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Capillary electrophoresis device with double UV detection and its application to the determination of effective mobilities of peptides

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Abstract

A new experimental device for high-performance capillary electrophoresis (HPCE) with a double UV detection system and with thermostating of the whole separation compartment was developed. UV detection is doubled by producing two apertures placed symmetrically close to the axis of the optical path of the single-beam UV detector and by the adjustment of the capillary loop on these two apertures. The double-detection system allows exact measurements of electrophoretic and electroosmotic flow velocities. A procedure for the determination of effective mobilities from the data obtained by the double UV detection system was developed and applied to determine the effective mobilities of synthetic peptides (diglycine, triglycine, growth hormone releasing peptide and its derivatives and fragments). The measurements are performed at constant temperature (25°C) and low input power at which temperature increase in the capillary can be neglected and temperature corrections of temperature-dependent magnitudes need not be included in the calculations.

1. Introduction

In the field of peptide chemistry capillary zone electrophoresis (CZE) is mostly used as a high-performance, high-sensitive technique for picoanalysis of both synthetic peptides and peptides isolated from natural material [1–3]. However, the application potential of CZE in this field is much broader. It can be used not only for purity determination of analyzed peptides but also for their more complex physico-chemical characterization. Important characteristics, e.g., effective charges, effective electrophoretic mobilities, dissociation constants, relative molecular masses and diffusion coefficients can be obtained from CZE data. Up to now only a few studies have been performed in which the effective electrophoretic mobilities of peptides have been measured by CZE and the correlation between the effective mobility, effective charge and relative molecular mass has been investigated [4–7].

All these measurements were performed with systems using single-beam UV detectors. This means the migration velocity was measured as an average velocity, with which the particle is moving from the injection end of the capillary to the detection position. It is obvious that not all such measurements are quite accurate, since the conditions are not always stable for the whole time of experiment.

In the beginning of the experiment the applied voltage, current and temperature are changing.

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Furthermore, depending on the sample solution and the background electrolyte (BGE) composition in the beginning of the experiment, different transient processes such as moving boundary electrophoresis or isotachophoresis can occur. Only after some time, which can be a significant part of the total time of an experiment, a real zone electrophoretic regimen is achieved and the separation conditions are stable and correct for mobility measurement.

Consequently, the electrophoretic mobilities obtained from such measurements represent average values, which are dependent on the given experimental device and conditions, which cannot be considered as qualitative characteristics of the analytes related to given background electrolyte and temperature only.

The aim of this work was to overcome these shortcomings of effective mobility determination in a single detector CZE device by a new design of the CZE device with a double UV detection system. In this device the migration velocity is measured on a well-defined part of the capillary between two UV detection positions, the distance of which is exactly known, where the sample components migrate under stable and equilibrated conditions [8].

This double-detection or multi-detection approach is suitable not only for effective mobility determination but also for monitoring the dynamics of electromigration separation processes. Recently some devices of such a type have been described in the literature. The system developed by Beckers et al. [9] uses two a.c. conductivity detectors, Terabe and Isemura [10] and Towns and Regnier [11] use two or several UV detectors placed along the capillary. The advantage of our system is that it uses, similarly as the system of Srichaiyo and Hjertén [12], only one UV detector and the capillary goes twice through it. In our device the whole separation compartment is thermostated to constant temperature (25°C). During the electrophoretic and electroosmotic flow velocities measurement the input power is lowered so that the temperature increase inside the capillary can be neglected and temperature corrections of temperature-dependent magnitudes need not be included in the calculation of electrophoretic mobilities.

2. Experimental

2.1. Chemicals

All chemicals were of analytical-reagent grade. Diglycine and triglycine were obtained from Reanal (Budapest, Hungary), phenol and acetic acid were from Lachema (Brno, Czech Republic).

Growth hormone releasing peptide and its derivatives and fragments were synthesized at our institute [13]. The list of analyzed peptides and their sequences are given in Table 1.

2.2. Capillary zone electrophoresis (CZE)

Separations were performed with the newly developed double UV detection CZE device, which is described in Section 3.

