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Capillary zone electrophoresis of organic acids in serum of critically ill children

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Abstract

Capillary zone electrophoresis with indirect UV detection was found to be suitable for the determination of organic acids in serum. Serum can be analysed directly without any deproteination in a capillary coated with linear polyacrylamide. With 10 m*M* ε-aminocaproic acid–10 m*M* mandelic acid (pH 3.8) as the operational electrolyte, anions such as pyruvate, phosphate, citrate, malate, acetoacetate and lactate can be determined in 12 min. In quantitative analysis, the calibration line for lactate is linear over the range 0–10 m*M*. The detection limit for citrate was 8 μ*M*. The effect of the chloride concentration on the migration times of minor peaks is discussed. The potential of the method was demonstrated by analysing sera from several critically ill children.

1. Introduction

A number of pathological states result in increases in the organic acid levels in blood. The increase in the level of some organic acids, so called acidaemia, can lead to a decrease in blood pH (i.e., acidosis), to acidotic coma and even to death. This is well described for lactic acidosis [1] and diabetic ketoacidosis [2,3]. The concentration of some acids need not necessarily influence the pH value of blood, but their values are related to some disorders, as shown for serum dicarboxylic acids in patients with Reye's syndrome [4], and have clinical significance as biochemical markers. For example, 2-ketoglutarate was shown to be a harbinger of hyperammonaemic coma [5]. In some cases, the concentration ratio between two organic acids gives more valuable information than the determination of a single acid: the concentration ratios of pyruvate to lactate [6,7] and acetoacetate to 3-hydroxybutyrate [8,9] are the best examples. The serum organic acid profiles, although studied by only few research groups, seem to be very promising for clinical chemistry [10,11].

Capillary electrophoresis has long been used for determination or organic acids in blood. Organic acids can be determined in serum directly by capillary isotachophoresis [12,13]. A good correlation has been found between the severity of disease and the isotachophoretic profile of serum organic acids [14]. Serum oxalate can be concentrated and detected by multi-column isotachophoresis [15]. Capillary isotachophoresis has been used for the determination of formate and glyoxalate in individuals intoxicated with methanol and ethylene glycol, respectively

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[16,17]. Capillary zone electrophoresis can separate acid metabolites of phenylalanine in phenylketonuric urine [18] and organic acids in cerebrospinal fluid [19] and urine [20]. It has been used to separate lactate and pyruvate in a single erythrocyte [21].

This paper describes the use of capillary zone electrophoresis with indirect UV detection for the determination of serum organic acids in blood of critically ill children.

2. Experimental

Samples of blood (1 ml) were collected from selected paediatric patients by venipuncture. After coagulation of the blood, the samples were centrifuged for 10 min at 3000 rpm. Sera were collected and stored at −26°C until analysis. In the initial experiments, sera were deproteinated by mixing with the same volume of acetone and centrifuged for 10 min at 10 000 rpm.

Vinylmagnesium bromide and furan were purchased from Aldrich (Milwaukee, WI, USA), ϵ-aminocaproic acid, β-alanine and citramalic acid from Sigma (St. Louis, MO, USA) and mandelic, 2-ketoisocaproic, 2-ketocaproic, 2-ketomethylvaleric, 2-hydroxyphenylacetic, hippuric and phenylpyruvic acid from Fluka (Buchs, Switzerland). All other chemicals were obtained from Lachema (Brno, Czech Republic). Sodium acetoacetate was prepared by alkaline hydrolysis of ethyl acetoacetate [22].

A fused-silica capillary (75 μm I.D., 360 μm O.D.) was a gift from J&W Scientific (Folsom, CA, USA). The total length of the capillary was usually 60 cm with 45 cm to the detection window. The capillary was coated with linear polyacrylamide after previous vinylation of the capillary inner surface with vinylmagnesium bromide [23] so that the electroosmotic flow was completely eliminated. To prevent damage of the coating, the detection window was made by manually cutting off the polymide layer [24].

