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Z-Shaped flow cell for UV detection in capillary electrophoresis

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ABSTRACT

A longitudinal (Z-shaped) flow cell for improved UV detection for capillary electrophoresis was examined and compared with perpendicular (on-column) detection. Bending of a small section of the capillary column into a Z-shaped flow cell has no adverse influence on the electrophoresis process. With enhancements in signal-to-noise ratio of up to 6-fold for 3-mm Z-shaped flow cells, a considerable improvement in detectability is possible. The increase in signal of up to 14-fold in comparison with on-column UV detection illustrates the potential of this new type of capillary flow cell. The loss in resolution caused by the extended path length is less pronounced than expected, and can be tolerated in many of the CE separation modes.

INTRODUCTION

A wide variety of detection techniques have been developed for capillary electrophoresis (CE) and have been reviewed recently [1–3]. Owing to its universal nature and its ease of use, UV detection is still the most popular method. In order to minimize band spreading and to cope with the nanolitre zone volumes, on-column UV detection is mainly used. Nevertheless, with capillaries of I.D. less than 100 μm, considerable loss in sensitivity is encountered. Recently, several attempts have been made to extend the path length for UV detection in CE. Tsuda et al. [4] described the use of rectangular tubing with widths of up to 1 mm, while Grant and Steuer [5] extended the path length up to 3 mm by axial illumination of the capillary using laser-induced fluorescence with indirect UV detection. Owing to the presence of high background noise, however, the advantage of an increased path length could not be fully exploited with this approach.

In capillary LC the successful use of longitudinal flow cells with volumes of $\leqslant$90 nl and path lengths of up to 20 mm resulting in enhancements in the signal-to-noise ratio of up to 100-fold have been described [6]. It was therefore of interest to know if similar types of flow cells with shorter path lengths (maximum 3-4 mm) could be used for UV detection in CE, where the maximum tolerable path length is determined by the width of the zone rather than the peak volume. Further, the influence of the bends (Z shape) on the electrophoretic process and the peak distortion caused by

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the extended path length have to been established.

The aim of this work was to evaluate the performance of longitudinal (Z-shaped) capillary flow cells. The advantages and drawbacks are discussed.

EXPERIMENTAL

Reagents

Nucleosides (2′-deoxycytidine, 2′-deoxyguanosine), nucleotides (2′-deoxyguanosine-5′-monophosphate, 2′-deoxycytidine-5′-monophosphate) and peptides (met-enkephalin, leu-enkephalin, val-5-angiotensin II, angiotensin II) were purchased from Sigma (St. Louis, MO, U.S.A.). Other reagents and solvents were of analytical-reagent grade.

The compounds were dissolved in water at concentrations of 50 μg/ml each. For electrophoresis of the nucleosides and nucleotides a 75 m*M* sodium dodecyl sulphate (SDS)-10 m*M* phosphate-6 m*M* borate buffer (pH 8.5) was used and for the peptides a 50 m*M* SDS-10 m*M* borate buffer (pH 8.5). Buffer solutions and samples were filtered through 0.2-μm membrane filters (Chromafil; Macherey, Nagel & Co., Düren, Germany) and were stored at 4°C.

Flow cell construction

In order to minimize zone dispersion and to facilitate handling, the Z-shaped flow cell is constructed as part of the capillary column by bending a small section of the capillary into a Z shape. The bending of fused-silica tubing into Z or U configurations has been described elsewhere [7].

The flow cell (Fig. 1) is prepared by sandwiching a shim (made of alumina) with the bent capillary between two plastic disks (black polyethylene). The thickness of the shim and the sharpness of the capillary bends determine the total path length of the flow cell. For shims of 1 mm thickness, and for sharp bends, flow cells of *ca. 3* mm path length can be obtained. The shim has a centred hole of 300 μm that is adapted to match the O.D. of the fused-silica capillary. Each plastic disk has a groove to fix the capillary with epoxy resin in order to obtain a stable capillary flow cell. For on-column detection the same type of shims and plastic disks was used. Thus, the 300-μm hole of the shim served as an aperture for on-column detection, placed directly in front of the perpendicular capillary. The distance between the flow cell and the photodiode was *ca.* 3-4 mm and was equal for both types of flow cell.

Capillary column pretreatment

Capillary columns were made of fused-silica tubing of 50 or 75 μm I.D. and 280 μm O.D. (Polymicro Technologies, Phoenix, AZ, U.S.A.). Typically, the columns had a length of 60 cm, with 40 cm to the detector.

