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Isotachophoretic separation of organic acids in biological fluids

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ABSTRACT

The operating conditions for the isotachophoretic separation of organic acids were evaluated. At pH values ranging from 2.90 to 4.25 both relative step heights and molar flow-rates were determined experimentally for 26 anions. Comparing the observed values with simulated data, highly significant ($p = 0.0001$) correlation coefficients of 0.993 and 0.920, respectively, were found at pH 3.50. Whereas the concentration of the leading electrolyte did not affect the relative step heights, it increased the molar flow-rates significantly. The same applied to the detection current. The time of analysis was observed to be a function of the concentration of the leading electrolyte. However, the time elapsed between injection of the analyte and its detection depended solely on the volume and not on the amount of analyte injected.

In isotachophoresis, incomplete separation of two compounds is indicated by the occurrence of a mixed zone which can hardly be distinguished from a pure zone. Thus, knowledge of the separation capacity is a prime prerequisite in optimizing the system for the analysis of biological fluids. The separability of nine equimolar pairs of anions was determined at pH values ranging from 2.90 to 4.25. Although two ionogenic constituents would separate only when their migration rates in the mixed state were different, no clear correlation was observed between separation capacity and difference in relative mobility. Separability, however, was found to increase with increasing concentration of the leading electrolyte. While the separation capacity was not influenced by the electric current, it was significantly affected by the volume injected. In subsequent analyses of serum, cerebrospinal fluid, seminal plasma and prostatic fluid, a variety of organic acids could be detected. Calibration graphs for the detected anions revealed a detection limit of 1 nmol and linearity over their biological concentration ranges. Further, the isotachophoretic results correlated well with high-performance liquid chromatographic and enzymatic analyses of citric acid and lactic acid in human seminal plasma and cerebrospinal fluid, respectively.

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INTRODUCTION

The usually small volumes of human body fluids, such as seminal plasma and prostatic fluid, have always hampered their investigation. Although the analysis of pooled samples proved useful in the basic determination of the chemical composition of biological fluids, such a procedure did not fulfil the requirements for the evaluation of the biochemical nature of diseases in the individual patient, research usually being confined to the analysis of single known substances. In recent years, the call for analytical methods that can provide sensitive and selective information on a broad range of compounds has resulted in the development of new techniques that permit the separation, identification, quantification and even isolation of a great variety of solutes contained in biological fluids.

Regarding the analysis of organic acids, various techniques are available. Isotachophoresis is certainly a powerful method, as it meets the basic requirements for screening procedures, namely multi-component information, rapid completion, reliability and low cost. The separation of organic acids by capillary isotachophoresis has been the topic of many theoretical and experimental papers in the last 15 years. The main reason for the extensive theoretical evaluation may be attributed to the large knowledge of dissociation constants of organic acids in aqueous solution[1–3]. Most publications dealing with the experimental evaluation of capillary isotachophoresis in the analysis of organic acids deal with their determination in food, microbiological samples, tissue and cell extracts, enzyme assays and in samples obtained during the hydrolytic degradation or oxidation of saccharides. Regarding human body fluids, most investigations have been restricted to the analysis of one specific organic acid, such as oxalic acid in urine[4–10] or trifluoroacetic acid, which is a major metabolite of the anaesthetic halothane, in urine and serum[11,12]. Only a limited number of papers have been published on the use of capillary isotachophoresis in obtaining profiles of organic acids in various body fluids[13–20].

In this study we investigated the experimental conditions for the determination of organic acids by means of capillary isotachophoresis, such as the influence of the pH and the concentration of the leading electrolyte on relative mobility, molar flow-rate and separation capacity. These data were then applied to the determination of organic acids in various biological fluids.