Acetic acid (0.5 mol/l, pH 2.5) was used as BGE. Sample was applied by at an overpressure of 0.006 bar for 5–15 s. Separations were performed at constant temperature 25°C.

Table 1
List of analyzed peptides and their sequences

Peptide	Sequence
His^1-GHRP:	H–His–D-Trp–Ala–Trp–D-Phe–Lys.NH_2
Tyr^1-GHRP:	H–Tyr–D-Trp–Ala–Trp–D-Phe–Lys.NH_2
Digly-GHRP:	H–Gly–Gly–His–D-Trp–Ala–Trp–D-Phe–Lys.NH_2
GHRP-frag.:	H–Ala–Trp–D-Phe–Lys.NH_2

GHRP = growth hormone releasing peptide.

3. Results and discussion

3.1. HPCE device with double UV detection

The scheme of the constructed double UV detection device for HPCE is shown in Fig. 1. The core of the device is a fused-silica capillary, C, with outer polyimide coating and with no modification of the inner surface (supplied by the Institute of Glass and Ceramics Materials of Czech Academy of Sciences, Prague, Czech Republic) with the following dimensions: I.D. 0.055 mm, O.D. 0.107 mm, total length 297 mm, effective length 1 (from the injection end to the first detector) 160 mm, effective length 2 (from the injection end to the second detector) 205 mm, distance of two detection positions 45 mm.

Polypropylene Eppendorf tubes (1.5 ml) serve as electrode vessels, EV. A high-voltage power supply, HV, (0.1–20 kV, 1–500 μA), developed earlier in our Institute was used in a constant-voltage mode.

Pneumatically formed overpressure, p, at the injection end of the capillary is used for filling and washing the capillary with BGE or other solutions ($\Delta p = 0.1$ bar) and for sample introduction ($\Delta p = 0.006$ bar).

The whole separation compartment (capillary, electrode vessels and detector) is thermostated by flowing air at 25°C. Combination of water cooling of the case of the separation compartment and heating wire inside the separation unit is used to quickly achieve the temperature equilibrium.

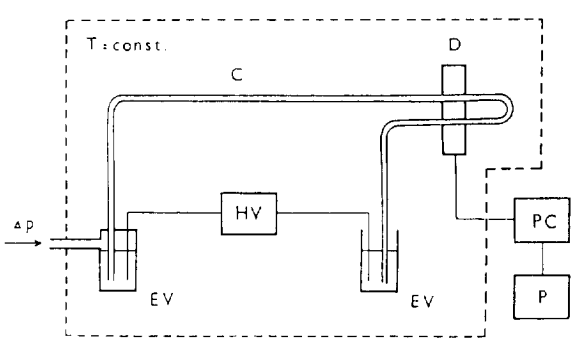

Fig. 1. Scheme of the HPCE device with double UV detection system. C = Fused-silica capillary; D = double UV detection system; HV = high-voltage power supply; EV = electrode vessels; PC = personal computer; P = printer; Δp = pneumatically formed overpressure, T = const. = thermostated space with constant temperature.

3.2. Double UV detection system

The device is equipped with a newly designed double UV detection system which allows measurement of UV absorption at two positions in the capillary. The schematic diagram of the total set-up of the detector is shown in Fig. 2a, the detail of the double passage of the capillary through the detector is shown in Fig. 2b. The detector uses some components of the previously developed device [14].

The UV light source is an iodine low-pressure electrodeless discharge lamp, I_2DL, (UV lamp type 1, LKB, Bromma, Sweden). The discharge lamp is excited by a high-frequency oscillator, HFO, with 100 MHz frequency and 6 W input