The experiments were performed with a Crystal CE System, Model 310 (ATI Unicam, Cambridge, UK). Some experiments were performed with laboratory-made instrumentation [25]. A Spectra 100 variable-wavelength detector (Thermo Separation Products, Palo Alto, CA, USA) was used to detect the separated zones. The highest sensitivity was found at a wavelength of 220 nm and therefore all analyses were monitored at this wavelength. The data were collected by using 4880 software (ATI Unicam) with reversed polarity of the signal.

3. Results and discussion

3.1. Selection of operational electrolyte

Effect of pH

The majority of organic acids of interest are weak acids with pK_a values in the range 2.5 (pyruvate) to 4.4 (3-hydroxybutyrate). Since it is well known that the best separation of weak acids and bases is achieved at pH values near to their pK_a values [12,18,26,27], this pH range represents the approximate pH interval in which the separation of the compounds of interest should be investigated. For indirect UV detection, a UV-absorbing co-ion is required, preferably with the pK_a value in the above estimated pH range. The initial experiments were performed with a model mixture; however, the electropherograms of real samples were found to be more useful for optimizing the separation conditions. Therefore, serum from a paediatric patient with severe respiratory insufficiency was used to find the optimum operational electrolyte. The identification of peaks was performed by simultaneous analysis of serum and the appropriate standards. The results are shown in Fig. 1. Since the polarity of the detection output was reversed, the non-UV-absorbing compounds provide positive peaks. The first peak behind the large skewed peak of chloride was not identified. It was shown, however, that it does not correspond to pyruvate, which is in this particular case overlapped by the chloride zone. The peak with a migration time of ca. 6 min corresponds to phosphate; as shown by detailed analysis, it also contains some other organic anions: 2-ketoglutarate, 2-ketoisovalerate (KIV), 2-ketomethylvalerate (KMV), etc. The next separated

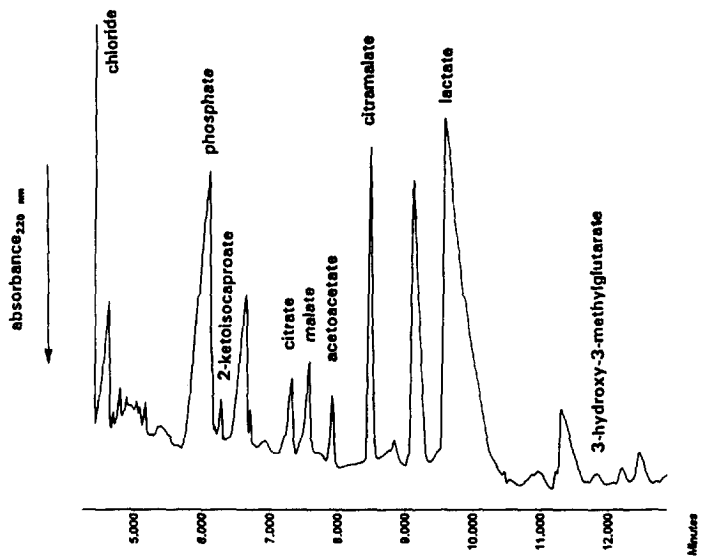

Fig. 1. Determination of acids in serum from a paediatric patient with respiratory insufficiency. Experimental conditions: linear polyacrylamide-coated capillary, 75 μm I.D., 360 μm O.D., total length 508 mm, effective length 413 mm; operational electrolyte, 10 m*M* ε-aminocaproic acid–10 m*M* mandelic acid; voltage, −20 kV; detection, absorption at 220 nm. Undiluted serum sampled by pressure of 100 mbar for 6 s.

peak corresponds to 2-ketoisocaproate (KIC) and may contain glyoxalate. The next peak was not identified. The triplet of peaks with migration times between 7 and 8 min are citrate, malate and acetoacetate. The peak with a migration time of ca. 8.5 min was identified as citramalate. The skewed peak with a migration time of 9.8 min corresponds to lactate and the minor peak with a migration time of 11.8 min is 3-hydroxy-3-methylglutarate.