EXPERIMENTAL

Sample collection and pretreatment

Cerebrospinal fluid specimens were obtained by lumbar puncture. No pretreatment was required. Blood samples were taken from the cubital vein and deproteinized immediately by ultrafiltration, using the disposable micropartition system Centrisart I (Sartorius, Göttingen, F.R.G.), which was filled with small glass beads to avoid coagulation. Volumes of 200–300 μl of ultrafiltrate were obtained from 2 ml of blood centrifuged with a swing-head rotor at 2000 *g* for 15 min at 4°C. In order to avoid both haemolysis and build-up of blood cells at the membrane surface, which would restrict the solvent flow considerably, blood samples were precentrifuged for about 2–3 min before inserting the floater with the membrane. Spontaneously liquefied semen was centrifuged at 1000 *g* for 10 min to remove spermatozoa. The samples were then

deproteinized by ultrafiltration, for which the disposable micropartition system Centrifree (Amicon, Danvers, MA, U.S.A.) was used. Volumes of 100–200 μl of ultrafiltrate were obtained from 400–500 μl of seminal plasma centrifuged in a fixed-angle rotor (25°) at 1500 *g* for 15 min at 4°C. Prostatic fluid was collected on routine rectal examination. Volumes of 20–100 μl of prostatic fluid could be obtained per patient. The samples were diluted 1:2 or 1:5 with doubly distilled water prior to ultrafiltration according to the conditions used for deproteinization of seminal plasma. All samples were stored at −30°C prior to their analysis.

Isotachophoretic conditions

Isotachophoretic analyses were carried out on an LKB (Bromma, Sweden) Model 2127 Tachophor equipped with a 250-mm Teflon capillary of I.D. 0.5 mm.

The leading electrolyte was 10 m*M* hydrochloric acid (Merck, Darmstadt, F.R.G.) adjusted to pH values ranging from 2.90 to 4.25 by the addition of β-alanine (Merck). Triton X-100 (Serva, Heidelberg, F.R.G.) was added to the leader to sharpen the zone boundaries by depressing electroendosmosis. The terminating electrolyte was 10 m*M* propionic acid (Sigma, St. Louis, MO, U.S.A.).

The samples were injected through the inlet membrane into the leading electrolyte by means of 5- and 10-μl Hamilton syringes (Hamilton, Bonaduz, Switzerland). Separations were started at a current of 200–225 μA, which was gradually reduced to 25–125 μA shortly before the separated anions could be detected on account of their conductivity. All analyses were carried out at 20°C.

High-performance liquid chromatographic (HPLC) determination of citric acid

The HPLC system, which was used for the determination of citric acid in human seminal plasma, consisted of a Model 112 pump (Beckman, Berkeley, CA, U.S.A.), a sample injection valve (Beckman Model 210) with a 20-μl loop, a Shimadzu (Kyoto, Japan) CTO-2A column oven unit, a differential refractive index detector (Altex, Berkeley, CA, U.S.A.) and a Shimadzu C-R2A-X integration system.

As stationary phase a pre-packed Aminex HPX-87H strong cation-exchange resin column (8% cross-linked, 300 × 7.8 mm I.D.) (Bio-Rad Labs., Richmond, CA, U.S.A.), fitted with an ion-exclusion micro-guard refill cartridge (Bio-Rad Labs.), was used. The eluent was 0.01 *M* sulphuric acid. The column temperature was maintained at 40°C. The flow-rate was adjusted to 0.6 ml/min[21,22].

Enzymatic determination of lactate

The L-lactate test provided by Boehringer (Boehringer, Mannheim, F.R.G.) was used for the enzymatic determination of lactate in human cerebrospinal fluid.

Colorimetric determination of inorganic phosphate

The inorganic phosphate test provided by Merck (Merckotest No. 3331) was used for the colorimetric determination of inorganic phosphate in human seminal plasma.

RESULTS

The relative step heights of 26 anions (Table I) in relation to propionic acid, which served as the terminating electrolyte, were obtained at seven pH values ranging

TABLE I

ZONE IDENTIFICATION OF THE INVESTIGATED COMPOUNDS AND THEIR p*K* VALUES[1–3]