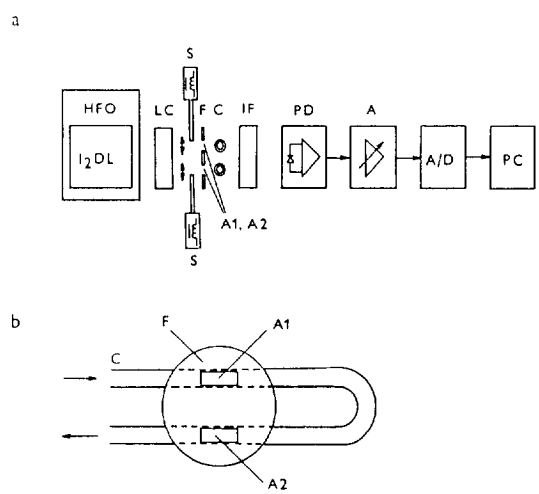

Fig. 2. Schematic diagram of the double UV detection system. (a) Total set-up: I_2DL = iodine discharge lamp; HFO = high-frequency oscilator; LC = light condensor; S = solenoids with needle cores; F = copper foil with two apertures A1, A2; C = cross-section of double passage of the capillary through the detector; IF = interference filter; PD = photodiode with built-in preamplifier; A = amplifier; A/D = analog–digital converter; PC = personal computer. (b) Detailed view of the double capillary passage through the detector: C = capillary loop; F = copper foil; A1, A2 = apertures to which capillary loop with two windows is fixed. Arrows indicate migration direction of an analyte through the double UV detection system.

power. The thermostating of the lamp at constant temperature (25°C) ensures the high stability of the light intensity and the detection system can work in a single-beam mode. The light condensor, LC, concentrates the light on two apertures with the dimensions 0.05×0.300 mm which are electrochemically produced on the copper foil, F, and which are placed symmetrically close to the optical axis of the detector. The capillary loop is attached to the foil in such a way that two windows of the capillary (short parts of capillary with removed polyimide coating) are placed exactly behind the two apertures. The adjustment and sealing of the capillary to the foil is performed under a microscope. The apertures can be alternatively opened or closed by the needle cores of two solenoids, S, situated between the light condensor and the foil.

After going through the capillary the light passes the interference filter, IF, (LKB, Bromma, Sweden) by which the detection wavelength 206 nm is selected. The light is detected by a silicone photodiode, PD, with a built-in preamplifier (type OP-AMP-Photodiode HUV 1000B, EG and G, Salem, MA, USA). The signal is further amplified by an operational amplifier, A, filtered and digitized by a 14-bit analog-digital converter, A/D. The data are collected during 100-ms sampling periods by a personal computer, PC (Xerius, 286/16, Elko, Prague, Czech Republic). Home-made software [15] is used for data acquisition, evaluation and presentation.

3.3. Determination of effective mobilities from the data of the double UV detection CZE device

A procedure has been developed for determination of effective electrophoretic mobilities of analytes separated by CZE with the double UV detection system. The procedure is based on exact measurement of migration velocity on a part of the capillary between the two detectors. Consider a mixture of a charged component, A, and a non-charged component (electroosmotic flow marker), M. The schematic electropherogram of CZE separation of this mixture in the device with double UV detection is shown in Fig.

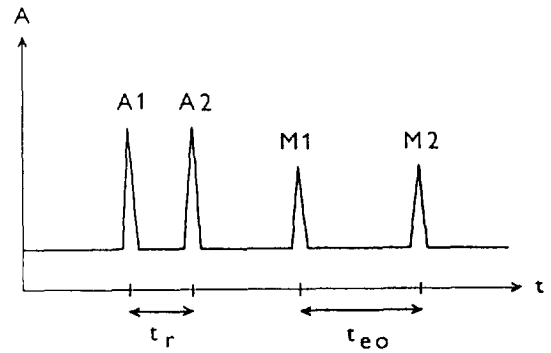

Fig. 3. Schematic electropherogram of the CZE separation of charged component A and non-charged component M obtained with the double UV detection system. A1 (A2) = peak of component A at the first (second) detection position; M1 (M2) = peak of component M at the first (second) detection position. A = absorbance; t = time, t_r (t_{eo}) = migration time of component A (component M) between two detection positions.

