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# Peak dispersion and separation efficiency in high-performance zone electrophoresis with gel-filled capillaries

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ABSTRACT

The Einstein–Nernst equation for the relationship between diffusion coefficient, $D$, and ionic mobility, $u$, was applied to describe the peak dispersion in zone electrophoresis with gel-filled capillaries. It follows that the effective charge number is the only analyte-specific parameter that influences the plate height or the plate number. Peak broadening is thus independent of $D$ and $u$, because these parameters can be substituted by physical and instrumental constants. The plate number depends linearly on the effective charge number of the separands. Plate numbers in the range of several millions are predicted for highly charged molecules. This theoretical approach is discussed on the basis of electropherograms of oligonucleotides.

## INTRODUCTION

Capillary zone electrophoresis in gel-filled tubes is a separation technique of very high efficiency. Especially for oligo- and polynucleotides, extremely high plate numbers in the range of several millions have been achieved. These high plate numbers are usually attributed to the reduced diffusion coefficients, $D_i$, of the molecules in the gel matrix, apparently leading to large values for the plate number, $N_i$, according to the expression [1–4].

$$N_i = u_i U / 2 D_i \quad (1)$$

where $U$ is the applied voltage and $u_i$ is the effective ionic mobility of the separand, $i$. This equation was derived for conditions where only diffusion, and no additional effects on band broadening, such as Joule heating, hydrodynamic or electroosmotic flow, migration dispersion or extra-column contributions, take place.

It must be taken into account, however, that the electrophoretic migration caused by the gradient of the electric potential is influenced by the gel matrix in a way similar to the corresponding migration caused by the gradient of the chemical potential, the diffusion. The diffusion coefficient and the mobility are in fact related according to the Einstein–Nernst equation [*e.g.*, 5,6]:

$$D_i = u_i k T / z_i e_0 \quad (2)$$

where $k$ is the Boltzmann constant, $T$ is the absolute temperature, $e_0$ is the electronic charge and $z_i$ is the effective charge number of the ion, $i$. In principle, this equation is valid only for infinite dilution. For finite concentrations, it has two main limitations. One is based on the different concentration dependence of the ionic mobility and the diffusion coefficient: the mobility is normally more strongly decreased than the diffusion coefficient by increasing concentration. The second limitation arises for weak electrolytes when the diffusion coefficient of the ionic species, which is migrating by electrophoresis as well as by diffusion, differs from that of the uncharged molecule, which does not undergo electromigration.

In previous papers [7–10], the consequences of the replacement of the ratio $u/D$ in the expression for the plate number in capillary zone electrophoresis (eqn. 1) by the Nernst–Einstein equation, leading to

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TABLE I

DIFFUSION COEFFICIENTS, $D$, AND ELECTROPHORETIC MOBILITIES, $u$, OF NUCLEOTIDES WITH DIFFERENT BASE NUMBERS, $n$

$z$ is the charge number of the separand; under the given electrophoretic conditions, $z$ is assumed to be $n+1$.

| $n$ | $D$ ($10^{-6}$ cm$^2$/s) | $u$ ($10^{-5}$ cm$^2$/V · s) | $u/z$ ($10^{-5}$ cm$^2$/V · s) | $D/(u/z)$ ($10^{-2}$ V) |
|---|---|---|---|---|
| 1 | 1.42 | 11.52 | 5.76 | 2.47 |
| 6 | 0.490 | 11.48 | 1.64 | 2.99 |
| 8 | 0.415 | 11.44 | 1.27 | 3.27 |
| 10 | 0.351 | 11.38 | 1.03 | 3.39 |
| 12 | 0.310 | 11.30 | 0.869 | 3.57 |
| 14 | 0.287 | 11.07 | 0.738 | 3.89 |
| 16 | 0.269 | 10.85 | 0.638 | 4.21 |
| 18 | 0.239 | 10.57 | 0.556 | 4.30 |
| 20 | 0.218 | 10.34 | 0.492 | 4.43 |
| 22 | 0.201 | 10.08 | 0.438 | 4.59 |
| 24 | 0.188 | 9.87 | 0.395 | 4.76 |
| 26 | 0.166 | 9.65 | 0.357 | 4.61 |
| 28 | 0.163 | 9.44 | 0.326 | 5.01 |
| 30 | 0.146 | 9.22 | 0.297 | 4.91 |
| 32 | 0.146 | 9.03 | 0.274 | 5.34 |