Zone No.	*Compound*	pK_1	pK_2	pK_3	pK_4
1	Pyrophosphate	0.85[a]	1.49[a]	5.77[a]	8.22[a]
2	2,3-Diphosphoglyceric acid				
3	Oxalic acid	1.27[b]	4.26[b]		
4	Maleic acid	1.97[c]	6.24[c]		
5	Pyruvic acid	2.49[c]			
6	Adenosine 5'-triphosphate				
7	L-Cysteic acid	1.89[c]	8.70[c]		
8	Phosphoric acid	2.12[c]	7.21[c]	12.67[c]	
9	2-Ketoglutaric acid	2.60[c]			
10	Fumaric acid	3.02[c]	4.38[c]		
11	L(+)-Tartaric acid	3.03[c]	4.37[c]		
12	Glyoxylic acid	3.34[c]			
13	Citric acid	3.14[b]	4.77[b]	6.39[b]	
14	Malic acid	3.40[c]	5.14[c]		
15	Acetoacetic acid	3.61[a]			
16	Glycolic acid	3.83[c]			
17	L(+)-Lactic acid	3.86[b]			
18	2-Hydroxybutyric acid	3.98[c]			
19	Hippuric acid	3.80[c]			
20	L-Aspartic acid	2.05[c]	3.87[c]	10.00[c]	
21	Succinic acid	4.22[b]	5.64[b]		
22	Benzoic acid	4.20[b]			
23	L-Ascorbic acid	4.17[b]	11.56[b]		
24	L-Glutamic acid	2.10[c]	4.07[c]	9.47[c]	
25	3-Hydroxybutyric acid	4.70[c]			
26	Acetic acid	4.76[b]			

[a] 18°C.
[b] 20°C.
[c] 25°C.

from 2.90 to 4.25 by injecting 15, 30 and 50 nmol of each anion. The observed relative step heights could be reproduced with intra- and inter-assay precisions of 0.37% and 3.84% R.S.D. (n = 6), respectively. Polynominal regression analysis was used to generate the curve fits through the data points. The relative step heights of almost all compounds increased gradually with increasing pH of the leading electrolyte, as shown in Fig. 1. However, as the change in mobility is not uniform, the analytical system may be adjusted to allow optimum separation of the organic acids of interest.

Molar flow-rates were evaluated under the same conditions as the relative step heights (Fig. 1). It is evident that the pH of the leading electrolyte does not exert a significant effect on the molar flow-rates for more than half of the investigated compounds within the pH range 3.3–4.0, indicating, that minor changes in pH will not influence the quantitative results. This is confirmed by the intra- and inter-assay precisions of 0.37% and 1.48% R.S.D. (n = 5), respectively.

Whereas the concentration of the leading electrolyte does not affect the relative step heights of various anions, the molar flow-rates were found to increase significantly with increasing concentration of hydrochloric acid in the leading electrolyte system

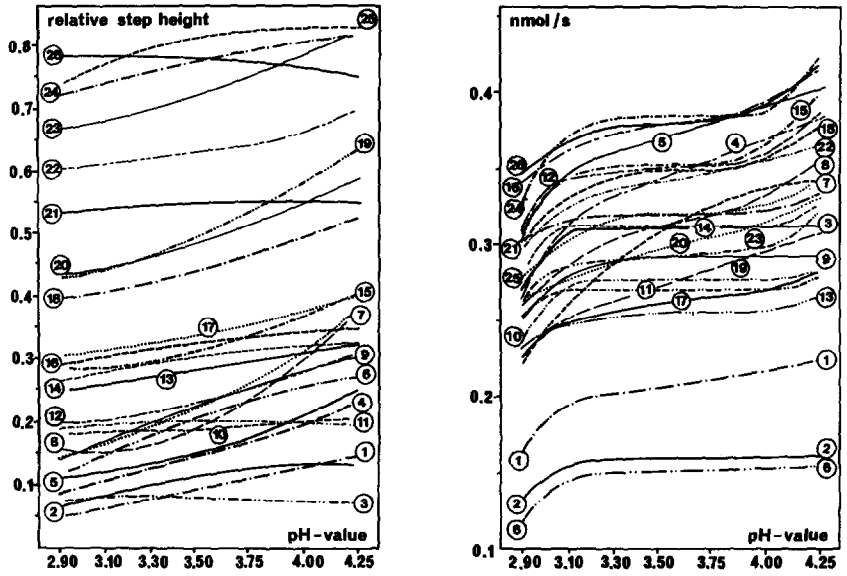


Fig. 1. Reltive step heights (propionic acid = 1.0) and molar flow-rates (nmol/s) of 26 anions at pH values ranging from 2.90 to 4.25. Leading electrolyte: 10 m*M* HCl–β-alanine–0.1% Triton X-100. Terminating electrolyte: 10 m*M* propionic acid. For identification, see Table I.

