Journal of Chromatography, 537 (1991) 407–428
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 753

Determination of absolute mobilities, p*K* values and separation numbers by capillary zone electrophoresis

Effective mobility as a parameter for screening

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(First received May 15th, 1990; revised manuscript received August 7th, 1990)

ABSTRACT

Migration times or apparent mobilities can never be used for the identification of ionic species in capillary zone electrophoresis if an electroosmotic flow (EOF) is present, because the velocity of this flow varies considerably with the "state" of the capillary. From the migration times of the EOF and the ionic species, the effective mobilities can be calculated. These effective mobilities are nearly independent of the concentrations of the sample ionic species. Although a large excess of one of the sample components can cause different values of the calculated effective mobility, they are reproducible if the matrix has a constant composition and in this way effective mobilities can be used for screening purposes. In the determination of effective mobilities the use of a "true" EOF marker is extremely important.

If effective mobilities are measured in two different electrolyte systems at different pH values, at which the degrees of dissociation differ sufficiently, the absolute ionic mobilities and p*K* values of ionic species can be calculated. Values obtained in this way, for mobility and p*K* were compared with data obtained isotachophoretically, showing good agreement.

Theoretically, the separation number in zone electrophoresis, defined as the number of components that can be separated within a unit of mobility, varies widely with the mobilities of the ionic species and the EOF. Experimentally obtained values of the separation number are significantly lower than the calculated values owing to the method of injection, temperature effects during analysis and amount of sample. For low-molecular-weight ionic species separations are possible if the effective mobilities differ by about one unit for cations and 0.2–0.3 for anions. A negative wall charge (at higher pHs) diminishes the separation number of cations considerably, especially on applying small diameter capillaries, owing to attractive forces between the wall and analytes.

INTRODUCTION

Since the availability of commercial apparatus, capillary zone electrophoresis (CZE) has been the subject of rapid development and is now applied in many areas, especially in the biological and biochemical fields.

For screening possibilities, the most important questions are as follows: (1) does the component migrate in a chosen electrolyte system and what parameter can be used to recognize it?; (2) is the separation capacity of the method sufficient to separate the

0021-9673/91/$03.50

component adequately from other components of a complex matrix and can it be identified in a simple way?; and (3) at what level can the component be detected?

In this paper we discuss the possibility of open capillary zone electrophoresis for screening purposes on a qualitative basis. When using open capillary zone electrophoresis, the electroosmotic flow (EOF) is a very important parameter, in addition to the effective mobility[a], determining the migration behaviour of components. As the EOF velocity strongly varies with the "state" of the capillary, the migration time (or apparent mobility[a]) can never be used in a proper way for screening purposes. The effective mobilities of components can be calculated from the experimentally obtained apparent mobilities and the mobility of the EOF. From the effective mobilities also the pK values and absolute mobilities[a] of the components can be calculated. These data are also important in the choice of a suitable electrolyte system for the separation of various components in complex matrices.

Until now, mobilities and pK values have often been determined by isotachophoresis (ITP) [1–8]. The calculation of mobilities and pK values in ITP is laborious, however, as in ITP all zones have different parameters such as pH, concentration and temperature, through which the data have to be calculated in an iterative way. The correction for the concentration dependence of mobilities and for activities is often troublesome for mixtures of ionic species with different charges. Further, in ITP the choice of the pH of the electrolyte systems is limited to about 3–11. Low pHs cannot be applied in the separation of cations owing to the great influence of hydrogen ions on the zone conductivity and high pHs can hardly be used in the separation of anions owing to the disturbance by carbonate. Especially at low pHs major problems can be expected in finding an appropriate slow terminator in the separation of cations because hydrogen ions can act as a terminator with relatively high effective mobilities [9,10]. Generally, the determination of the mobilities of weak acids and bases with low mobility and also those of the subspecies from multivalent acids and bases is difficult.

In CZE, on the other hand, many of these limitations are not present. Background electrolytes at low and high pHs can be used easily, there is no need for a terminator and corrections for several effects are relative easy because all parameters in the background electrolyte can be considered to be nearly constant, such as ionic strength, pH, temperature and electric field strength.

Effective mobilities for low-molecular-weight ionic species determined in CZE and, from these values, calculated pK values and absolute ionic mobilities are presented, and compared with ITP data. In the CZE experiments special attention was paid to the reproducibility and the effect of the EOF, taking into account the influence of the composition of the samples, such as the concentrations of the sample ions and the effect of the presence of background electrolyte in the sample solution.

Using CZE as a screening method, the effective mobility ("yes or no", in combination with, *e.g.*, the use of UV absorbance ratios at different wavelengths) can be the only way to identify substances, stressing the importance of the determination of mobilities from electropherograms.

For the experiments, representatives of several classes of compounds were

[a] Note that the apparent mobility refers to the net migration behaviour, including the velocity of the EOF, and the effective and absolute mobility refer to the pure electrophoretic migration behaviour.

chosen, such as procaine (an anaesthetic) and some antibiotics used in cattle-breeding. Antibiotics are often administered on a large scale to food-producing animals in order to guarantee safe products such as milk, meat and eggs. Their presence in food is forbidden, however, and for this reason there is a need for screening facilities for all these components. The β_2-agonists clenbuterol and fenoterol were studied. In addition to the use of β_2-agonists for the treatment of asthmatic deseases, they have a positive effect on the fat/meat ratio in cattle, which explains their improper use. We also measured the effective mobility of levamisol, an anthelmintic or vermicide used against maggots. As an example of coccidiostats, used to treat parasitic diseases especially in the intestines of cattle, sheep, goats, dogs, cats, rabbits and poultry, amprolium was studied. In Fig. 1 some characteristic structural formulae are given.

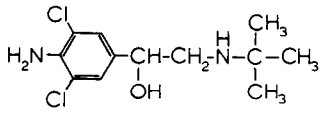
THEORETICAL

Determination of mobilities and pK values

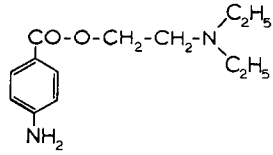
If in CZE the applied voltage V and the length of the capillary L_c are known, the electric field strength is

$$E = V/L_c \text{ (V/m)} \tag{1}$$

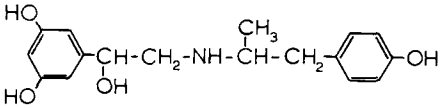
If the distance from the injection point to the detector, L_d, and the migration time t of



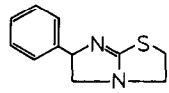
clenbuterol



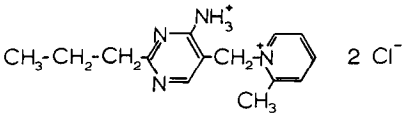
procaine



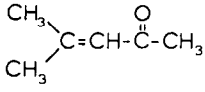
fenoterol



levamisol



amprolium



mesityl oxide

Fig. 1. Some characteristic structural formulae.

a component are known, the velocity v and the apparent mobility m_{app} can be calculated using

$$m_{app} = v/E = L_d/tE \ (m^2/V \cdot s) \tag{2}$$

In CZE, a high EOF can generally act in the direction of the cathode using silica capillaries. The velocity of the EOF can be determined using the "migration" time, t_{EOF}, of an uncharged substance and, because this velocity shows a linear relationship with E, the m_{EOF} can be defined as

$$m_{EOF} = L_d/t_{EOF}E \ (m^2/V \cdot s) \tag{3}$$

and the effective mobility of a component can be obtained from

$$m_{eff} = m_{app} - m_{EOF} \tag{4}$$

If the absolute values of the effective mobilities of anionic species are smaller than m_{EOF}, they can be determined in the upstream mode (UM) simultaneously with cations migrating in the downstream mode (DM). From the effective mobility, the absolute ionic mobility can be calculated, correcting for activities, dissociation and concentration dependence of the mobility.

