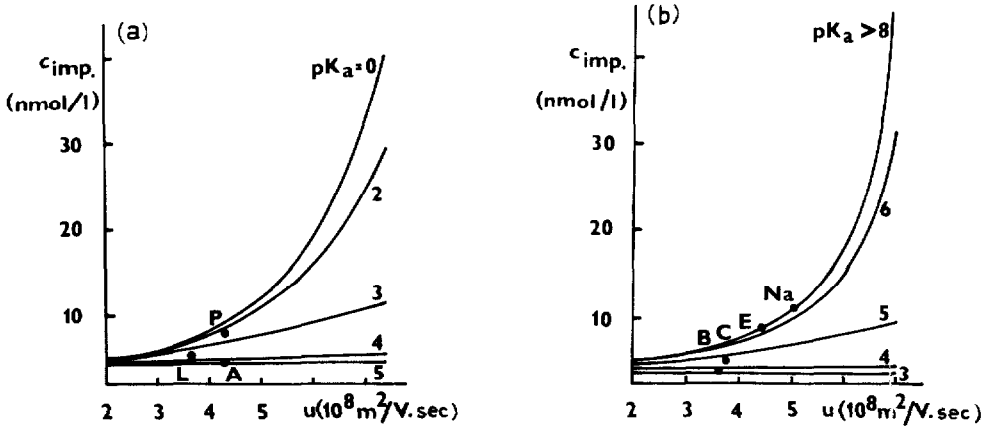

Fig. 3. Dependence of the limiting concentration of impurity in the leading electrolyte on pK_a and the ionic mobility of the impurity. Leading electrolytes: a, 10 mmol/l HCl + β-alanine, pH 3.3; b, 10 mmol/l potassium acetate + acetic acid, pH 4.7. A = Acetate; L = lactate; P = pyruvate; B = β-alanine; C = creatinine; E = ethanolamine.

EXPERIMENTAL

All chemicals were supplied by Lachema (Brno, Czechoslovakia) except for Cyanogum 41 and γ-aminobutyric acid (Serva, Heidelberg, F.R.G.).

The synthetic resin ChS Polyester 104 (Spolek pro chemickou a hutní výrobu, Ústí nad Labem, Czechoslovakia) was used for preparation of the migration unit block.

Granulated polyacrylamide gel for stabilization of zones in the separation channel was prepared as follows: 5% Cyanogum 41 and 1% triethanolamine were dissolved in water and impurities were removed by filtration. 1% ammonium persulphate was added and after its dissolution the solution was overlayered by parafffin oil and left for *ca.* 60 min to polymerize. After complete polymerization the gel was forced through a sieve (mesh dimension 0.2 mm). Very small particles of gel were removed by washing on a sieve (mesh dimension 0.1 mm) and by repeated decantation. The remaining polyacrylamide gel was washed with distilled water and deionized water until the conductivity of the gel suspension was *ca.* 10^{-4} S m^{-1}. Then chemicals used for preparation of the leading electrolyte were added to the required concentration. This procedure was also applied for regeneration of gels.

The isotachophoretic migration unit[10] developed on the basis of the theoretical considerations described above is shown in Fig. 4. The migration unit consists of a metal cooling block and a synthetic resin block equipped with electrode chambers and valves. The migration channels are placed inside the resin block. The terminating electrode chamber is connected to the separation channel via a four-way tap. Buffer volumes contained in auxiliary electrode chambers are connected to the tapered channels via cylindrical valves. When closed, the cores of these valves create a channel wall so that no dead-volumes are formed. The channels are filled with gel suspension by a syringe via the buffer volumes and cylindrical valves. The separation channel is rectangular with cross-section 20 × 1.3 mm, and the re-establishing channel is also rectangular, with cross-section 1 × 1.3 mm. The detection capillary is a circular tube (0.2 mm I.D.). The distance between the rectangular channels and metal cooling block is 0.2 mm.

Prior to the analysis the detection capillary is filled with leading electrolyte, and the remaining channels are filled with a suspension of granulated polyacrylamide in the leading electrolyte. The sample valve is filled with a sample, and the electrode chambers with the corresponding electrolytes. The first cylindrical valve is opened, and the second one is closed.

To start a separation, the power supply is connected to the first auxiliary elec-

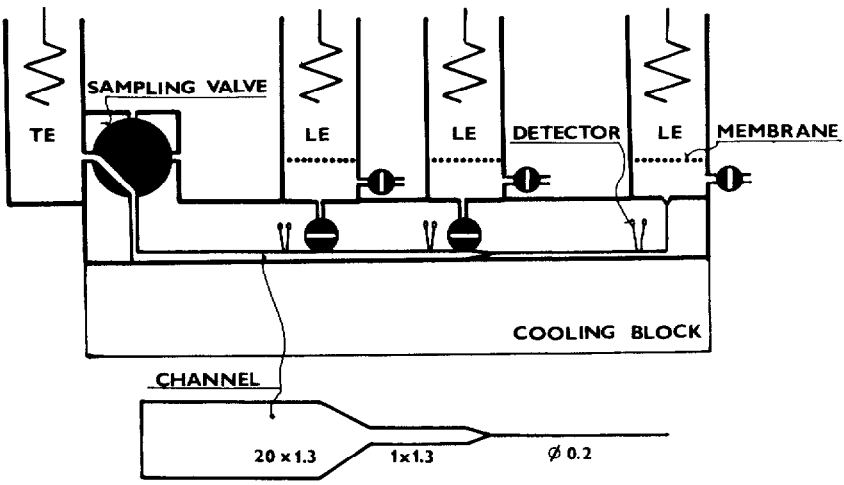

Fig. 4. Diagram of migration unit. TE = Terminating elecrolyte; LE = leading electrolyte.