The effect of various operational electrolytes on the separation of the acids of interest is shown in Fig. 2. With an operational electrolyte containing 10 m*M* ε-aminocaproic acid (EACA) and 10 m*M* 2-hydroxyphenylacetic acid (pH 4.35), several peaks of acids with higher pK_a appear in the electropherogram, including 3-hydroxybutyrate with a migration time of 15.8 min. However, the peaks between phosphate and lactate are only partially resolved (trace a). Trace b shows electropherogram of the test serum, when 10 m*M* ε-aminocaproic acid (EACA)–10 m*M* mandelic acid (pH 3.83) was used as the operational electrolyte (this trace is almost identical with the electropherogram in Fig. 1). The peaks between phosphate and lactate are well resolved, although the phosphate peak still contains some other acids. 3-Hydroxybutyrate has a migration time >35 min at this pH (not shown). When EACA ($pK_a = 4.3$) is replaced with β-alanine ($pK_a = 3.55$), so that the operational electrolyte contains 10 m*M* β-alanine–10 m*M* mandelic acid (pH 3.51), trace c is obtained. In this case, the migration times of lactate and other anions increase and the shape of their peaks is more deformed owing to electromigration dispersion. However, the separation in the vicinity of phosphate is not improved in comparison with trace b. The electropherogram of serum acids in the operational electrolyte 10 m*M* β-alanine–10 m*M* hippuric acid (pH 3.6) is shown as trace d. In this case, the migration times are not changed significantly, only the skewed shape of the slower peaks is reversed as a result of a higher mobility of the co-ion. Even more acidic operational electrolytes were tested (e.g., 10 m*M* picric acid). Unfortunately, the capillary frequently became plugged with these operational electrolytes, probably as a result of denaturation of serum proteins. We

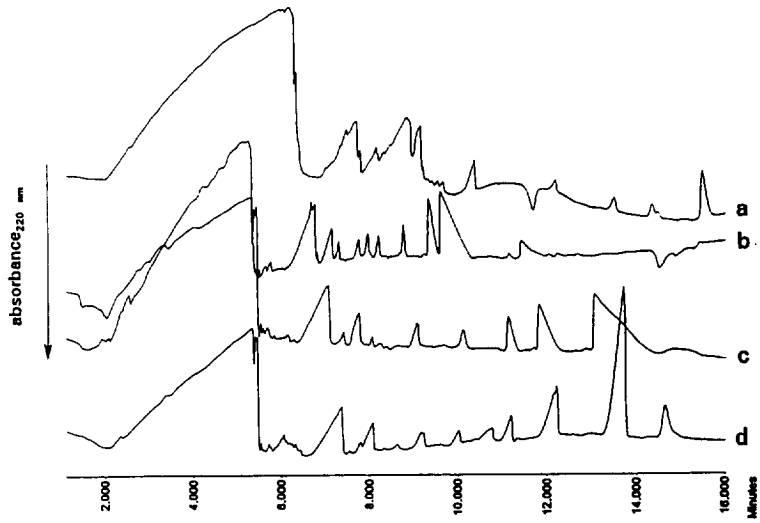

Fig. 2. Effect of pH of the operational electrolyte on the separation of serum acids. Experimental conditions: linear polyacrylamide-coated capillary, 75 μm I.D., 360 μm O.D., total length 550 mm, effective length 455 mm; operational electrolyte, (a) 10 m*M* ε-aminocaproic acid–10 m*M* 2-hydroxyphenylacetic acid (pH 4.35); (b) 10 m*M* ε-aminocaproic acid–10 m*M* mandelic acid (pH 3.83); (c) 10 m*M* β-alanine–10 m*M* mandelic acid (pH 3.51); (d) 10 m*M* β-alanine–10 m*M* hippuric acid (pH 3.6); voltage, −24 kV; detection, absorption at 220 nm. Undiluted serum as in Fig. 1 sampled by pressure of 100 mbar for 6 s.

consider the possibility of determining serum acids directly without any previous deproteinization to be one of the major advantages of capillary electrophoresis and therefore more acidic operational electrolytes were not tested further.