If the effective mobility of a component is known for two different electrolyte systems at different pHs, at which the component shows a different degree of dissociation, both its pK value and its absolute mobility can be calculated. For a monovalent acid the calculation is as follows. The thermodynamic equilibrium constant for the equilibrium

$$HZ \rightleftharpoons H^+ + Z^- \tag{5}$$

is defined as

$$K_{th} = \gamma_{Z^-}\gamma_{H^+} \frac{[H^+]\,[Z^-]}{[HZ]} \tag{6}$$

with the assumption that for HZ the activity coefficient $\gamma = 1$. Hence,

$$pK_{th} = -\log \gamma_{Z^-} - \log \gamma_{H^+} + pH - \log \frac{[Z^-]}{[HZ]} \tag{7}$$

Because the effective mobility $m_{eff} = \alpha m_c$, where m_c is the ionic mobility at a specific equivalent concentration c, it follows that

$$\frac{[Z^-]}{[HZ]} = \frac{\alpha}{1 - \alpha} = \frac{m_{eff}}{m_c - m_{eff}} \tag{8}$$

The value of m_c can be calculated from the ionic equivalent conductance λ_c using Faraday's constant, F. For the ionic equivalent conductance in mixed solutions we

used the expression according to Bennewitz, Wagner and Kuchler as described by Falkenhagen [11]:

$$\lambda_c = \lambda_0 - (0.229\lambda_0 + 30.1)\sqrt{c} \tag{9}$$

where λ_c and λ_0 are the ionic equivalent conductances at an equivalent concentration c and infinite dilution, respectively. This relationship can be used for very dilute solutions of a particle in a bulk of the background electrolyte.

If the effective mobilities are known in two different electrolyte systems, we have

$$-\log \gamma_{Z,1}^{-} - \log \gamma_{H,1}^{+} + pH_1 - \log\left(\frac{m_{eff,1}}{m_{c,1} - m_{eff,1}}\right) = -\log \gamma_{Z,2}^{-} - \log \gamma_{H,2}^{+} + pH_2 - \log\left(\frac{m_{eff,2}}{m_{c,2} - m_{eff,2}}\right) \tag{10}$$

The activity coefficients γ can be calculated by

$$-\log \gamma = \frac{0.5085\, z^2 \sqrt{\mu}}{1 + 0.3281 a\sqrt{\mu}} \tag{11}$$

where z is the valency of the component, μ is the ionic strength of the solution as determined by the background electrolyte and a is the effective hydrated diameter of the ion in Å. If the effective hydrated diameter was unknown, 5 Å is assumed.

If the ionic strengths and the equivalent concentrations of two electrolyte systems are known, all activity coefficients can be calculated with eqn. 11. Using Faraday's constant and eqn. 9, $m_{c,1}$ and $m_{c,2}$ can be replaced by the absolute ionic mobility and thus the only unknown parameter in eqn. 10 is the absolute ionic mobility, which can be calculated. With eqn. 7, pK_{th} can be obtained. Analogous derivations can be given for multivalent anions and cations.

Effect of the electroosmotic flow

From eqn. 4, it can be concluded that the effect of the EOF is extremely Iimportant in the determination of effective mobilities. In Fig. 2 the calculated relationship between migration time and effective mobility for an E gradient of 25 kV/m and L_c and L_d of 1 m is given for several values of m_{EOF}. It can be clearly seen that at low EOF only cations can be determined whereas at high EOF simultaneously anions in the UM can be determined with $|m| < m_{EOF}$. A disadvantage at high EOF is, however, that the separation power for cations diminishes.

Separation number

In gas chromatography the separation number (SN) [12]

$$SN = \frac{t_{R(z+1)} - t_{R(z)}}{w_{h(z)} + w_{h(z+1)}} - 1 \tag{12}$$

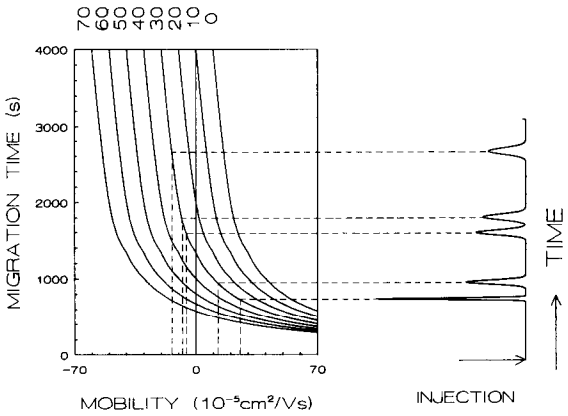


Fig. 2. Calculated relationship between migration time and effective mobility applying an E gradient of 25 kV/m. The different lines are marked with numbers representing the mobility ($\times 10^5$) of the EOF ($cm^2/V \cdot s$). At large EOF velocities negative ions can be analysed in the upstream mode. From this relationship electropherograms can be deduced, as shown for an m_{EOF} of $30 \cdot 10^{-5}$ $cm^2/V \cdot s$.

where t_R is the retention time and w_h the peak width at half-height, is used in order to calculate the number of component peaks which can be placed between peaks of two consecutive homologous standards with z and $z+1$ carbon atoms with a resolution of $R_s = 1.177$.

In CZE, an analogous expression can be used as a parameter for the separation power. We can define the separation number SN_m as

$$SN_m = \left| \frac{t_{m-0.5} - t_{m+0.5}}{2\,(\sigma_{m+0.5} + \sigma_{m-0.5})} \right| \tag{13}$$

This number indicates how many components can be separated at an effective mobility m within a unit of effective mobility. Using the absolute value, this equation can be used for both cations and anions, independent of the direction of the EOF.

Using eqns. 2 and 4, the migration time can be calculated from the effective mobility and m_{EOF} and, using for σ the expression

$$\sigma^2 = 2Dt \ (m^2) \text{ or } \sigma^2 = 2Dt/v^2 \ (s^2) \tag{14}$$

SN_m can be calculated. In the calculations other effects of zone broadening, such as σ_{inj}, are neglected.

In Fig. 3 the calculated relationship between SN_m values and effective mobilities is given for several values of EOF (L_c and L_d = 1 m, E = 25 kV/m). For the diffusion constant D we used in the calculations the Einstein expression

$$D = mkT/ez \tag{15}$$

It can be seen from Fig. 3 that if the mobility is tending to zero SN_m is strongly increasing because D is decreasing to zero. Very low diffusion constants will only act

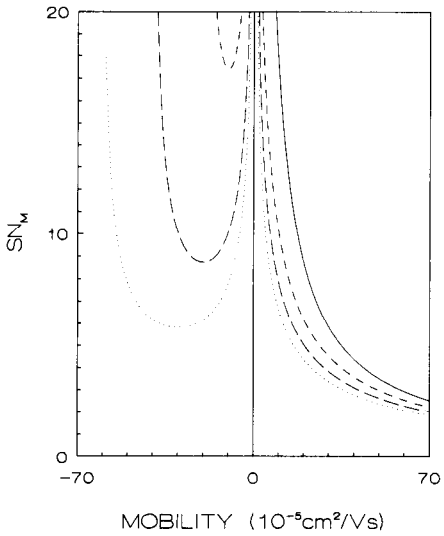


Fig. 3. Calculated relationship between SN_m values and effective mobilities for $m_{EOF} \cdot 10^5$ (cm^2/V · s) of 0 (———), 20 (---------), 40 (– – –) and 60 (· · ·). For further explanation, see text.

for very large molecules such as DNA fragments, causing a high peak capacity. For small molecules this will be not true as, generally, a very low mobility means that an ionic species for the greater part will be present as a neutral molecule with a large diffusion constant. Therefore, we recalculated SN_m as a function of the effective mobility, under the assumption that D is determined by the Stokes–Einstein relationship:

$$D = kT/6\pi\eta a \tag{16}$$

taking arbitrarily an average value for D of $5 \cdot 10^{-10}$ m^2/s.