The columns were pretreated with 0.1 *M* sodium hydroxide solution for 5 min *(ca. 25* × capillary volume), followed by a 5-min rinse with water. After each injection, the column was rinsed with 0.1 *M* sodium hydroxide solution for 1 min *(ca. 5* × capillary volume), followed by a 1-min rinse with water.

Instrumentation

The basic electrophoretic apparatus was similar to that described in detail by

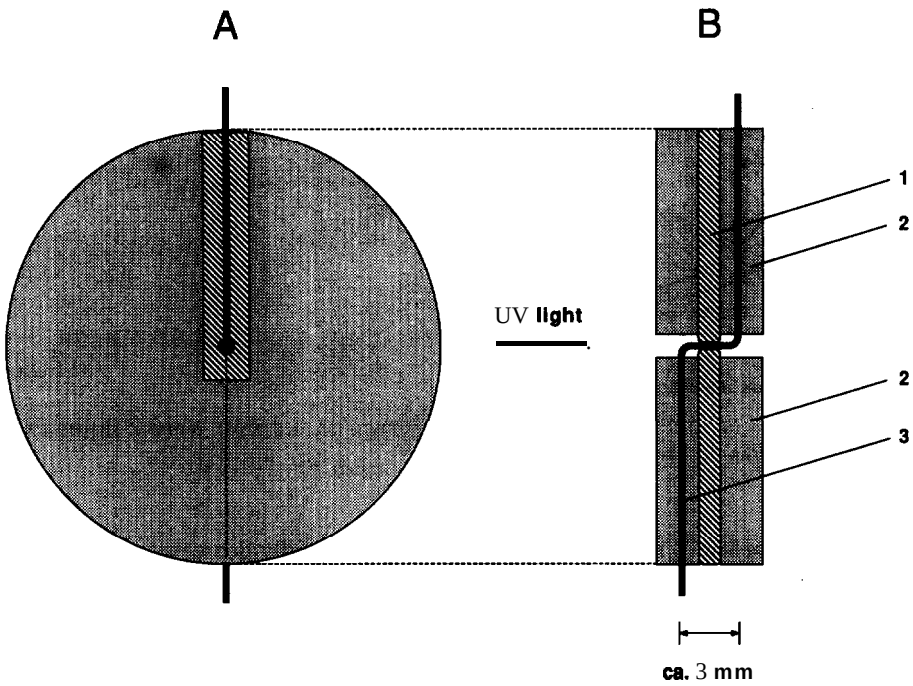


Fig. 1. Schematic diagram of the 3-mm Z-shaped capillary flow cell. (A) Front view; (B) cross-sectional view. 1 = Shim (alumina) with centred 300 μm I.D. hole; 2 = plastic disks (black polyethylene or Plexiglas); 3 = fused-silica capillary of 50 or 75 μm I.D., 280 μm O.D.

Jorgenson and Lukacs [8,9]. A variable-wavelength UV detector (Model 433; Kontron Instruments, Milan, Italy) equipped with a flow-cell holder was used for all measurements. The holder guarantees optimum positioning and alignment of the flow cell (Z shape and on-column). It minimizes the distance between the flow cell and the photodiode [10] and allows for easy capillary column replacement.

A d.c. power supply capable of delivering up to 30 kV (Alpha III, Model 3807; Brandenburg, Surrey, U.K.) was used to provide the high voltage. Sample introduction was accomplished manually by hydrostatic injections with a height of 10 cm for 5 s. All data were collected with a Kontron Datasystem 450, using MT-2 chromatography software (Kontron Instruments).

THEORY

UV detection

In *W* detection, flow cells of longer path length (l_c) exhibit increased sensitivities. If, however, the volume of the flow cell has to be minimized, the I.D. of the flow cell must be reduced, which results in a smaller aperture width (d_c). As a consequence, the optical transmittance of the flow cell decreases rapidly, resulting in poor linearity and increased noise levels. For *W* detectors with common cylindrical flow cells, the ideal optics have numbers for the optical aperture (d_c/l_c) within the range 1/10 to 1/5 [11]. Fig. 2 illustrates qualitatively this optimum aperture range for micro flow cells with volumes ranging from 2 to 20 nl.