(Fig. 2). As regards the driving current, there is a direct proportionality between molar flow-rate and the current fed through the system. The relative step heights, however, are not affected by the electric current.

From the results shown in Fig. 3, it is evident that the time elapsed between the injection of the analyte and its detection depends solely on the injection volume and not on the amount injected.

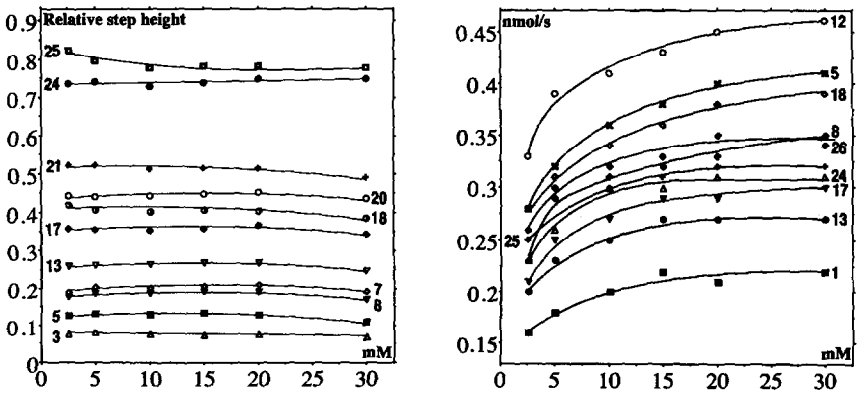


Fig. 2. Impact of the concentration of hydrochloric acid in the leading electrolyte system on relative step heights and molar flow-rates of different anions at pH 3.50. The concentration of the terminating electrolyte was the same as that of hydrochloric acid. Current: 75 μA. For identification, see Table I.

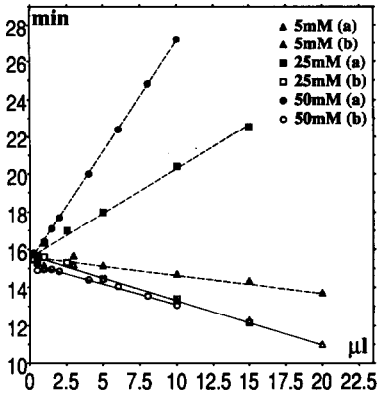


Fig. 3. Impact of injection volume and concentration of malonic acid on time of analysis: a = overall time of analysis; b = time elapsed between injection of the analyte and its detection. Leading electrolyte: 10 m*M* HCl–β-alanine–0.1% Triton X-100 (pH 3.50). Terminating electrolyte: 10 m*M* propionic acid. Current: 125 µA.

In isotachophoresis, incomplete separation of two compounds is indicated by the occurrence of a mixed zone which can hardly be distinguished from a zone containing only one constituent (see Fig. 10b).

The data in Fig. 4 show the maximum separable amounts of nine equimolar pairs of anions within the pH range 2.90–4.25. As preliminary experiments had indicated that the separation capacity might vary with time, probably owing to changes in the composition of the leading electrolyte, determinations were carried out on five consecutive days for every pH value. This period was chosen because under routine conditions both leading and terminating electrolyte solutions would be prepared only once a week in amounts sufficient to allow the continuous analysis of samples from Monday to Friday. Triton X-100, the addition of which had been observed to make