Based on these results, the operational electrolyte containing ε-aminocaproic acid as counter ion and mandelic acid as the UV-absorbing co-ion was used in all subsequent experiments. The main disadvantages when using this operational electrolyte are the long migration time of 3-hydroxybutyrate, especially in comparison with isotachophoresis, where all serum acids including 3-hydroxybutyric acid are determined in a reasonable time [12], and significant asymmetry of some peaks. However, preliminary experiments indicated that this problem may be solved by introducing a pH gradient or pH step into the separation capillary [28,29].

Effect of concentration

To achieve a reasonable detection sensitivity in capillary zone electrophoresis with indirect UV detection, the co-ion of the operational electrolyte must absorb UV light, whereas the counter ion should be transparent at the given wavelength. The detected substance partially replaces the co-ion in its zone, which results in a reduced UV absorption when the ω-function is kept constant.

The effect of the concentration of the operational electrolyte was investigated using an equimolar mixture of ε-aminocaproic acid as counter ion and mandelic acid as co-ion, analysing the serum as above. The results are shown in Fig. 3. The concentration of the operational electrolyte determines the value of the ω-function and thus the maximum concentration of chloride which can be reached in its zone. Because of this limit, the volume of the chloride zone and the migration time of the rear boundary of the chloride zone decrease with increasing electrolyte concentration.

For the detection sensitivity, it holds that the lower the concentration of the operational electrolyte, the higher is the relative concentration of the detected substances in their zones. Simul-

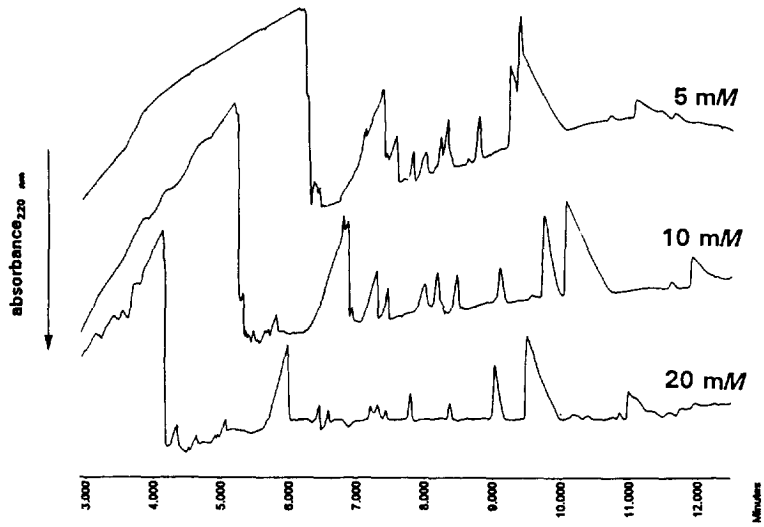

Fig. 3. Effect of concentration of the operational electrolyte on the separation of serum acids. Experimental conditions as in Fig. 2. Operational electrolyte, 5–20 m*M* ϵ-aminocaproic acid–5–20 m*M* mandelic acid.

taneously, some disturbing effects such as noise and baseline drift are more pronounced at lower concentrations of the operational electrolyte. This is demonstrated in Fig. 3: in 5 m*M* operational electrolyte, a high detection sensitivity is achieved when a significant baseline drift is observed. In 20 m*M* operational electrolyte, the baseline drift is almost negligible, but the detection sensitivity is reduced. A concentration of 10 m*M* seems to be an acceptable compromise.