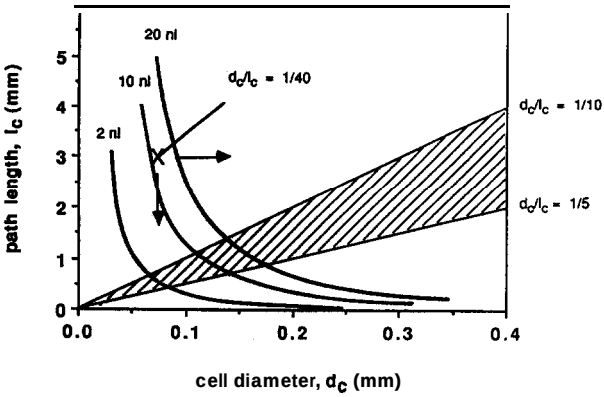


Fig. 2. Aperture ratios (d_c/l_c) for cylindrical flow cells. Optimum (shaded area), 3-mm Z-shaped (×); arrows indicate directions for aperture optimization.

For values below 1/10 the utilizable light energy rapidly decreases, resulting in increased noise and poor linearity. For values above 1/5 the path length becomes too short, resulting in poor sensitivity. Optimum values for d_c/l_c are in the shaded area. In principle, longitudinal (Z-shaped) capillary flow cells have the same cylindrical geometry as common flow cells used in *W* detection. Assuming that the same aperture ratios (d_c/l_c) are valid for the capillary flow cells, values far beyond the optimum of 1/10 result, e.g., 1/40 for a 3 mm × 75 μm I.D. capillary flow cell. However, the substantial gain in sensitivity with Z-shaped flow cells counterbalances the drawbacks of increased noise and loss in linearity. Nevertheless, ideal optical d_c/l_c ratios can only be accomplished with capillaries of larger I.D. and/or shorter path lengths.

Dispersion in CE

In CE, the dispersion of a zone can be expressed by its total variance [12,13]. Assuming an ideal Gaussian distribution of the zone, the total variance can then be summed in terms of its individual variances according to the well known relationship

$$\sigma^2_{tot} = \sigma^2_{col} + \sigma^2_{inj} + \sigma^2_{det} \quad (1)$$

where σ^2_{col} is the variance generated during the migration of the zone in the capillary column and σ^2_{inj} and σ^2_{det} are variances originating from the injection and detection system, respectively.

The spreading of the zone in the capillary (σ^2_{col}), which includes the longitudinal molecular diffusion, dispersion by Joule heating and deviation from the ideal electroosmotic plug flow, is usually the major contributor to σ^2_{tot}. For longitudinal flow cells with a path length longer than 3 mm, the detection system starts to exhibit a sizable contribution to the total peak distortion and therefore should be considered.

For an ideal rectangular zone, the dispersion of the detector cell (σ^2_{det}) can be expressed as

$$\sigma^2_{det} = l^2_c/12 \quad (2)$$

where l_c is the path length of the detector cell [14]. Hence the dispersion of a zone in a flow cell is determined mainly by the path length. Since in CE the separation is still in progress in the flow cell, it is advisable to define the influence of the path length as zone distortion, rather than dispersion.

Assuming that the injection and other factors that contribute to the total dispersion of the zone are relatively small in comparison with σ^2_{col} and σ^2_{det}, and by replacing σ^2_{tot} by l_c^2/N in eqn. 1, the following equation can be derived for the maximum tolerable path length ($l_{c\,max}$) of the flow cell [5]:

$$l_{c\,max} = [(12/N)^{1/2}]\,\{[f/(1-f)]^{1/2}\}\,L \qquad (3)$$

where f is the fraction of theoretical plates lost that is tolerated, N the number of theoretical plates and L the length of the capillary column (from column inlet to detector).

If a moderate loss in efficiency can be tolerated, path lengths of up to 3 mm are possible (see Fig. 3). For example, if a loss of 30% in plate numbers ($f = 0.3$) can be tolerated, we can calculate for a l-m column and an efficiency of 600 000 theoretical plates a maximum path length of almost 3 mm. In agreement with the Beer-Lambert law, the gain in sensitivity would be substantial.

RESULTS AND DISCUSSION

Influence of the bends (Z shape)

To examine the influence of the 3-mm Z-shaped flow cell on the electrophoretic process, without interfering with the UV detection, two similar capillary columns with on-column detection were used (see Table I). One of the capillaries had an additional Z-shaped flow cell (bends) in the separation part of the column. The two capillary columns were tested under identical conditions. Both capillaries showed identical electropherograms. The resulting efficiencies and asymmetries for the nucleoside–nucleotide test mixture are summarized in Table I.