3. Peak A1 represents the passage of component A through the first detection position, peak A2 represents the passage of A through the second detection position. Similarly M1 and M2 represent the first and the second passage of M through the detector. From the time intervals t_r and t_{eo} and from the known distance of the detectors, d, the resulting migration velocity of charged component, v_r, and electroosmotic flow velocity, v_{eo}, are calculated.

$$v_r = d/t_r \tag{1}$$

$$v_{eo} = d/t_{eo} \tag{2}$$

Since the resulting migration velocity, v_r, is a sum of electrophoretic velocity, v_{ep}, and electroosmotic flow velocity, v_{eo}, the electrophoretic velocity, v_{ep}, can be obtained as a difference between v_r and v_{eo}:

$$v_{ep} = v_r - v_{eo} = d(t_{eo} - t_r)/t_r \cdot t_{eo} \tag{3}$$

The effective electrophoretic mobility, m_{ef}, is defined as electrophoretic velocity related to unit intensity of electric field, i.e.

$$m_{ef} = v_{ep}/E = v_{ep} \cdot l/U \tag{4}$$

where E is the intensity of electric field, U is the

voltage on the capillary and l is the total length of the capillary.

Combining Eqs. 3 and 4 we can obtain for m_{ef}

$$m_{ef} = \frac{l \cdot d \cdot (t_{eo} - t_r)}{U \cdot t_{eo} \cdot t_r} \quad (5)$$

i.e. effective electrophoretic mobility can be calculated from the capillary parameters l and d, from the voltage, U, applied on the capillary and from experimentally measured migration times t_{eo} and t_r.

The electrophoretic mobilities are relatively strongly dependent on temperature (ca. 2.5% change of mobility per 1°C). Therefore, the obtained values of effective mobilities have to be related to a defined temperature (mostly 25°C). This problem is solved in our procedure in the following way. The whole separation compartment, i.e., capillary and electrode vessels, is thermostated at a constant temperature of 25°C and the measurement of migration velocities is performed at such a low input power that the temperature increase inside the capillary due to Joule heat can be neglected. Consequently, the measured data are directly related to 25°C and no corrections of temperature-dependent magnitudes have to be included in the calculations of the electrophoretic mobilities.

In order to find out the level of input power up to which the temperature increase inside the capillary due to Joule heat can be neglected the dependence of driving current on the applied voltage was measured (see Fig. 4). In the linear part of this dependence the Ohm's law is valid, i.e., the resistance of BGE inside the capillary is constant. The deflection from linearity starts at voltages (input power), at which the Joule heat causes temperature increase inside the capillary and lowers the BGE resistance.

Consequently, the working conditions for CZE measurement of electrophoretic mobilities were chosen in the linear part of the current–voltage dependence. Most of separations were performed at 7.0 kV, 6.3 μA (0.15 W/m input power) which is sufficiently lower than the values at which the deflection of linearity starts (11 kV, 0.5 W/m). Obviously, using the double UV detection device it is not necessary to perform the whole experiment at such low input power. In order to speed up the measurements at the beginning of the experiment the higher voltage can be applied and only when the first analyte is nearing the first detection position the voltage is decreased to a lower value.

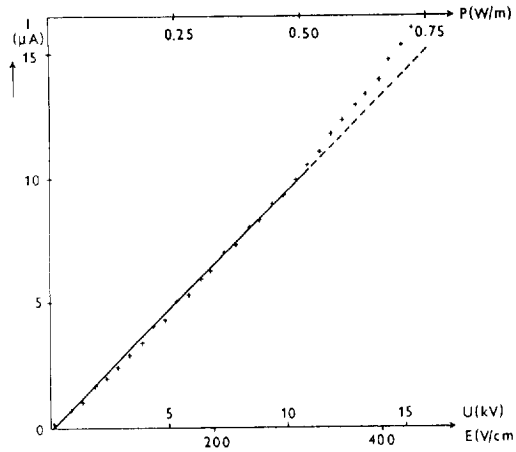

Fig. 4. Dependence of current, I, on the voltage, U, applied to the capillary in the flowing-air-thermostated separation compartment. E = intensity of electric field corresponding to applied voltage; P = input power corresponding to applied voltage and driving current in the capillary.