This relationship is shown in Fig. 4 (L_c and L_d = 1 m, E = 25 kV/m). It can be clearly seen that the separation number increases at lower effective mobilities because the difference in migration time is increasing at an equal diffusion constant.

In Fig. 5, the relationship between the separation number SN_{20} (at an effective mobility of $2 \cdot 10^{-4}$ cm^2/V · s, L_c and L_d = 1 m) as a function of the applied voltage is given, showing that an increasing separation power is obtained by applying higher voltages.

EXPERIMENTAL

For all CZE experiments the P/ACE™ System 2000 HPCE (Beckman, Palo Alto, CA, U.S.A.) was used. All experiments were carried out at 25°C in the constant-voltage mode at 25 kV, unless mentioned otherwise. Several different capillaries were applied. Further information concerning the apparatus is given elsewhere [13]. For all ITP experiments the apparatus described previously [14] was used.

Table I gives the compositions of all the electrolyte systems used.

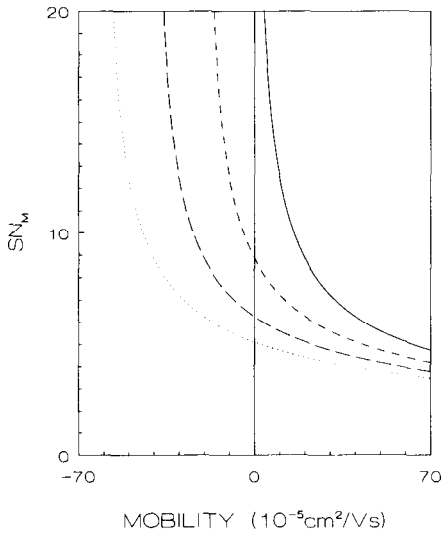


Fig. 4. Calculated relationship between SN_m values and effective mobilities for $m_{EOF} \cdot 10^5$ (cm^2/V · s) of 0 (———), 20 (---------), 40 (– – – –) and 60 (· · · ·), assuming a diffusion constant of $5 \cdot 10^{-10}$ m^2/s.

RESULTS AND DISCUSSION

Choice of the EOF marker

For the calculation of effective mobilities from the apparent mobility and the EOF, the velocity of the EOF has to be precisely known. There are several ways to

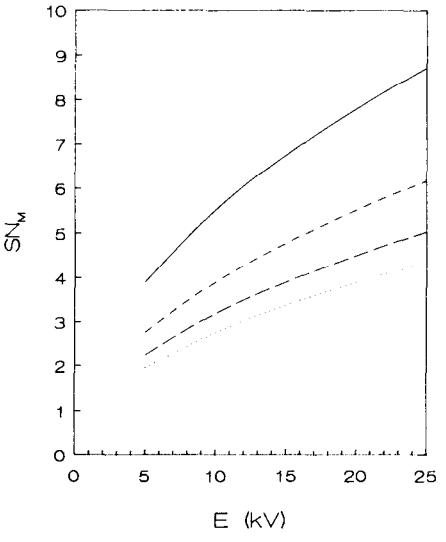


Fig. 5. Calculated relationship between SN_m values and E gradient for $m_{EOF} \cdot 10^5$ (cm^2/V · s) of 0 (———), 20 (---------), 40 (– – – –) and 60 (· · · ·) at an effective mobility of $2 \cdot 10^{-4}$ cm^2/V · s.

TABLE I

COMPOSITIONS OF BACKGROUND ELECTROLYTES AT DIFFERENT pH VALUES

All buffer solutions were prepared by adding the buffering counter ion to the cations until the desired pH was reached. All phosphate buffers were prepared by adding orthophosphoric acid to 0.01 M KOH until the desired pH was reached.

Cation[a]	Buffering counter ion[a]	pH
0.02 M β-Alanine	Formic acid	3.5
0.02 M β-Alanine	Formic acid	3.8
0.02 M β-Alanine	Acetic acid	3.9
0.02 M EAC	Formic acid	4.0
0.02 M EAC	Acetic acid	4.4
0.02 M β-Alanine	Acetic acid	4.7
0.02 M EAC	Acetic acid	5.0
0.02 M HIST	MES	6.1
0.02 M HIST	MES	6.2
0.01 M KOH	MES	6.2
0.02 M TEA	MOPS	7.0
0.04 M Imidazole	MOPS	7.5
0.02 M TRIS	MOPS	7.9
0.02 M TRIS	MOPS	8.2
0.02 M DEA	BICINE	9.0

[a] BICINE = N,N-Bis(2-hydroxyethyl)glycine; DEA = diethanolamine; EAC = ε-aminocaproic acid; HIST = histidine; MES = 2-(N-morpholino)ethanesulphonic acid; MOPS = morpholinopropanesulphonic acid; TEA = triethanolamine; TRIS = tris(hydroxymethyl)aminomethane.

measure the EOF. In Fig. 6 some possibilities are shown schematically. The real EOF displacement is indicated with an arrow. Using a neutral EOF marker, it is possible that this marker indicates the EOF displacement (2). If the marker, however, meets

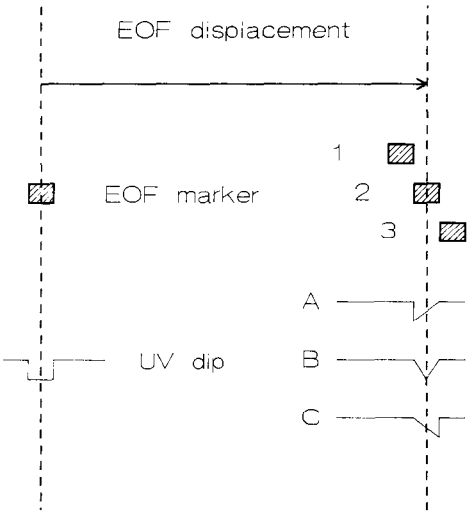


Fig. 6. Several possibilities of measuring EOF. For further explanation, see text.

a power of attraction from the capillary or if it is partially negatively charged by complexation with negative ions of the background electrolyte it will be too slow (1), or it will be too quick if it is positively charged by complexation (3).

Because many electrolyte systems (*e.g.*, the system HIST–MES at pH 6.2; see Table I) absorb UV light at a wavelength of 214 nm, sample solutions with a lower buffer concentration than the background electrolyte show a dip in the UV signal, because the local concentration of the buffer is lower. If an aqueous sample solution is introduced, the original concentration (UV) dip can indicate the correct EOF displacement (B), but because the shape of the concentration dip can change owing to diffusion effects, the shape can become asymmetric [15,16] to one of the sides (A or C), depending on the mobilities of the background ionic species, indicating the wrong EOF.