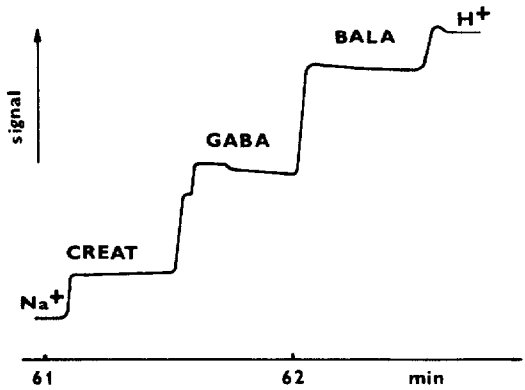

Fig. 5. Analysis of minor components in 1.2 ml of a model mixture containing 0.14 mol/l Na^+ and 10^{-6} mol/l of creatinine (CREAT), γ-aminobutyric acid (GABA), and β-alanine (BALA). Leading electrolyte: 10 mmol/l potassium acetate. Terminating electrolyte: 100 mmol/l acetic acid. Driving current in analytical step: 20 μA. A potential gradient detector was used to record the analysis.

trode and to the terminating electrode, and maintained until the first zone of interest reaches the first detector. Then the electric circuit is disconnected, the first cylindrical valve is closed, the second one is opened and the second step is started by connecting the power supply to the terminating electrode and to the second auxiliary electrode. After recording the first zone on the second detector, the electric circuit is disconnected again, the second cylindrical valve is closed and analysis is completed in the usual way by recording the signal of the analytical detector.

RESULTS AND DISCUSSION

The stabilization effect of polyacrylamide suspension was checked visually by migrating small quantities of dyes [4,5-dihydroxy-3-(*p*-sulphophenylazo)-2,7-naphthalenedisulphonic acid in anionic electrolyte systems, ferroin in cationic electrolyte systems]. The dyes migrated as sharp and symmetrical zones throughout the bore of the separation channel.

With respect to the small bore of the detection capillary, the gel suspension could not be used in it. The capillary was filled with a free electrolyte solution. A migrating concentration boundary, which can be formed at the boundary between the gel and free electrolyte during analysis[11], was not observed.

The analytical possibilities of the device described were demonstrated by analyzing 1.2 ml of a model mixture containing 0.14 mol/l Na^+ and 10^{-6} mol/l β-alanine, γ-aminobutyric acid and creatinine (Fig. 5). Using a driving current of 15–5 mA in the separation step, 1 mA in the second step and 20 μA in the detection step, the analysis was complete after 60–70 min, when the bulk of the sodium was removed from the separation channel by migration to the first auxiliary electrode. Minor components were separated and quantitated without any problems.

The theoretical detection limit calculated for a detection cell of comparable size, 30 pmol[12], represents the minimum detectable concentration of 25 nmol/l substance in a sample in our apparatus using an universal detector.

The major advantage of separation in a wide-bore channel packed with gel

suspension together with detection in a narrow-bore tube in free electrolyte is the potential for further reduction of the detection limit by applying larger and larger volumes of sample, if disposable. However, the strict requirements of electrolyte purity even more crucial in this case. Probably this factor will limit further decreases in the detection limit.

REFERENCES

1 L. Arlinger and R. Routs, *Sci. Tools,* 17 (1970) 21.
2 J. C. Reijenga, Th. P. E. M. Verheggen and F. M. Everaerts, *J. Chromatogr.*, 283 (1984) 99.
3 L. Arlinger, *J. Chromatogr.*, 91 (1974) 785.
4 Th. P. E. M. Verheggen, F. E. P. Mikkers and F. M. Everaerts, *J. Chromatogr.*, 132 (1975) 205.
5 P. Boček, M. Deml, B. Kaplanová and J. Janák, *J. Chromatogr.*, 160 (1978) 1.
6 F. M. Everaerts, J. Vacík, Th. P. E. M. Verheggen and J. Zuska, *J. Chromatogr.*, 49 (1970) 262.
7 P. Boček, M. Deml and J. Janák, *J. Chromatogr.*, 156 (1978) 323.
8 F. M. Everaerts, Th. P. E. M. Verheggen and F. E. P. Mikkers, *J. Chromatogr.*, 169 (1979) 21.
9 Th. P. E. M. Verheggen and F. M. Everaerts, *J. Chromatogr.*, 249 (1982) 221.
10 V. Dolník, M. Deml and P. Boček, *Czech. Pat., Pending,* PV 5782-84.
11 M. Spencer, *Electrophoresis,* 4 (1983) 36.
12 J. C. Reijenga, Th. P. E. M. Verheggen and F. M. Everaerts, *J. Chromatogr.*, 245 (1982) 120.
13 *Clinical Laboratory*, Merck, Darmstadt, 1974.
14 T. Hirokawa, M. Nishino, N. Aoki, Y. Kiso, Y. Sawamoto, T. Yagi and J. Akiyama, *J. Chromatogr.*, 271 (1983) D 1.
15 W. J. Moore, *Physical Chemistry,* Prentice Hall, Englewood Cliffs, 1972.