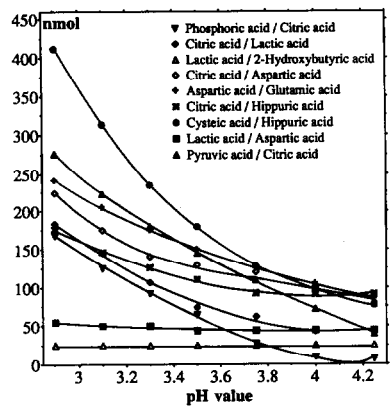


Fig. 4. Impact of the pH of the leading electrolyte on the separability of nine equimolar pairs of anions. Leading electrolyte: 10 m*M* HCl–β-alanine–0.1% Triton X-100. Terminating electrolyte: 10 m*M* propionic acid. Current: 75 µA. The capillary tubing was 25 cm × 0.5 mm I.D.

solutions visibly turbid within a few days depending on the amount added, was dissolved in the leading electrolyte approximately 1 h before the first isotachophoretic run on day one.

As illustrated in Fig. 5, the data obtained for an equimolar mixture of phosphoric acid and citric acid also apply to a non-equimolar mixture. Additionally, a linear relationship was observed between the concentration of hydrochloric acid in the leading electrolyte system and the separation capacity.

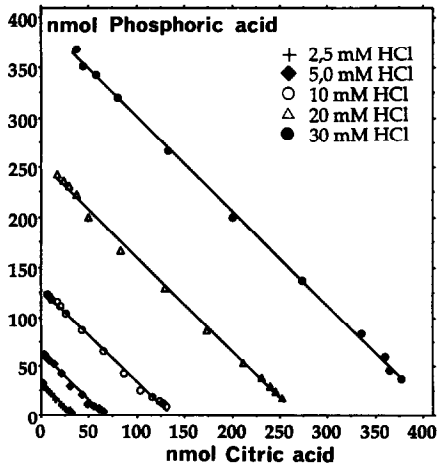


Fig. 5. Impact of the concentration of hydrochloric acid in the leading electrolyte system on the separability of phosphoric acid from citric acid, which were injected at different molar ratios. The concentration of the terminating electrolyte was the same as that of hydrochloric acid. pH of the leading electrolyte = 3.50. Current: 75 μA. The capillary tubing was 25 cm × 0.5 mm I.D.

Whereas the electric current chosen does not influence the separation capacity, the latter is affected by the sample volume injected. This was assessed by preparing mixtures of equal amounts of seven pairs of anions at concentrations of 5, 10, 20, 40, 60, 80 and 100 mM, respectively. The injected volumes of a certain concentration were then increased until mixed zones occurred and the maximum values defined the points in Fig. 6. The vertical axis denotes the maximum number of nanomoles of each of the sample ions which separated completely and the horizontal axis gives the total volume that was injected. It can be concluded that the injection volume should be as low as possible in order to avoid distortion and, thus, a reduced separation capacity by overloading.

Fig. 7a shows the isotachophoretic analysis of a sample of human seminal plasma that had been deproteinized by ultrafiltration prior to analysis. Phosphate, citric acid, lactic acid, aspartic acid and glutamic acid are the main constituents. Their identities were confirmed by the injection of an additional small amount of each compound, which resulted in an increase in the length of the respective zones. Further, the step heights were characteristic of the detected compounds.

Fig. 7b shows an isotachopherogram of ultrafiltered human prostatic fluid.

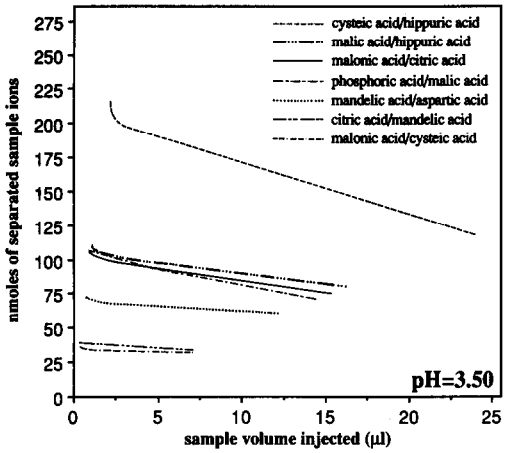


Fig. 6. Effect of sample volume injected on the separability of seven equimolar pairs of anions. Leading electrolyte: 10 m*M* HCl–*β*-alanine–0.2% Triton X-100 (pH 3.50). Terminating electrolyte: 10 m*M* propionic acid. Current: 75 *µ*A. The capillary tubing was 25 cm × 0.5 mm I.D.