As expected, the bending of a small portion of the capillary column into a Z

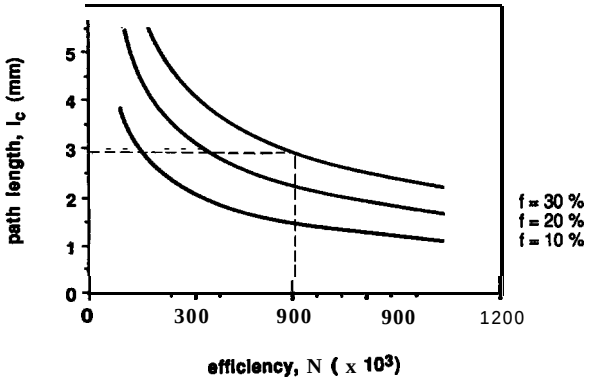
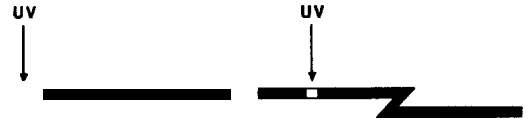


Fig. 3. Maximum tolerable path length vs. column efficiency for different fractional losses (f), in efficiency for a capillary column of 1 m length.

TABLE I

INFLUENCE OF BENDS (Z SHAPE) ON EFFICIENCY (*N*) AND ASYMMETRY (*s*)



Compound	On-column		On-column + bends	
	N ($\times 10^3$)	s	N ($\times 10^3$)	s
2′-Deoxycytidine	142	0.93	119	0.90
2′-Deoxyguanosine	166	1.09	155	1.07
2′-Deoxyguanosine-5′-MP	173	0.82	180	0.79
2′-Deoxycytidine-5′-MP	162	0.78	166	0.74

shape has virtually no influence on the electrophoretic process. The data for both types of capillary column are fairly similar with respect to our instrumentation (manual injection, no thermostating). No difference in the resulting current could be observed.

Noise

The Z-shaped flow cell is more susceptible to detection of impurities and contamination (so-called "chemical noise") than less sensitive on-column detection. Capillary washing and pretreatments that have been described for improving the reproducibility of the electrophoretic process [15,16] become important for stabilizing the baseline. The effect of capillary washing using a highly sensitive 5-mm Z-shaped flow cell is depicted in Fig. 4. In general, extended washing of the capillary with water (to

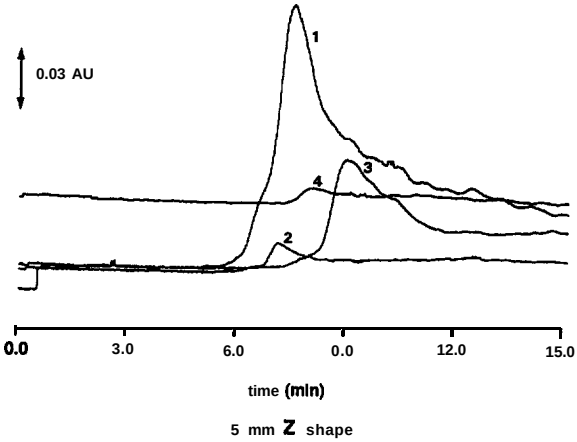


Fig. 4. Influence of capillary washing on baseline stability in MEC: 60 cm (40 cm to detector) × 75 μm I.D. fused-silica capillary; 6 m*M* borate–10 m*M* phosphate-75 m*M* SDS (pH 8.5) buffer; 11 kV, 30 μA; detection at 254 nm, rise time 2 s. All flushing steps are *ca.* 5 column volumes in 1 min. 1 = Flushing with 0.1 *M* NaOH and buffer; 2 = flushing with 0.1 *M* NaOH, water and buffer; 3 = flushing with 0.1 *M* HCl and buffer; 4 = flushing with 0.1 *M* HCl, water and buffer.

remove the acid or base) before flushing with buffer solution improves the baseline considerably. When no water was used after the acid or base treatment, variations in the baseline appeared.