3.4. CZE analysis of peptides and determination of their effective mobilities.

The developed double UV detection CZE device was used for analysis of several synthetic peptides and for measurement of their effective electrophoretic mobilities.

The CZE separation of a test mixture containing synthetic peptides diglycine and triglycine and an electroosmotic flow marker, phenol, is shown in Fig. 5. As expected, separation of this three-component mixture by the double UV detection CZE device provides an electropherogram with six peaks. Generally, n-component mixtures will generate electropherogram with $2n$ peaks.

The higher peak heights of the same sample components at the first detection position (aperture A1, peaks 1, 2, 3 in Fig. 5) than at the second one (aperture A2, peaks 1′, 2′, 3′ in Fig.

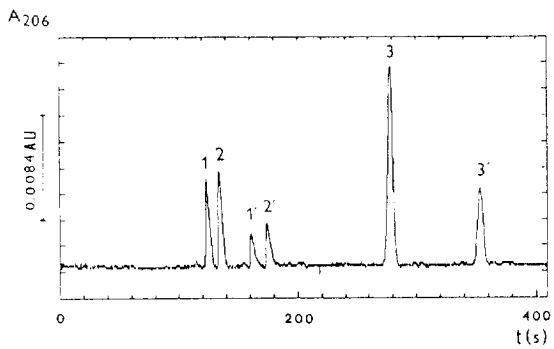

Fig. 5. Separation of a test mixture of diglycine, triglycine and phenol by the double UV detection CZE device. 1 (1′) = diglycine (1 mg/ml) in the first (second) detection position; 2 (2′) = triglycine (1 mg/ml) in the first (second) detection position; 3 (3′) = phenol (0.3 mg/ml) in the first (second) detection position. A = absorbance at 206 nm; t = migration time. Sample components dissolved in BGE (0.5 mol/l acetic acid, pH 2.5). Sample introduction overpressure 0.006 bar applied for 5 s. Voltage 7.0 kV, current 7.3 μA. The other separation conditions are given in the text.

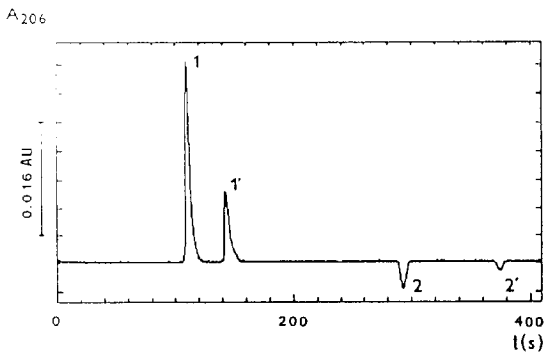

Fig. 6. Analysis of diglycyl growth hormone releasing peptide by the double UV detection CZE device. 1 (1′) = peptide peak in the first (second) detection position; 2 (2′) = peak of electroosmotic flow marker in the first (second) detection position. A = absorbance at 206 nm; t = migration time. Sample (1 mg/ml) dissolved in BGE. The other experimental conditions are given in the text and in Fig. 5.

5) reflect better adjustment of the capillary window on the aperture A1 than on the aperture A2. The signal of the detector is very sensitive on this adjustment. A small change in the relative position between the capillary and the aperture causes that some light is not going through the core of the capillary but through its walls which results in a signal decrease of the detector. However, the sensitivity of both detection positions of the developed system is sufficient for our measurements (noise at the level $4 \cdot 10^{-4}$ AU).

The double UV detection system was mostly used in a mode with simultaneous measurement of UV absorption at both detection positions, i.e., both apertures are opened and the sum of the light coming in the two light beams is measured by the photodiode. With respect to the relatively low light amount going through the miniature apertures, this mode is more suitable than the mode with alternatively opened and closed apertures, since the photodiode noise is relatively lower, i.e., the signal-to-noise ratio is higher at higher light intensities. If linearity of detection is preferred over its sensitivity, then the mode with alternatively opened apertures should be used.