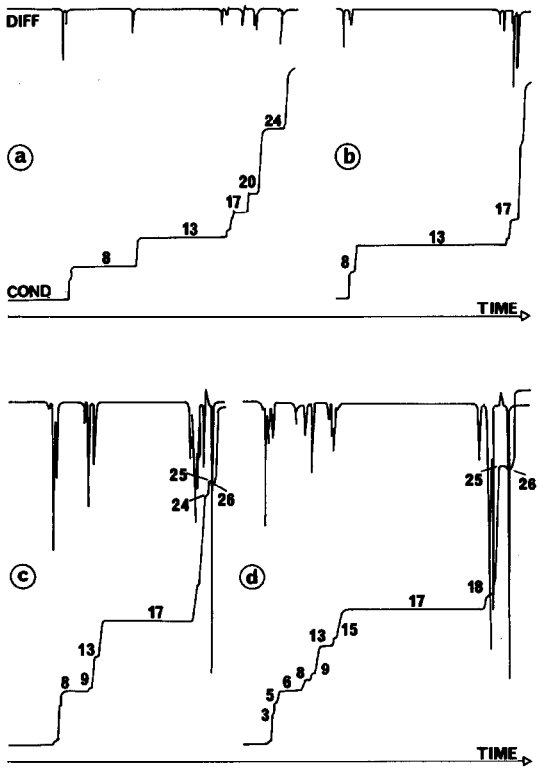


Fig. 7. Isotachophoretic analyses of (a) seminal plasma, (b) prostatic fluid, (c) serum and (d) cerebrospinal fluid. Leading electrolyte: 10 m*M* HCl–*β*-alanine–0.2% Triton X-100 (pH 3.30). Terminating electrolyte: 10 m*M* propionic acid. The capillary tubing was 25 cm × 0.5 mm I.D. Detection: conductivity (COND) and differential conductivity (DIFF). Current: 75 *µ*A. Chart speed: 0.5 mm/s. Injection volumes: 2.5, 5, 10 and 10 *µ*l, respectively. For zone identification, see Table I.

Citric acid is the main constituent, with concentrations ranging from 53 to 126 m*M*. In contrast to seminal plasma, only small amounts of phosphate could be detected. Based on the investigation of semen samples obtained from vasectomized patients, it can be concluded that phosphate is mainly derived from the seminal vesicles.

Phosphate, 2-ketoglutaric acid, citric acid, lactic acid, glutamic acid, 3-hydroxybutyric acid and acetic acid were detected routinely in ultrafiltered samples of serum (Fig. 7c). The isotachopherogram of cerebrospinal fluid shown in Fig. 7d reflects the significantly increased metabolic activity of cerebral tissue in a patient who had just experienced an epileptic seizure. As organic acids diffuse only slowly across the blood–brain barrier as long as it is intact, the prompt determination of organic acids in cerebrospinal fluid and blood is of great value in assessing the cerebral acid–base status and the integrity of the blood–brain barrier.

Quantitative information from isotachophoretic analyses was obtained by measuring the zone lengths. The calibration graphs for the detected anions revealed a detection limit of *ca.* 1 nmol and linearity over their biological concentration ranges. The reproducibility of the measurements was checked by at least seven repeated determinations for which different samples of biological fluids were used. A precision of 1.5% was obtained, the main errors resulting from the injection of the samples and the measurements of the zone lengths.

A linear relationship was found between isotachophoretic and HPLC determinations of citric acid in ultrafiltered human seminal plasma ($r = 0.998, p = 0.0001$). A comparison of the enzymatic assay of lactic acid in cerebrospinal fluid and the isotachophoretic results also showed a good correlation ($r = 0.997, p = 0.0001$). For phosphate, however, the zone length seen with seminal plasma was about three to five times greater than that expected based on colorimetric analyses of inorganic phosphate. This suggested that the phosphate zone contained additional components which had about the same mobility. No conditions could be found that caused a further separation of this zone, but several phosphorus-containing compounds commonly occurring in biological materials were observed to migrate within the respective zone.