In the first instance we compared (UV detection at 214 and 254 nm) as EOF markers acetone, benzene, crotonaldehyde, mesityl oxide (MO) and paracetamol in a background electrolyte at pH 8.2. The best results (high absorbance and symmetrical peaks) were obtained using MO as EOF marker. In all further experiments we always measured at a wavelength of 214 nm. In Fig. 7 the measured UV signal for a sample solution of (a) 0.0001 *M* MO in 100% buffer and mixtures of (b) 1% water and 99% buffer and (c) 50% water and 50% buffer are shown. In Table II the measured migration times and calculated m_{EOF} are given for the 0.0001 *M* MO solution in 100% buffer and the mixture of 1% water in 99% buffer. In the latter instance the observed UV dip is used for the determination of the EOF. The background electrolyte was HIST–MES at pH 6.2. It can be concluded that the reproducibility of the experimental values is good and MO can be used as a true EOF marker in this system.

In Fig. 8, (a) the UV dip on injecting water as a sample and (b) the UV signal on injecting an aqueous solution of 0.0005 *M* MO are shown for the system KOH–MES at

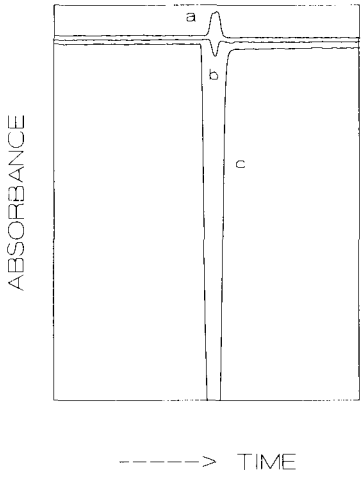


Fig. 7. Measured UV signal for a sample solution of (a) 0.0001 *M* MO in 100% buffer, (b) a mixture of 1% water and 99% buffer solution and (c) 50% water and 50% buffer solution. Background electrolyte, HIST–MES at pH 6.2. Capillary from Scientific Glass Engineering, I.D. 72 μm, L_c = 56.55 cm and L_d = 49.60 cm. Pressure injection time, 5 s.

TABLE II

MEASURED MIGRATION TIME t (min) AND $m_{EOF} \cdot 10^5$ ($cm^2/V \cdot s$) USING A HIST–MES BACKGROUND ELECTROLYTE AT pH 6.2

Capillary from Scientific Glass Engineering, I.D. 72 μm, L_c = 56.55 cm and L_d = 49.60 cm. Pressure injection time, 5 s. Applied voltage, 25 kV.

No.	0.0001 M MO in 100% background electrolyte		1% water and 99% background electrolyte	
	t	m_{EOF}	t	m_{EOF}
1	3.55	52.67	3.55	52.67
2	3.54	52.82	3.55	52.67
3	3.56	52.53	3.56	52.53
4	3.56	52.53	3.55	52.67
5	3.56	52.53	3.55	52.67

pH 6.2. In this instance the EOF marker lies behind the water dip. In Table III the m_{EOF} and effective mobilities of clenbuterol and benzoic acid are given, using for the calculation of the m_{EOF} (1) the beginning of the UV dip, (2) the lowest point of the UV dip (with MO present), (3) the middle of the UV dip (without MO) and (4) the UV peak of MO. The importance of the use of a "true" EOF marker will be clear considering the differences in the effective mobilities. The experiments with the system KOH–MES were carried out applying 10 kV, in order to avoid temperature effects, as this system shows much higher electric currents than HIST–MES owing to the higher con-

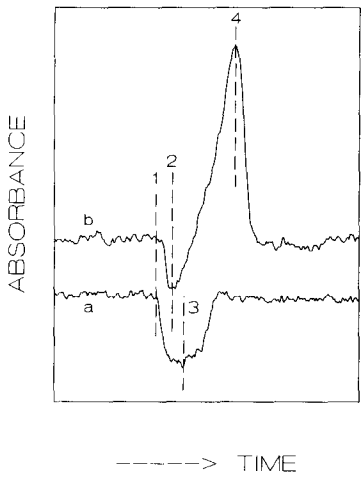


Fig. 8. Measured UV signal for a background electrolyte of 0.01 M KOH at pH 6.2 adjusted by adding MES, for a sample consisting of (a) 100% water and (b) 0.0005 M MO in water. (1) Beginning and (3) middle of the UV signal for 100% water and (2) lowest point and (4) top of the UV signal for 0.0005 M MO in water. Capillary from Scientific Glass Engineering, I.D. 72 μm, L_c = 56.43 cm and L_d = 49.83 cm. Pressure injection time, 1 s.

TABLE III

MEASURED MIGRATION TIME t (min) AND $m \cdot 10^5$ ($cm^2/V \cdot s$) USING A KOH–MES BACKGROUND ELECTROLYTE AT pH 6.2

Capillary from Scientific Glass Engineering, I.D. 72 μm, L_c = 56.43 and L_d = 49.83 cm. Pressure injection time, 5 s. Applied voltage, 10 kV.

EOF marker point	EOF		Clenbuterol		Benzoic acid	
	t	m	t	m	t	m
1	8.167	57.38	6.274	17.31	18.172	−31.59
2	8.240	56.88	6.274	17.82	18.172	−31.09
3	8.368	56.01	6.274	18.69	18.172	−30.22
4	8.406	55.75	6.274	18.95	18.172	−29.96

ducitivity of the system. If an EOF marker was used, it was carefully checked whether the migration times of the water dip and EOF marker were identical.

Electroosmotic flow

In Fig. 9 the measured velocity of the EOF, v_{EOF}, as a function of the applied voltage is given for the apparatus used at two different times. As expected, a linear relationship is obtained, although the values differ in time. The background electrolyte was the TRIS–MOPS system at pH 8.2. In both instances the EOF marker was dissolved in both water and buffer and identical values were obtained in each case.

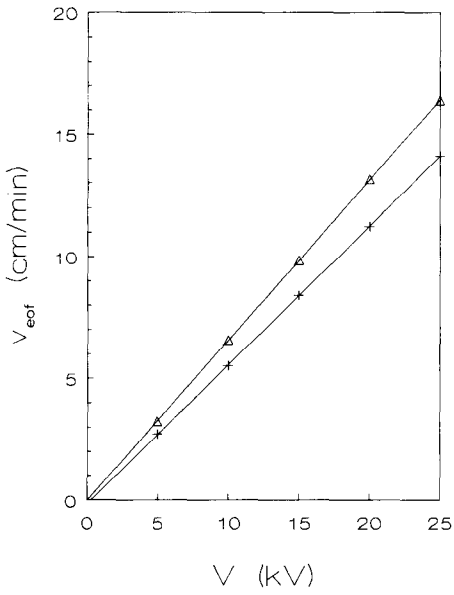


Fig. 9. Measured relationship between the velocity of the EOF and applied voltage at two different times. Background electrolyte, TRIS–MOPS at pH 8.2. Capillary from Scientific Glass Engineering, I.D. 72 μm, L_c = 56.95 cm and L_d = 50.05 cm. Pressure injection time, 5 s.