3.2. Effect of chloride concentration on migration times

Several effects of increasing concentration of the major compound (chloride) in the sample were observed which influence the migration of minor compounds. Chloride has a higher mobility than the co-ion (mandelate) so that during analysis there is a lower electric field strength in the chloride zone. At constant voltage, since the electric current is constant through the whole capillary, the electric field out of the chloride zone is higher than the average calculated value. An inhomogeneous electric field strength results in shorter migration times and higher absolute values of the apparent mobilities of the anions of interest with increasing amount of injected chloride. The effect is demonstrated in Fig. 4 in the chloride concentration range 0–15 m*M*. (Obviously, a reversed effect of low-mobility anions can be expected and actually was observed in sera with a higher content of lactate.) When the concentration of chloride increases further, other effects appear. Starting from a certain amount of chloride, sample self-stacking occurs [30] and

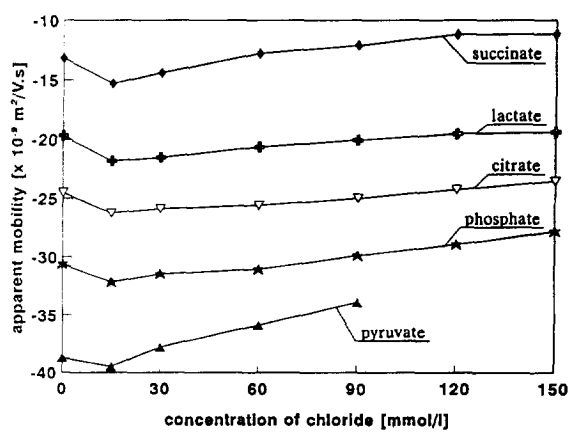

Fig. 4. Effect of concentration of chloride in sample on the apparent mobility of selected anions at constant voltage. Separation conditions as in Fig. 1. Apparent mobilities calculated as $\mu_{app} = l_t l_{eff}/Vt_m$.

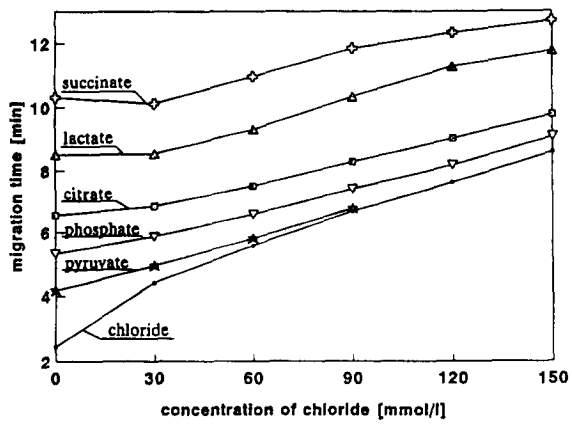

Fig. 5. Effect of concentration of chloride in sample on the migration times of selected anions at constant driving current. Experimental conditions: linear polyacrylamide-coated capillary, 75 μm I.D., 360 μm O.D., total length 600 mm, effective length 450 mm; operational electrolyte, 10 m*M* ϵ-aminocaproic acid–10 m*M* mandelic acid; driving current, −11 μA; detection wavelength, 220 nm. Model mixture sampled by pressure of 100 mbar for 6 s.

minor compounds are retained in the chloride zone. This is why the pyruvate peak does not appear in all runs. Some other effects can be expected which contribute to the mobility shift, such as a pH decrease in the chloride zone in comparison with the operational electrolyte and a lower electric field in the chloride zone. The larger the amount of chloride, the longer the compounds of interest stay in the mixed zone with chloride and, because of the lower pH value, they acquire lower ionization and, thus, a lower absolute value of the apparent mobility. Slower compounds with higher pK_a (e.g., succinate) leave the chloride zone sooner than those with lower pK_a, but the effect of slower pH (resulting in a more pronounced decrease in ionization) still influences their effective mobility. Simultaneously, the lower electric field strength in the chloride zone results in lower absolute values of the apparent mobilities of the anions of interest.