DISCUSSION

Owing to their further metabolization, several organic acids are known to be unstable in blood and to a lesser extent in other body fluids after their collection. Therefore, immediate inhibition of enzyme activities is a prime prerequisite for accurate analyses. This is accomplished either by the addition of enzyme inhibitors, such as sodium fluoride, or through protein-precipitating agents, such as organic solvents and anions. However, both fluoride and anionic precipitants, such as perchloric acid, trichloroacetic acid and metaphosphoric acid, interfere with the isotachophoretic determination of organic acids, as they exhibit similar migration behaviours to the compounds under investigation. Moreover, low-molecular-mass constituents of the sample may be trapped in the precipitate. With blood, the addition of the anticoagulants most often used in clinical laboratories, such as citrate, oxalate and heparin, again results in a large increase in the time of analysis and in a considerable reduction in separation capacity. Further, these compounds may also mask constituents of the sample under investigation. Recently, ultrafiltration has been

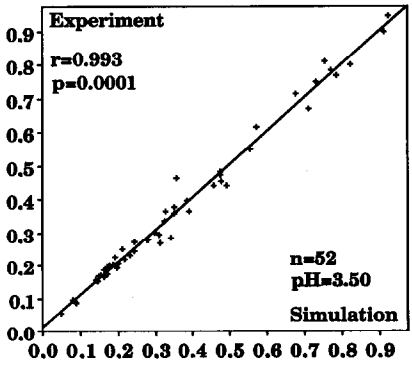
established to be a reliable and convenient method for the preparation of protein-free samples[18–20,22,23]. As the concentration of the sample solutes remains constant and none of the ultrafilterable constituents are partially or totally coprecipitated, deproteinization can be accomplished within a few minutes with an efficiency of more than 99.5%[24]. Moreover, organic acid have been shown to be stable in protein-free ultrafiltrates[25].

For several years, computer simulation of isotachophoretic equilibria has been used for the determination of qualitative and quantitative indices and as for the establishment of optimum separation conditions for organic acids[26–29]. Therefore, it was considered of interest to compare the observed values of relative mobililty and time-based zone length with simulated data. For this purpose, the indices evaluated by Hirokawa *et al.*[29] for 287 anionic substances in the pH range 3.0–10.0 were used. Prior to simple regression analysis, the R_E values, which define the ratio of the potential gradient of the sample zone to that of the leading zone, had to be converted into the indices given in this paper using the R_E values of the sample, $R_E(S)$, and terminating ion, $R_E(T)$:

$$\text{relative step height} = [R_E(S) - 1]/[R_E(T) - 1]$$

Highly significant ($p = 0.0001$) correlation coefficients of 0.993 and 0.920 were found between observed and simulated values of relative step height and molar flow-rate at pH 3.50, respectively (Figs. 8 and 9).

For the identification of unknown sample zones, the addition of standards has usually been employed. However, this may prove cumbersome if possible candidates are not known. Based on data in this paper and published by Hirokawa *et al.*[29], qualitative identification may also be achieved by comparing the observed and simulated values. At least the possible candidates for the components detected in



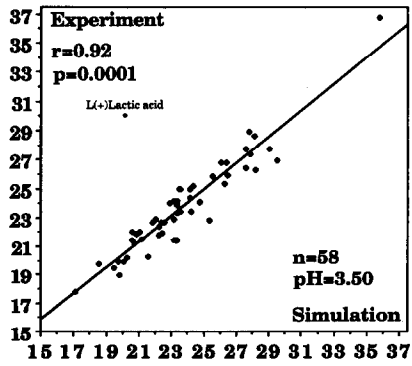


Fig. 8. Correlation between simulated and experimentally obtained values for the relative step heights of 52 anions at pH 3.50. The solid line was generated by simple linear regression analysis. The leading electrolyte system was 10 m*M* HCl, the pH having been adjusted by the addtion of β-alanine.