$$N_i = z_i e_0 U / 2 k T \quad (3)$$

for systems without electroosmosis, was discussed. The same equation was derived by Giddings [1–3], setting equal the friction coefficients occurring in the transport equations for diffusion and electromigration such as in the derivatization of the Nernst–Einstein equation.

It can be seen from eqn. 3 that (within the limitations mentioned above) the plate number is dependent on the effective charge number as the only analyte-specific parameter, and not on diffusion coefficient and mobility. The validity of this transformation was demonstrated experimentally with ions with different charges in free capillary zone electrophoresis, carried out without electroosmotic flow [10]. In this work, this theoretical approach was applied to zone electrophoresis in gel-filled capillaries. Its validity is discussed based on diffusion coefficients of oligonucleotides, published by Yin *et al.* [11], and the corresponding mobilities, and also by comparison of the actual plate numbers with the maximum obtainable value.

## EXPERIMENTAL

Ionic mobilities were calculated from published electropherograms [11]. These electropherograms were obtained with oligonucleotides as separands [the 8-, 10-, 12-, 14-, 16-, 18-, 20-, 22-, 24-, 26-, 28-, 30- and 32-mers of a size marker mixture of the sequence d(GACT)$_n$ and d(GACT)$_n$GT]. Separation was carried out in a gel-filled capillary (100 $\mu$m I.D., 69 cm total length, 46 cm length to the detector; polyacrylamide gel made from 6% T, 5% C[a]; buffer 0.1 *M* Tris–0.25 *M* boric acid–7 *M* urea). The mobilities of adenosine-5′-monophosphate and pd(A)$_6$ were determined by extrapolation from the observed relationship between the mobility and the base number. Diffusion coefficients determined by the stopped-flow method were taken from the literature [11].

The second moments (variances), $\sigma^2$, caused by the finite volumes of injection and the detector cell were calculated from the corresponding rectangular functions with the width, $w$, by $\sigma^2 = w^2/12$. For the injection zone of the electropherogram under consideration, a length of about 0.25 mm was calculated from the given injection voltage and time [11]. For the length of the detector cell, a value of 0.7 mm was taken [12]. Based on the additivity of the variances, a value of 0.22 mm was derived in this in-

[a] C = g N,N′-methylbisacrylamide (Bis)/%T; T = g acrylamide + g Bis per 100 m of solution.

stance for the standard deviation, $\sigma_{z,ex}$, caused by both extra-column effects.

The peak widths based on time, $\sigma_t$, of the oligonucleotides were measured from the published electropherogram. [11]. From these values, the corresponding standard deviations based on length, $\sigma_z$, were calculated taking into account the different migration velocities, also derived from the electropherogram.

## RESULTS AND DISCUSSION

### *Relationship between diffusion coefficient and ionic mobility*

The ionic mobilities and the diffusion coefficients of the solutes under discussion are given in Table I. The diffusion coefficient and the normalized mobilities ($u_i/z_i$, the mobility of the ion with unit charge number) exhibit a similar dependence on the base number, $n$, namely an almost linear relationship between log $D$ and log $(u/z)$, respectively, and log $n$, as shown in Fig. 1.

According to the Nernst–Einstein equation, the ratio of the diffusion coefficient and the normalized mobility should be constant, as eqn. 2 can be transformed into

$$D_i/(u_i/z_i) = kT/e_0U \quad (4)$$

At 20°C, this ratio has the value 0.0255 V. It can be seen from Table I (assuming that $z$ equals $n + 1$) that this theoretical value is in fact observed for the 1- and 6-mers. Values for the other separands are in the same range, but they increase with increasing base number. The higher oligonucleotides show a deviation from the theoretically predicted value up to a factor of 2.