In the constant-current regime, the applied voltage varies during analysis, which is why migration times are used to describe the following experiments. In this case, the electric field strength out of the chloride zone is not affected by the amount of chloride injected. However, at lower concentrations of chloride, we can see acceleration of slower anions (i.e., anions with higher pK_a, such as succinate and partially lactate) with increasing concentration of chloride (Fig. 5). This may be caused by faster migration of these anions in the zone of the original sample if this has higher pH. At higher concentrations of chloride, the same effects are observed as at constant voltage.

These effects have a significant practical impact: in samples of biological origin, the content of the major compound chloride varies, which results in variation of the migration times of the same compound when analyzing different samples. This has to be taken into account when identifying the particular peaks in the sample. Since the time period during which the zone stays in detector is also affected by mobility variations, these effects can also influence the quantitative analysis.

3.3. Quantitation and detection limit

Quantitative analysis was performed only for lactate and the calibration line is given in Fig. 6. It is linear in the range 0–10 m*M*. Although the lactate peaks are significantly skewed in the

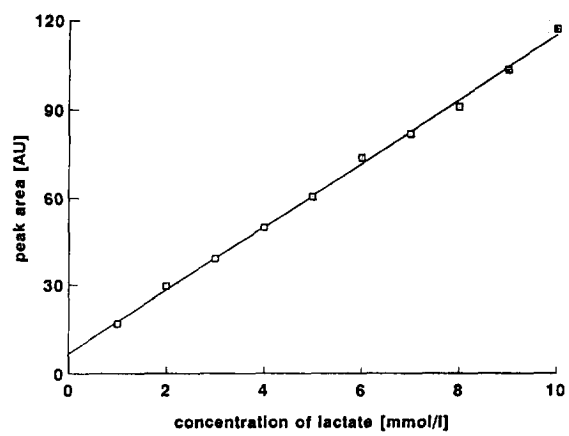

Fig. 6. Calibration graph for determination of lactate. Experimental conditions: linear polyacrylamide-coated capillary, 75 μm I.D., 360 μm O.D., total length 650 mm, effective length 500 mm; operational electrolyte, 10 m*M* ϵ-aminocaproic acid–10 m*M* mandelic acid; voltage, −26 kV; detection wavelength, 220 nm. Samples injected by pressure of 100 mbar for 6 s.

operational electrolyte EACA–mandelic acid, the correlation coefficient of this line is 0.998. This permits the determination of lactate in real samples to be performed.

Of the minor anions found in sera, citrate was selected as a model compound for the determination of the detection limit. Citric acid was determined in the concentration range 1–10 μM. The lowest concentration of citrate providing an observable peak was 8 μM, and this value was taken as the detection limit.

3.4. Practical examples

We analysed sera from patients hospitalized in the Intensive Care Unit, J.G. Mendel Faculty Children's Hospital, Brno, during September–December 1994. Sera were analysed without prior deproteination. No significant plugging of the capillary by serum proteins precipitated during analysis was observed if the capillary was rinsed sufficiently between runs. Depending on the patients' condition, we found various acid profiles in the sera analysed. Typical peaks, which were present in all the samples analysed, were phosphate and lactate. Pyruvate frequently did not appear in the electropherogram since it was overlapped by the chloride peak. Citrate, malate and acetoacetate, which form a triplet of peaks in the centre of the chloride–lactate area, were sometimes incomplete, as some of these anions (most frequently malate) were present at levels below the detection limit.