TABLE IV

$m_{EOF} \cdot 10^5$ (cm^2/V · s) AS A FUNCTION OF pH FOR THE ORIGINAL BECKMAN CAPILLARY (I) AND A SCIENTIFIC GLASS ENGINEERING CAPILLARY (II–V) AT DIFFERENT TIMES

For composition of background electrolytes, see Table I. V: Phosphate buffers.

pH	I	II	III	IV		V	
	m_{EOF}	m_{EOF}	m_{EOF}	pH	m_{EOF}	pH	m_{EOF}
3.8	33.8	30.2	28.0	3.8	27.8	2.5	15.1
4.4	36.2	37.1	35.0	5.0	52.4	3.0	26.8
5.0	47.5	–	–	6.1	45.3	4.0	36.1
6.2	55.6	56.2	53.1	7.0	44.5	5.0	47.1
7.5	61.7	61.8	60.4	7.9	62.4	6.0	56.9
8.2	69.9	72.1	73.1	9.0	59.4	7.0	65.0
						8.0	70.6
						9.0	73.1

In order to measure the effect of the pH on the EOF, measurements were carried out in several background electrolytes. In Table IV the m_{EOF} values for some electrolytes are given for the original Beckman capillary cartridge (I). Series II and III and series IV and V were measured with two different Scientific Glass Engineering (SGE) capillaries.

On running ultracentrifuged serum samples, a dramatic change in the EOF resulted. For a pH of 3.8, the migration time of the EOF changed from about 5.6 to 21.2 min for an SGE capillary. In order to examine what happens with time we measured the migration times of a mixture of amprolium, levamisol, clenbuterol (all

TABLE V

MEASURED MIGRATION TIMES t (min) AND EFFECTIVE MOBILITIES $m \cdot 10^5$ (cm^2/V · s) FOR A SAMPLE OF AMPROLIUM, LEVAMISOL, CLENBUTEROL, MESITYL OXIDE AND BENZOIC ACID WITH β-ALANINE–FORMIC ACID BACKGROUND ELECTROLYTE AT pH 3.8.

Capillary from Scientific Glass Engineering, I.D. 72 μm, L_c = 57 cm and L_d = 50 cm. Pressure injection time, 5 s. Applied voltage, 25 kV.

No.	Amprolium		Levamisol		Clenbuterol		EOF (MO)		Benzoic acid	
	t	m	t	m	t	m	t	m	t	m
1	4.206	36.22	5.444	25.95	6.780	19.07	21.229	8.95		
2	4.012	36.56	5.126	26.27	6.299	19.36	17.589	10.80		
3	3.855	36.80	4.881	26.45	5.938	19.52	15.231	12.47		
4	3.791	36.79	4.771	26.50	5.777	19.56	14.257	13.33		
5	3.740	36.84	4.692	26.55	5.666	19.59	13.624	13.96		
6	3.598	36.92	4.451	26.76	5.316	19.81	11.927	15.93		
7	3.498	36.89	4.306	26.69	5.110	19.75	10.900	17.43	23.139	−9.22
8	3.433	36.84	4.211	26.61	4.975	19.68	10.265	18.51	20.570	−9.27
9	3.390	36.75	4.140	26.59	4.875	19.67	9.844	19.30	18.917	−9.26
10	3.336	36.77	4.063	26.58	4.768	19.67	9.414	20.18	17.394	−9.26

TABLE VI

EFFECTIVE MOBILITIES $m \cdot 10^5$ ($cm^2/V \cdot s$) FOR SEVERAL IONIC SPECIES IN A HIST–MES BACKGROUND ELECTROLYTE AT pH 6.2

The sample components were dissolved in water (W) and background electrolyte (B). If the capillaries were rinsed with 0.1 *M* KOH the measurements are indicated as WR and BR. Capillary from Scientific Glass Engineering, I.D. 72 μm, L_c = 56.55 cm and L_d = 49.60 cm. Pressure injection time. 5 s. Applied voltage, 25 kV. Variances are given in parentheses.

Compound	Concentration (*M*)	W (5 expts.)	B (25 expts.)	WR (5 expts.)	BR (5 expts.)	Average
Procaine	0.0001	20.87 (0.07)	20.68 (0.02)	20.74 (0.06)	20.82 (0.09)	20.72 (0.17)
	0.00005	20.96 (0.00)	20.82 (0.11)	20.87 (0.06)	20.86 (0.05)	20.84 (0.10)
	0.00001	20.82 (0.00)	20.80 (0.19)	20.94 (0.05)	20.86 (0.27)	20.83 (0.18)
Clenbuterol	0.0001	18.13 (0.07)	18.30 (0.17)	18.15 (0.04)	18.10 (0.09)	18.06 (0.15)
	0.00005	18.21 (0.00)	18.10 (0.09)	18.12 (0.08)	18.11 (0.06)	18.12 (0.11)
	0.00001	18.09 (0.00)	18.10 (0.14)	17.93 (0.43)	18.07 (0.07)	18.07 (0.20)
Fenoterol	0.0001	16.12 (0.10)	16.03 (0.14)	16.06 (0.03)	16.06 (0.08)	16.05 (0.13)
	0.00005	15.91 (0.35)	16.08 (0.10)	16.14 (0.08)	16.10 (0.06)	16.08 (0.17)
	0.00001	16.05 (0.00)	16.09 (0.16)	16.19 (0.09)	15.92 (0.15)	16.07 (0.15)
Mesityl oxide	0.0001	52.17 (0.07)	51.04 (0.92)	48.83 (0.49)	51.89 (0.19)	51.01 (1.17)
	0.00005	52.09 (0.00)	50.64 (0.95)	47.68 (0.18)	51.40 (0.06)	50.33 (1.38)
	0.00001	51.94 (0.00)	50.55 (0.96)	47.36 (0.05)	51.34 (0.16)	50.43 (1.47)
Uric acid	0.0001	−22.40 (0.06)	−22.53 (0.10)	−22.35 (0.03)	−22.53 (0.06)	−22.49 (0.11)
	0.00005	−22.48 (0.02)	−22.58 (0.10)	−22.43 (0.03)	−22.52 (0.04)	−22.54 (0.09)
	0.00001	−22.58 (0.02)	−22.70 (0.11)	−22.62 (0.04)	–	−22.66 (0.10)
p-Hydroxyphenylacetic acid	0.0001	−26.41 (0.60)	−26.89 (0.54)	−26.57 (0.04)	−26.55 (0.27)	−26.73 (0.15)
	0.00005	−26.81 (0.02)	−26.88 (0.10)	−26.70 (0.04)	−26.82 (0.03)	−26.84 (0.10)
	0.00001	−26.95 (0.02)	−27.02 (0.09)	−26.94 (0.05)	–	−26.99 (0.08)
Benzoic acid	0.0001	−29.28 (0.05)	−29.41 (0.10)	−29.13 (0.04)	−29.31 (0.04)	−29.35 (0.13)
	0.00005	−29.41 (0.01)	−29.53 (0.12)	–	–	−29.51 (0.11)
	0.00001	−29.59 (0.03)	−29.69 (0.11)	–	–	−29.66 (0.11)

positive ions), MO (EOF marker) and benzoic acid (negative ion) ten times. After each run we rinsed the capillary repeatedly: 10 min with 0.1 *M* KOH, 10 min with water and 10 min with the background electrolyte. The result of the measurements are given in Table V. Although there appears to be a dramatic course of EOF with time, all the effective mobilities of the sample components were nearly constant.