These deviations of the ratio $D_i/(u_i/z_i)$ of the separands with higher base numbers can be caused, besides a systematic error in the determination of the diffusion coefficients (the changes in peak widths, observed by the stopped-flow method, become very small for the higher oligomers), by the limitations of the Nernst–Einstein equation. For dilute aqueous solutions and simple electrolytes these deviations are, however, much lower, of the order of only about 10% [5,6].

On the other hand, the different experimental conditions in determining the diffusion coefficients

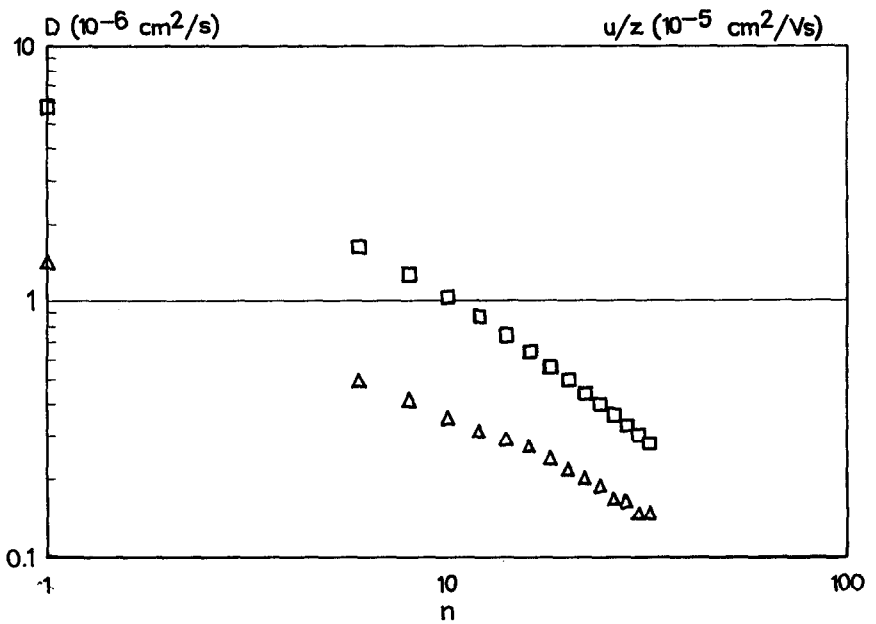


Fig. 1. Logarithmic plots of the dependence of (△) diffusion coefficient, $D$, and (□) normalized ionic mobility, $u/z$, on the base number, $n$, of the oligonucleotides. The normalized mobility is the mobility of the ion with unit charge. The charge number, $z$, is assumed to be $n+1$.

compared with mobilities may also be responsible for this deviation, briefly discussed as follows. The migration of a charged polymer in a gel is explained in the short-chain limit by a theory introduced by Ogston [13], where a sieving mechanism is assumed. The movement of large polymer chains, on the other hand, is best described by the so-called reptation theory [14–17]. This theory was modified, as additional stretching of the large polymer chain must be considered, resulting in field-dependent mobilities [15].

As in capillary zone electrophoresis high electric fields are applied (several hundred V/cm), possibly the conformation also of small fragments (even several-ten-mers) is influenced by the electric field. Hence it may be assumed that diffusion coefficients determined by the stopped-flow method, where no electric potential is applied along the capillary, will differ from the diffusion coefficients established under separation conditions, at least for higher oligomers, where this deviation was in fact observed.

Another reason for the observed deviation may be the fact that the effective charge number differs from the nominal charge number, as calculated from the base number, especially when large polymers are considered.

However, as diffusion and electrophoretic migration are influenced by the gel matrix in a similar way, the Einstein–Nernst relationship (which is generally applied in reptation theory) is also applied for the given conditions.