Fig. 7 shows two electropherograms of serum from the same patient suffering from meningoencephalitis and from a perforated duodenal ulcer and the resulting shock. Trace a corresponds to the analysis of the blood taken shortly after a repeated cardiopulmonary resuscitation. The large peak of lactate, resulting from insufficient oxygenation, which corresponds to a lactate concentration of 14.8 mM, confirms clearly the severity of the patient's state. Trace b corresponds to the analysis of blood taken 2 days later when the patient's state had stabilized; the peak of lactate is reduced, corresponding to a concentration of 2.1 mM. Two peaks migrating in front of lactate were not observed in trace a

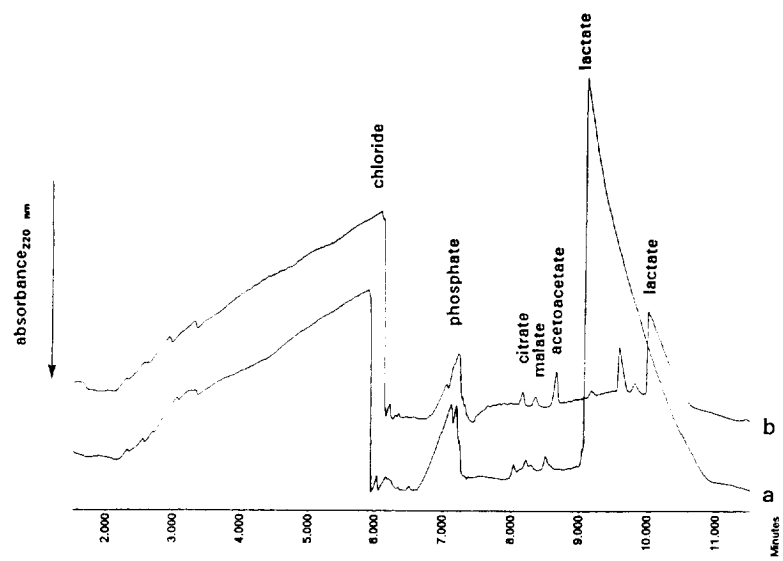

Fig. 7. Determination of acids in serum from a paediatric patient in shock. Experimental conditions: linear polyacrylamide-coated capillary, 75 μm I.D., 360 μm O.D., total length 600 mm, effective length 500 mm; operational electrolyte, 10 mM ϵ-aminocaproic acid–10 mM mandelic acid; voltage, −24 kV; detection, absorption at 220 nm. Undiluted serum sampled by pressure of 100 mbar for 6 s. (a) Analysis after repeated cardiopulmonary resuscitation; (b) analysis 2 days later, when the patient's state had stabilized.

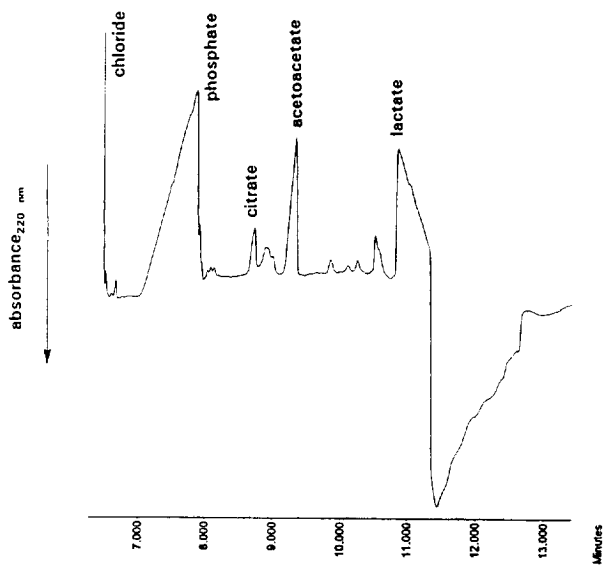

Fig. 8. Determination of acids in serum from a paediatric patient with respiratory insufficiency. Experimental conditions as in Fig. 7.