CZE analysis of diglycyl growth hormone releasing peptide is shown in Fig. 6. From the differences of migration times of the peptides at the second and at the first detection position the resulting migration time of their movement between the two detectors is obtained and using the other experimental data (see Eq. 5) the effective electrophoretic mobilities of peptides were calculated. The results are summarized in Table 2.

The better reproducibility of effective mobilities obtained from shorter series (five experiments performed in series during few hours, RSD = 0.4–0.8%) than from the longer series (twenty experiments during few days, RSD = 1.2–1.3%) can be explain by the fact that local micro changes of the double-layer composition (electrokinetic potential) at the inner surface of the capillary, resulting in electroosmotic flow velocity variation, are smaller during shorter periods of time. Also the other sources of variance, such as e.g. slight changes of pH and ionic strength of BGE, can be expected to be smaller during the shorter time period than during the longer one. The uncertainty caused by a sampling period of 0.1 s for migration time, e.g. $t = 40$ s, $(0.1/40) \times 100 = 0.25\%$ should be also taken into account as a source of variance of the measured data.

Table 2
Calculated effective electrophoretic mobilities of peptides analyzed by the double UV detection CZE device

Peptide	$m_{ef} \cdot 10^9$ ($m^2 V^{-1} s^{-1}$)	z_{ef}	RSD (%)	n
Diglycine	25.6 ± 0.3	0.78	1.2	20
Triglycine	22.3 ± 0.3	0.84	1.3	20
Diglycine	25.1 ± 0.2	0.78	0.8	5
Triglycine	21.9 ± 0.1	0.84	0.4	5
His^1-GHRP	35.6 ± 0.3	3.0	0.8	5
Tyr^1-GHRP	23.5 ± 0.2	2.0	0.6	5
Diglycyl-GHRP	34.0 ± 0.2	3.0	0.6	5
GHRP-frag.	30.0 ± 0.2	2.0	0.7	5

m_{ef} = Effective electrophoretic mobility at 25°C (in 0.5 mol l acetic acid, pH 2.5), RSD = relative standard deviation, n = number of measurements.

Effective mobility, m_{ef}, of the peptide is a function of its effective charge and size (relative molecular mass, M_r). Several relations have been suggested to describe this dependence [7]. Among them the Offord equation [16] is the most frequently used:

$$m_{ef} = k \cdot z_{ef} \cdot (M_r)^{-2/3} \quad (6)$$

where k is constant of proportionality and z_{ef} is the effective charge of peptide. From this relation it follows that the ratio m_{ef}/z_{ef} should be directly proportional to $(M_r)^{-2/3}$.

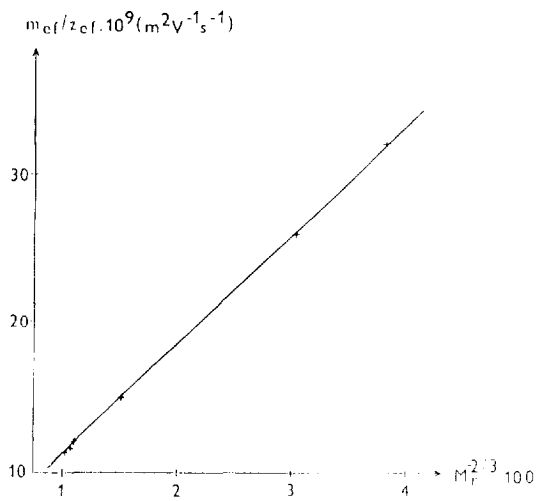

Fig. 7. Dependence of the ratio of effective mobility and effective charge, m_{ef}/z_{ef}, on the relative molecular mass, $(M_r)^{-2/3}$, of analyzed peptides.

In Fig. 7 the ratio of determined effective mobilities (see Table 2) and their calculated effective charges is plotted against relative molecular mass of peptides, $(M_r)^{-2/3}$. For calculation of the effective charges our previously developed program was used [17]. The very good agreement of our data with the Offord equation is evident.

4. Conclusions

The developed HPCE device with the double UV detection system was shown to be a suitable tool for the fast and accurate measurement of electrophoretic mobilities and potentially of other physico-chemical characteristics of the peptides analyzed or other substances.

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