It can be concluded from Table V that migration times (or apparent mobilities) can never be used for the identification of sample components without problems. Although in the above experiments severe changes in EOF occurred and hence also in the apparent mobilities of sample ionic species, we noticed that the effective mobilities were fairly constant.

Effective mobility

To investigate the reproducibility with time of the effective mobility, several experiments were carried out with a sample consisting of the positive ions procaine, clenbuterol and fenoterol and the negative ions of uric, *p*-hydroxyphenylacetic and benzoic acid. As EOF marker we always used MO. All measurements were always carried out several times on different days and the variance is given in parentheses (see Table VI). In order to study the effect of the sample concentrations we measured at three concentrations, *viz.*, $1 \cdot 10^{-4}$, $5 \cdot 10^{-5}$ and $1 \cdot 10^{-5}$ *M*. Further, we measured the sample components dissolved both in water and in background electrolyte. Between all measurements we only rinsed the capillary with background electrolyte for 5 min, except where the columns are headed WR and BR. In that case there was an extra rinsing step with 0.1 *M* KOH for 5 min and with water for 5 min. In Table VI all effective mobilities, calculated from the apparent mobilities, are given. Although sometimes the EOF differs, the effective mobilities are remarkably constant.

In Table VII all effective mobilities (in duplicate, calculated from the measured apparent mobilities) for the same components as in Table VI are given for several background electrolytes at different pHs (note that all electrolyte systems have a different ionic strength and equivalent concentration). The negative ions show smaller effective mobilities at low pHs (not fully ionized) and so do the positive ions at high pHs. Fenoterol is even negative, possibly owing to the ionization of phenolic groups at high pH.

Calculation of pK values and absolute mobilities

If effective mobilities are known in two different electrolyte systems, the absolute ionic mobility and the p*K* value of a component can be calculated using eqns. 9, 10 and 11.

In Table VIII the results of the calculations of p*K* values and absolute mobilities for several acids are given using the effective mobilities of the systems at pH 4 and 6.2 (HIST–MES) using CZE. The results are compared with data given by Hirokawa *et al.* [3] and with data obtained isotachophoretically using the concept of the isoconductor and specific zone resistance [17]. The CZE experiments were carried out using 50-μm capillaries. Compared with 75-μm capillaries, the peaks obtained for negative ions were much more gaussian, owing to the repulsive forces between anions and the negative wall charge. For cations, smaller diameters led to strongly tailing peaks. The ITP experiments were carried out with a leading electrolyte of 0.01 *M* HCl with EAC at pH 4 in combination with a terminator of 0.01 *M* pivalic acid, whereas for the system of

TABLE VII

EFFECTIVE MOBILITIES $m \cdot 10^5$ ($cm^2/V \cdot s$) FOR SEVERAL IONIC SPECIES IN SEVERAL BACKGROUND ELECTROLYTES AT DIFFERENT pH VALUES

Capillary from Scientific Glass Engineering, I.D. 72 μm, L_c = 56.55 cm and L_d = 49.60 cm. Pressure injection time, 5 s. Applied voltage, 25 kV. For the composition of the background electrolytes see Table I. 1 = Procaine; 2 = clenbuterol; 3 = fenoterol; 4 = mesityl oxide; 5 = uric acid; 6 = *p*-hydroxyphenylacetic acid; 7 = benzoic acid.

pH	1	2	3	4	5	6	7
3.9	22.49	19.25	17.42	29.45	− 0.81	−23.24	−11.35
	22.04	19.01	17.13	26.15	− 0.85		−11.07
5.0	21.39	18.87	17.02	52.23	− 7.60	−27.17	−26.93
	21.09	18.57	16.99	52.53	− 7.79	−27.29	−27.05
6.1	20.57	17.90	16.03	45.28	−20.48	−26.46	−29.06
	20.69	18.00	15.92	45.39	−20.55	−26.59	−29.25
7.0	19.96	17.19	14.28	44.52	−24.96	−26.42	−29.17
	19.84	17.30	14.39	44.42	−24.75	−26.30	−29.08
7.9	18.62	17.24	9.87	62.33	−25.30	−26.09	−28.94
	18.41	17.03	9.66	62.54	−25.44	−26.23	−29.03
9.0	11.20	14.55	− 1.11	59.36	−26.15	−26.38	−29.20
	11.20	15.43	− 1.11	59.36	−26.09	−26.33	−29.20

TABLE VIII

CALCULATED pK VALUES AND ABSOLUTE MOBILITIES $m \cdot 10^5$ ($cm^2/V \cdot s$) FOR SEVERAL ACIDS USING EXPERIMENTAL DATA FOR TWO DIFFERENT ELECTROLYTE SYSTEMS WITH (I) ISOTACHOPHORESIS AND (II) OPEN CAPILLARY ZONE ELECTROPHORESIS AND (III) LITERATURE VALUES

Capillary from Siemens, I.D. 50 μm, L_c = 77.33 cm and L_d = 70.53 cm. Pressure injection time, 1 s. Applied voltage, 25 kV.

Compound	(I) ITP		(II) CZE		(II) Ref. 3	
	pK	m	pK	m[a]	pK	m
m-Aminobenzoic acid	4.79	−31.49	4.74	−31.64[b]	–	–
Benzoic acid	4.18	−33.26	4.16	−33.40	4.19	−32.9
Hippuric acid	3.63	−27.50	3.60	−27.77	2.70	−25.3
p-Methoxyphenylacetic acid	4.37	−28.75	4.37	−29.03	4.36	−29.7
Nicotinic acid	4.85	−33.71	4.82	−33.44	4.82	−34.6
p-Nitrobenzoic acid	3.38	−31.92	3.49	−31.94[c]	3.52	−32.3
α-Dinitrophenol	4.01	−32.33	4.04	−32.39	4.02	−31.3
2,6-Dinitrophenol	3.65	−33.96	3.73	−33.99[c]	3.71	−31.3
Phenylacetic acid	4.29	−30.84	4.28	−31.10	4.41	−31.7
Propionic acid	4.89	−37.41	–	–	4.87	−37.1
Sulfanilic acid	3.12	−33.81	3.27	−33.93[c]	3.23	−33.7
Uric acid	5.55	−31.08	5.41	−29.99[d]	–	–

[a] Marked values were determined using electrolyte systems at pH values of [b] 4.7 and 6.2, [c] 3.5 and 6.2 and [d] 5.0 and 6.2.

pH 6 a leading electrolyte of 0.01 *M* HCl with HIST and 0.01 *M* MES as terminator was used.

From Table VIII, it can be concluded that absolute mobilities and p*K* values can be obtained in this way, provided that a good set of effective mobilities is available. Note that for this reason, for some ionic species (see Table VIII) pHs other than 4 were chosen in order to obtain larger differences between the degrees of dissociation (effective mobilities) for these components in the two electrolyte systems. For *m*-aminobenzoic acid a pH of 4.7 was chosen as the lowest pH in order to avoid the possibility that this component partially dissociates to a positive ionic form. For uric acid a pH of 5 was chosen as the lowest pH in order to obtain a real electrophoretic migration.

Zone electrophoresis for screening purposes

For qualitative screening, the most important question is whether the component of interest can be recognized from the matrix. As already shown, the effective mobility, which can be calculated from absolute mobility, p*K* value and EOF, can be used as a parameter. A complicating factor in the analysis of complex matrices is often the presence of an excess of one of the components such as sodium chloride in urine or serum. Beckers and Everaerts [15,16] showed that this can lead to different migration behaviour during the analysis. Schoots *et al.* [18] showed that if the composition of the sample (uraemic serum samples) is nearly constant, reproducible migration times are found, although the migration times differ considerably compared with those of the pure components.