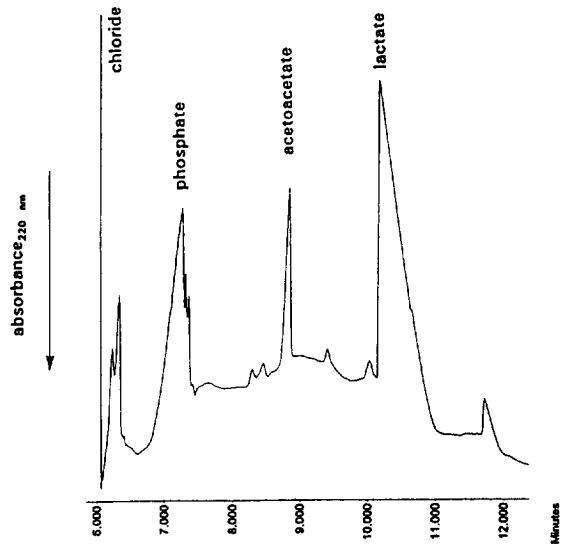

Fig. 9. Determination of acids in serum from a paediatric patient with severe head injury. Experimental conditions as in Fig. 7.

since they were probably overlapped by the lactate peak.

Fig. 8 shows an electropherogram of serum from a patient suffering from congenital heart malformation, bronchopneumonia, overall dystrophy and respiratory insufficiency. Several peaks are significant, e.g., the increased peak of acetoacetate and especially the UV-absorbing peak migrating behind lactate, which was not identified (hippurate migrates in model mixtures in this position; however, analysis of this sample at various pH values showed that this UV-absorbing peak is not hippurate).

Fig. 9 shows an electropherogram of serum from a patient with severe craniocerebral injury. There is a pair of unidentified peaks migrating in front of phosphate, several peaks co-migrating with phosphate and increased peaks of acetoacetate and lactate.

Analysis of serum from a patient with multiple trauma and brain injury is shown in Fig. 10. A large peak of phosphate dominates this electropherogram and obviously it is not clear how many compounds may be hidden by it. It is likely that phosphate is a minor compound in this peak. A similar profile was observed when analysing uraemic sera by capillary isotachophoresis [14].

4. Conclusion

Capillary zone electrophoresis has been demonstrated to be a suitable method for the determination of organic acids in real samples of serum. It does not require deproteination of sera prior to analysis and provides valuable results in ca. 12 min.

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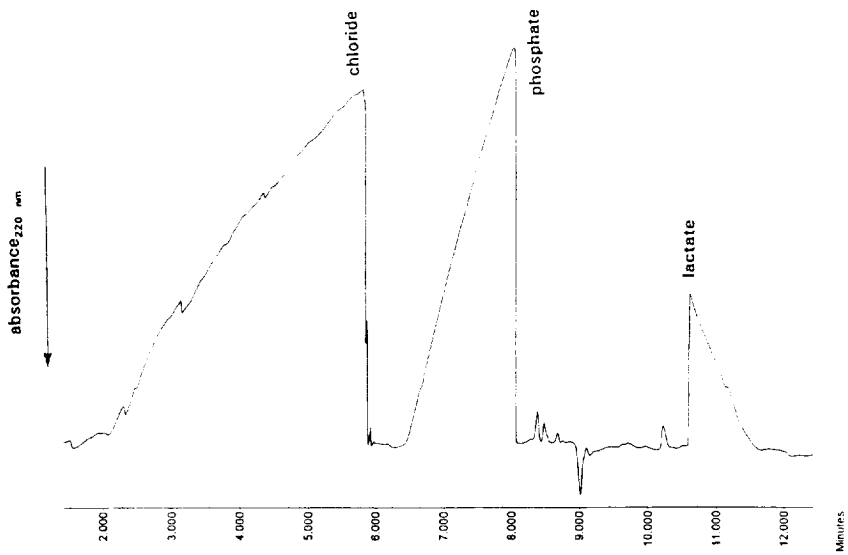

Fig. 10. Determination of acids in serum from a paediatric patient with multiple trauma. Experimental conditions as in Fig. 7.

Gebauer for discussions of the sample self-stacking phenomena.

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