Fig. 9. Correlation between simulated and experimentally obtained values for the time-based zone lengths of 10-nmol samples of 58 anions at pH 3.50. The solid line was generated by simple linear regression. The leading electrolyte was 10 m*M* HCl. The pH was adjusted by the addition of β-alanine. Current: 100 μA. Capillary length: 25 cm × 0.5 mm I.D.

actual samples can be limited to several kinds, and their number may be reduced further by considering the relative mobilities measured at other pH values. The identification may even be made easier and more accurate if the time-based zone lengths are measured at different pH values.

As it is difficult to establish whether two compounds have been separated completely or not, it is important to know the maximum separable amounts of two analytes under the conditions used. However, the values obtained for equal amounts of two compounds do not necessarily apply to a more complex mixture of organic acids, as is shown in Fig. 10. By injecting equimolar amounts of phosphoric acid and citric acid, for instance, it was possible to separate a maximum amount of 65 nmol of each anion (Fig. 10a and b), but when aspartic acid and glutamic acid were added to the mixture, only 40 nmol of phosphoric acid and citric acid could be separated (Fig. 10c and d). As regards the separability of aspartic acid and glutamic acid, however, the separation capacity was not affected by the addition of other analytes (Fig. 10h–j).

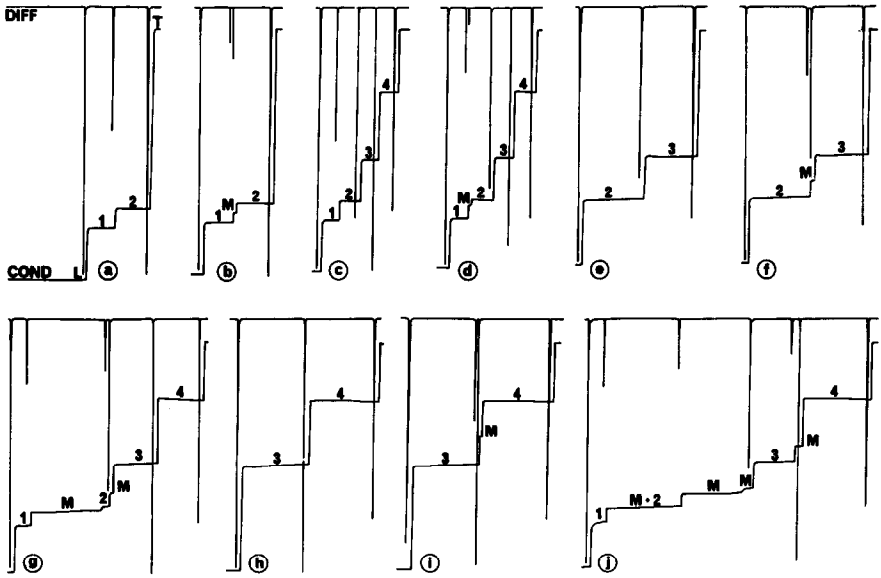


Fig. 10. Evaluation of the separability of equimolar amounts of (1) phosphoric acid, (2) citric acid, (3) aspartic acid and (4) glutamic acid. The appearance of a mixed zone (M) indicates the end of separability under the conditions used. (a) 65, (b) 72.5, (c) 40, (d) 45, (e) 120, (f) 125, (g) 105, (h) 150, (i) 155 and (j) 155 nmol. Leading electrolyte (L): 10 m*M* HCl–*β*-alanine–0.1% Triton X-100 (pH 3.50). Terminating electrolyte (T): 10 m*M* propionic acid. Capillary: 25 cm × 0.5 mm I.D. Detection: conductivity (COND) and differential conductivity (DIFF).

The separation capacity can be enhanced in various ways, such as by increasing the concentration of the leading electrolyte or the length of the capillary. All these measures, however, result in proportional increase in the time of analysis. Therefore, it will be necessary to adjust the experimental conditions to the requirements of a specific analysis in order to permit the separation of analytes with a maximum resolution and separability within the shortest period possible.

By allowing the rapid and reproducible determination of a vast range of organic acids in body fluids, isotachophoresis may serve as a valuable technique in the diagnosis, prognosis and monitoring of various pathological conditions.

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