*Dependence of plate number on electrical charge*

It was discussed above that the substitution of the ratio of mobility and diffusion coefficient (eqn. 2) in eqn. 1 for the plate number leads to an expression that depends on, besides non-specific parameters such as voltage and temperature, only the effective charge number as an analyte-specific parameter (eqn. 3). At 20°C and with an effective voltage of 12 000 V (used in the experiments), the plate number may thus be calculated from $N_i = 237\,700 z_i$.

In the derivation of this expression, contributions to the peak width other than diffusion, namely temperature gradients or extra-column effects due to injection and detection, are ignored, in addition to the limited validity of the Nernst–Einstein relationship at higher concentrations, and the possible de-

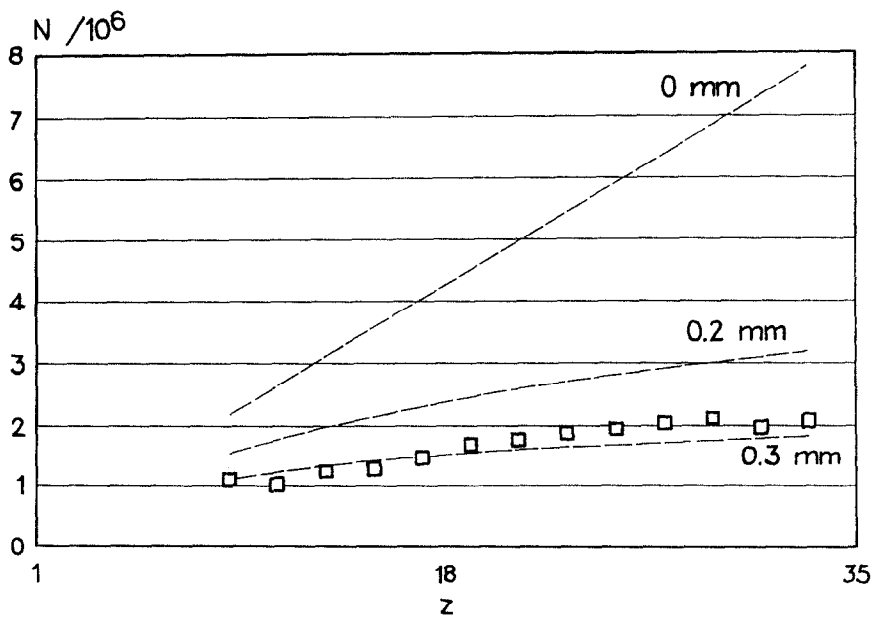


Fig. 2. Dependence of the plate number, $N$, on the effective charge number, $z$, of the separands. Conditions: effective voltage, 12 000 V; temperature, 20°C. Broken lines: theoretical dependence according to eqn. 3, for different extra-column contributions, expressed by the standard deviation, $\sigma_{z,ex}$ = 0 mm (without extra-column effects), 0.2 mm and 0.3 mm. □ = Experimental values obtained from the electropherogram of the oligonucleotides; $z$ is assumed to be $n + 1$.

viation of the effective from the nominal charge number. Therefore, this plate number represents the maximum obtainable value. It is shown in Fig. 2 as a function of the charge number of the separands, assuming that the charge originates from the dissociation of the phosphate groups only. In this instance, $z$ is connected to the base number, $n$, by $z=n+1$. This maximum plate number is, *e.g.*, 2 140 000 for the 8-mer and increases linearly to 7 840 000 for the 32-mer.

Owing to the finite injection volume and the volume of the detector cell, extra-column band broadening occurs, this effect being the greater the higher is the efficiency of the separation capillary. The theoretical dependence of the plate number was calculated for different extra-column contributions, namely for realistic values of 0.2 and 0.3 mm for $\sigma_{z,ex}$, the particular standard deviation based on length. The result of this calculation is shown in Fig. 2: the plate number increases with increasing charge number in both instances, but this increase is not as steep as for $\sigma_{z,ex} = 0$, and it is not linear. For the 32-mer, for example, only $2 \cdot 10^6$–$3 \cdot 10^6$ plates can be reached theoretically for an extra-column contribution corresponding to about 0.2–0.3 mm for $\sigma_{z,ex}$. This value for $\sigma_{z,ex}$ is realistic, taking into account the experimental conditions as discussed above.