To investigate the effect of the presence of a sample component in excess, we determined the effective mobilities of the mixture in Tables VI and VII (all components were 10^{-4} *M*, either in water or dissolved in buffer) and added increasing amounts of sodium chloride. In Table IX all effective mobilities, calculated from the measured apparent mobilities, are given. It can be concluded that up to about 0.01 *M* NaCl the effective mobilities are nearly constant, except for potassium and sodium, as they are not migrating in a proper CZE way. At higher concentrations of NaCl the effective mobilities decrease, although these values are reproducible. Using higher background concentrations this effect will, of course, diminish.

An interesting point in these experiments was that on adding larger amounts of NaCl to the sample, in the first instance a UV dip was obtained, but at a certain NaCl concentration the UV signal of the EOF marker increased rapidly. The explanation is that if a high concentration of NaCl is present, at the point of the sample injection (note: the EOF position) the local ionic strength is very high, and according to Kohlrausch an adaptation to this original concentration always takes place. This means that at the point of the EOF later in the analysis a higher background concentration will be found, giving a high UV signal if the background electrolyte shows UV absorption.

In Fig. 10 this effect is shown for samples of aqueous NaCl solutions (without EOF marker). For higher concentrations of NaCl there is an increasing UV signal at the point of the EOF. The consequence of this effect for complex matrices can be that uncharged components migrating at the EOF position are covered by this effect. The choice of a non-UV-absorbing background electrolyte will be important.

As an example of screening possibilities, we added the same components (0.0001

TABLE IX

EFFECTIVE MOBILITIES $m \cdot 10^5$ ($cm^2/V \cdot s$) DETERMINED IN AN INCREASING AMOUNT OF SODIUM CHLORIDE DISSOLVED IN WATER AND BUFFER SOLUTION WITH HIST–MES AS BACKGROUND ELECTROLYTE AT pH 6.2

Capillary from Scientific Glass Engineering, I.D. 72 μm, L_c = 56.43 cm and L_d = 49.83 cm. Pressure injection time, 5 s. Applied voltage, 25 kV. 1 = Procaine; 2 = clenbuterol; 3 = fenoterol; 4 = mesityl oxide; 5 = uric acid; 6 = *p*-hydroxyacetic acid; 7 = benzoic acid.

Solution	NaCl (*M*)	K	Na	1	2	3	4	5	6	7
Water	0	66.97	47.52	20.96	18.27	16.18	45.28	−21.70	−26.96	−29.53
	0.0001	68.00	47.65	20.86	18.15	16.26	45.61	−21.82	−27.11	−29.74
	0.0005	67.54	46.50	20.85	18.16	16.27	45.39	−21.78	−27.08	−29.69
	0.001	67.32	48.12	20.86	18.15	16.26	45.61	−21.76	−27.09	−29.68
	0.005	67.32	48.12	20.86	18.15	16.26	45.61	−21.70	−27.03	−29.62
	0.01		47.19	20.63	17.94	16.05	45.61	−21.64	−26.94	−29.53
	0.05		50.74	19.48	17.10	15.47	45.39	−21.33	−26.53	−29.09
	0.075		52.36	18.92	16.79	15.19	45.28	−21.22	−26.38	−28.92
	0.1		54.01	18.16	16.29	14.72	45.17	−21.20	−26.31	−28.81
Buffer	0	68.44	47.17	20.84	18.16	16.09	45.17	−21.77	−27.02	−29.63
	0.0001	70.44	47.52	20.96	18.27	16.18	45.28	−21.79	−27.06	−29.68
	0.0005	71.99	47.63	20.84	18.16	16.29	45.17	−21.71	−27.01	−29.58
	0.001	69.02	48.45	20.96	18.27	16.38	45.38	−21.70	−26.97	−29.55
	0.005		47.87	20.85	18.16	16.07	45.39	−21.75	−27.03	−29.62
	0.01		48.81	20.62	17.94	16.07	45.39	−21.63	−26.94	−29.50
	0.05		51.24	19.48	17.10	15.28	45.39	−21.42	−26.57	−29.15
	0.075		53.38	18.92	16.79	15.00	45.28	−21.31	−26.46	−28.98
	0.1		54.65	18.48	16.40	14.83	45.06	−21.21	−26.30	−28.79
10-Fold diluted urine spiked with components 1–7	–	69.99	47.08	20.29	17.82	15.94	45.72	−21.34	−26.71	−29.25

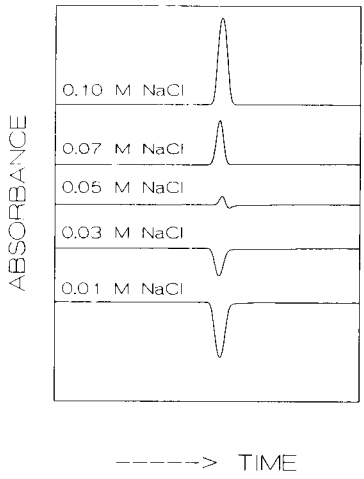


Fig. 10. Measured UV signal for the background electrolyte HIST–MES at pH 6.2 for different aqueous solutions of NaCl without EOF marker. For further explanation, see text. Capillary from Siemens, I.D. 50 μm, L_c = 77.33 cm and L_d = 70.53 cm. Pressure injection time, 1 s.

M) to 10-fold diluted ultrafiltered human urine (with about 0.015 M NaCl) and determined the effective mobilities of the components. These values are also given in Table IX, and it can be seen that the components can be easily recognized from the effective mobilities. Potassium and sodium are indicated by negative UV dips in the electropherogram. Using capillaries with small diameters (50 μm), cations showed increasing tailing peaks owing to wall attraction forces.

Separation number

An important aspect in screening is the separation number. This number indicates how many peaks can be distinghuished within a unit of effective mobility. As already indicated under Theoretical, this number can theoretically be about 4–8 for cations with mobilities of 20–30 and different EOF. In practice, this number will be much smaller because the total variance will be affected by the variances of the injection and detection and by several other effects causing zone broadening. To obtain an impression of the order of magnitude in practice we measured an electropherogram of a mixture of nineteen ionic species in a HIST–MES electrolyte system at pH 6.2 and calculated the separation number for the effective mobilities by

$$SN_m = \left| \frac{t_{m-0.5} - t_{m+0.5}}{4\sigma_m} \right| \tag{17}$$

In Fig. 11 the electropherogram for this separation is given and in Table X all data are presented.

In Fig. 12 the relationship between the separation numbers (both those according to eqn. 17 and the theoretical values, assuming a diffusion constant of $5 \cdot 10^{-10}$ m^2/s) and the effective mobilities is given for an m_{EOF} of $47.97 \cdot 10^{-5}$ cm^2/V · s.