The experimentally observed peak widths $\sigma_{z,i}$ (based on length) of the oligonucleotides are presented in Table II. They decrease with increasing base number. This means that the plate heights in fact decrease with increasing $n$, as was predicted from theory (eqn. 3).

TABLE II

PEAK STANDARD DEVIATIONS, $\sigma_z$, BASED ON LENGTH, OF THE OLIGONUCLEOTIDES WITH DIFFERENT BASE NUMBERS, $n$

The values were calculated from published electropherogram [11].

| $n$ | $\sigma_z$ (mm) | $n$ | $\sigma_z$ (mm) |
|---|---|---|---|
| 8 | 0.437 | 22 | 0.335 |
| 10 | 0.454 | 24 | 0.329 |
| 12 | 0.413 | 26 | 0.321 |
| 14 | 0.405 | 28 | 0.315 |
| 16 | 0.379 | 30 | 0.326 |
| 18 | 0.352 | 32 | 0.318 |
| 20 | 0.344 | | |

The decrease in the peak width with increase in base number cannot be attributed to the decrease in the diffusion coefficients, an argument which often occurs in the literature, because the resulting diffusion must not be considered independently from electromigration, and therefore from the ionic mobility, as discussed above. Taking the case that the ions of a species have identical diffusion coefficients, but different charges, and hence different electrophoretic velocities, they will then exhibit different migration times, $t$, in a column with (effective) length, $l$, resulting in different plate heights, $H$, according to $2\,Dt = Hl$. The ions with the higher charge are migrating faster, and therefore have a shorter time available for diffusion, which leads to a smaller plate height, despite the identical diffusion coefficients. This example demonstrates clearly that a discussion on plate numbers that is focused on diffusion coefficients alone must lead to a misinterpretation of the electrophoretic results.

From the standard deviations given in Table II, the plate numbers for the particular analytes were calculated according to the equation $N_i = l^2/\sigma^2_{z,i}$, for an effective length of 460 mm. The values are shown in Fig. 2, assuming that the charge number, $z$, is correlated with the base number, $n$, by $z=n+1$. The plate numbers in fact increase with increasing charge number, from about $1.1 \cdot 10^6$ for the 8-mer ($z=9$) to $2.1 \cdot 10^6$ for the 32-mer ($z=33$). It can be seen that the experimental values are in good agreement with those predicted from theory, taking a value of 0.22 mm for the extra-column contribution to $\sigma_z$ as derived under Experimental.

Further confirmation of the theory discussed here will be obtained by reducing the extra-column peak broadening, which should lead to higher plate numbers, approaching closer the maximum obtainable values, as expected by theory. This goal should be reached by decreasing the aperture of the detector cell, because this cell width has the most pronounced extra-column contribution on the total variance; this work is in progress in cooperation with Schomburg's group.

SYMBOLS

$D$ diffusion coefficient ($cm^2/s$)
$e_o$ electronic charge
$E$ electric field strength (V/cm)

$H$ plate height
$k$ Boltzmann constant
$L$ total length of the separation capillary
$l$ effective length; electrophoretic migration distance; distance from injector to detector
$n$ base number of oligonucleotide
$N$ plate number
$\sigma_{ex}$ peak standard deviation caused by extra-column effects
$\sigma_t$ peak standard deviation based on time
$\sigma_z$ peak standard deviation based on length; $\sigma_z = \sigma_t v$
$t$ migration or retention time
$T$ absolute temperature (K)
$u$ effective ionic mobility ($cm^2/V \cdot s$) of a charged particle, given by $u = vE$
$u/z$ reduced ionic mobility; mobility of the particle with unit charge number
$U$ effective voltage (V); potential drop from injector to detector across effective length
$v$ electrophoretic velocity (cm/s), given by $l/t$
$z$ effective charge number ($z$ also indicates the axis of migration)

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