From Fig. 12 it can be concluded that the experimentally obtained separation numbers are smaller than the theoretical values owing to several zone broadening

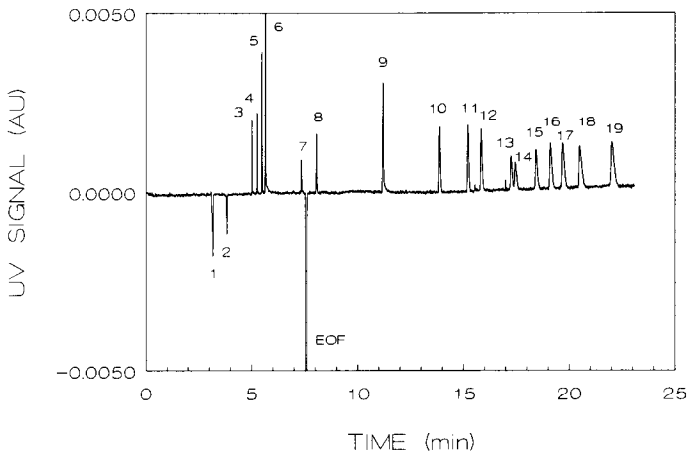


Fig. 11. Electropherogram of a mixture of nineteen components in a HIST–MES background electrolyte at pH 6.2. See Table X for the composition of the sample. Capillary from Siemens, I.D. 50 μm, L_c = 77.33 cm and L_d = 70.53 cm. Pressure injection time, 5 s.

TABLE X

MIGRATION TIMES t (min), EFFECTIVE MOBILITIES $m \cdot 10^5$ ($cm^2/V \cdot s$), PEAK WIDTH AT HALF-HEIGHT w (min), SEPARATION NUMBER SN AND THEORETICAL PLATE NUMBERS N FOR SEVERAL COMPONENTS IN A HIST–MES BACKGROUND ELECTROLYTE AT pH 6.2 AND MIGRATION TIMES t (min) AND EFFECTIVE MOBILITIES $m \cdot 10^5$ ($cm^2/V \cdot s$) OF THE COMPONENTS IN SPIKED HUMAN URINE

A and B: peak numbers of the components in Figs. 11 and 13, respectively. Peaks 8, 11 and 12 in Fig. 13 are unknown. Capillary from Siemens, I.D. 50 μm, L_c = 77.33 cm and L_d = 70.53 cm. Applied voltage, 25 kV.

Component	A	B	Sample mixture					Human urine	
			t	m	w	SN	N ($\times 10^{-5}$)	t	m
Potassium	1	1	3.19	66.01	0.045	0.37	0.278	3.16	65.26
Sodium	2	2	3.85	46.47	0.024	1.00	1.42	3.86	44.39
Levamisol	3	3	5.02	24.46	0.019	2.15	3.86	4.91	24.25
Procaine	4	4	5.27	21.03	0.018	2.49	4.74	5.14	20.93
Clenbuterol	5	5	5.49	18.26	0.017	2.87	5.77	5.35	18.15
Fenoterol	6	6	5.66	16.27	0.020	2.59	4.43	5.51	16.18
Creatinine	7	7	7.36	1.43	0.030	2.92	3.33	7.09	1.48
EOF			7.58	47.97	0.050	–	–	7.30	49.81
o-Nitrophenol	8		8.07	− 2.91	0.034	3.10	3.12		
Bromothymol blue	9		11.21	−15.53	0.043	4.73	3.76		
Uric acid	10	9	13.87	−21.75	0.065	4.79	2.52	12.94	−21.71
Hippuric acid	11	10	15.21	−24.06	0.074	5.06	2.34	14.09	−24.00
p-Methoxyphenylacetic acid	12		15.84	−25.01	0.081	5.02	2.11		
p-Hydroxyphenylacetic acid	13		17.26	−26.90	0.089	5.42	2.08		
Phenylacetic acid	14		17.45	−27.13	0.092	5.36	1.99		
p-Nitrobenzoic acid	15		18.44	−28.25	0.094	5.86	2.13		
Orotic acid	16		19.12	−28.95	0.110	5.38	1.67		
Benzoic acid	17		19.69	−29.50	0.123	5.11	1.42		
Sulphanilic acid	18		20.50	−30.23	0.129	5.28	1.40		
Aspirin	19		22.02	−31.46	0.150	5.24	1.19		

effects, especially for cations. In Fig. 13 the electropherogram of 10-fold diluted human urine spiked with levamisol, procaine, clenbuterol and fenoterol is shown. The migration times and calculated effective mobilities are given in Table X. Using the effective mobilities, the components (1) potassium, (2) sodium, (3) levamisol, (4) procaine, (5) clenbuterol, (6) fenoterol, (7) creatinine, (9) uric acid and (10) hippuric acid can easily be recognized.

CONCLUSION

It can be concluded from the foregoing experiments that in open capillary zone electrophoresis with EOF for low-molecular-weight substances, migration times or apparent mobilities can never be used for identification of the components. The effective mobility, however, which can be calculated from the migration time and the EOF velocity, can be used as a parameter for identification. The choice of a "true" EOF marker is extremely important.

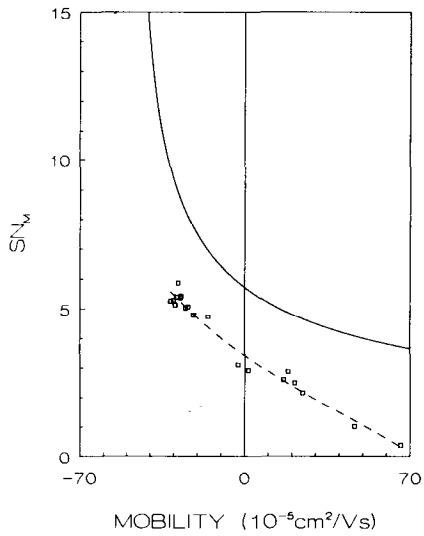


Fig. 12. Relationship between theoretical separation numbers (solid line) and experimentally determined separation numbers (dashed line) and effective mobility. The experimentally determined numbers were calculated from the electropherogram in Fig. 11.

If the ionic strength of the matrix is high compared with that of the background electrolyte, differences in effective mobilities can be expected, although they are reproducible if the matrix is of constant composition. Hence effective mobilities can be used for screening purposes.

From effective mobilities, measured in two different electrolyte systems at pH values where the degrees of dissociation of a component differ sufficiently, the absolute mobility and pK value can be calculated. The separation number, indicating how many components can be separated within a unit of mobility, is, however, much smaller than the theoretical values.

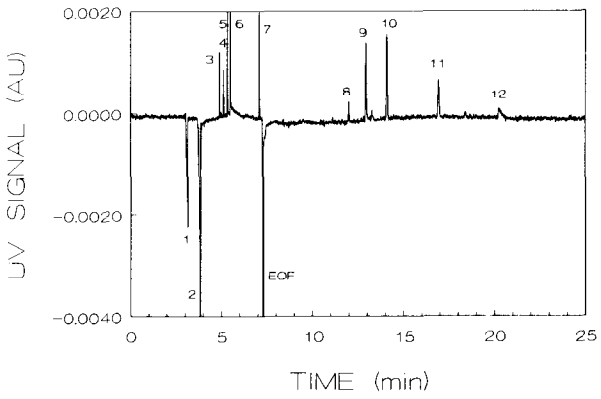


Fig. 13. Electropherogram of 10-fold diluted human urine, spiked with levamisol, procaine, clenbuterol and fenoterol (0.0001 M) in a HIST–MES background electrolyte at pH 6.2. See Table X for the composition of the sample. Capillary from Siemens, I.D. 50 μm, L_c = 77.33 cm and L_d = 70.53 cm. Pressure injection time, 1 s.

ACKNOWLEDGEMENTS

The authors express their gratitude to the State Institute for Quality Control of Agricultural Products (RIKILT, The Netherlands) for financial support of this investigation and gifts of several chemicals.

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