Under static conditions (no voltage applied) both type of flow cell showed similar noise levels with values ranging from 0.040 to 0.062 mAU at 254 nm (see Table II). As soon as a high voltage is applied the noise increases in proportion to the voltage, as reported by Walbroehl and Jorgenson [17]. This increase in noise is even more pronounced with Z-shaped flow cells. At a moderate voltage of 11 kV the noise level is nearly twice that for on-column detection (0.125 vs. 0.067 mAU) utilizing a 50 μm I.D. capillary column. The results of the noise measurements for capillaries of 50 and 75 μm I.D. are given in Table II. Further, Table II lists data obtained using 75 μm I.D. capillaries at different wavelengths. At lower wavelengths, such as 210 nm, a considerable increase in noise was observed for the Z-shaped flow cell (0.40 vs. 0.15 mAU), caused by the larger, less UV-transparent volume segment of buffer solution captured in the longitudinal flow cell.

Signal and signal-to-noise ratio (S/N)

Figs. 5 and 6 show the micellar electrokinetic chromatography (MEC) [18–20] of nucleosides-nucleotides and peptides, respectively, using the two types of flow cell.

TABLE II

SIGNAL AND NOISE VALUES FOR Z-SHAPED AND ON-COLUMN FLOW CELLS

Parameter	λ = 254 nm			
	On-column flow cell		Z-shaped flow cell	
	50 pm I.D.	75 pm I.D.	50 pm I.D.	75 μm I.D.
Static noise (mAU)	0.041	0.040	0.057	0.062
Dynamic noise[a] (mAU)	0.067	0.062	0.125	0.105
Signal, S[b] (mAU)	4.1	6.6	29.0	67.0
$S/S_{on\text{-}column}$	1	1	7.1	10.2
S/N	62	106	232	638
$(S/N)/(S/N)_{on\text{-}column}$	1	1	3.1	6.0
	λ = 210 nm			
	On-column flow cell (75 μm I.D.)		Z-shaped flow cell (75 pm I.D.)	
Dynamic noise[a] (mAU)	0.15		0.40	
Signal, S[c] (mAU)	2.3		32.5	
$S/S_{on\text{-}column}$	1		14.1	
S/N	15		81	
$(S/N)/(S/N)_{on\text{-}column}$	1		5.4	

[a] At 11 kV.
[b] Measured with 2′-deoxyguanosine, peak No. 2, Fig. 5.
[c] Measured with val-5-angiotensin II, peak No. 3, Fig. 6.

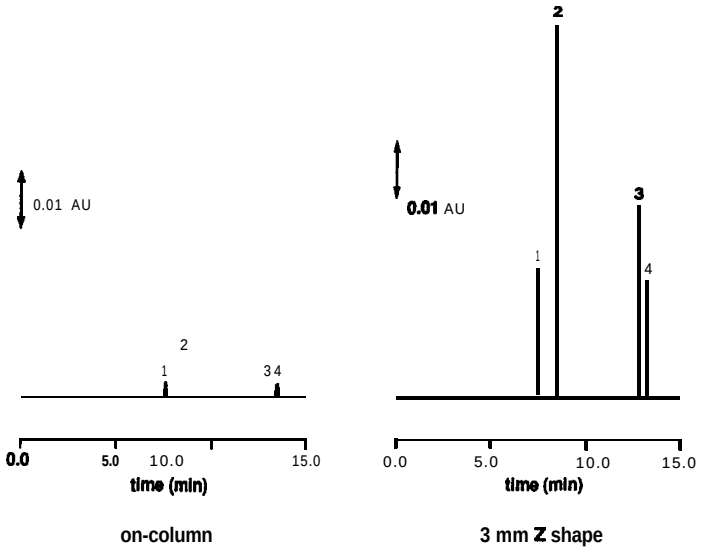


Fig. 5. Separation of nucleosides and nucleotides by MEC: 60 cm (40 cm to detector) × 75 μm I.D. fused-silica capillary; 6 m*M* borate–10 m*M* phosphate75 m*M* SDS (pH 8.5) buffer; 11 kV, 30 μA; hydrostatic injection 10 cm, 5 s; detection at 254 nm, rise time 2 s; 50 μg/ml (0.35 ng absolute) each, in water. Peaks: 1 = 2'-deoxycytidine; 2 = 2'-deoxyguanosine; 3 = 2'-deoxyguanosine-5'-monophosphate; 4 = 2'-deoxycytidine-5'-monophosphate.

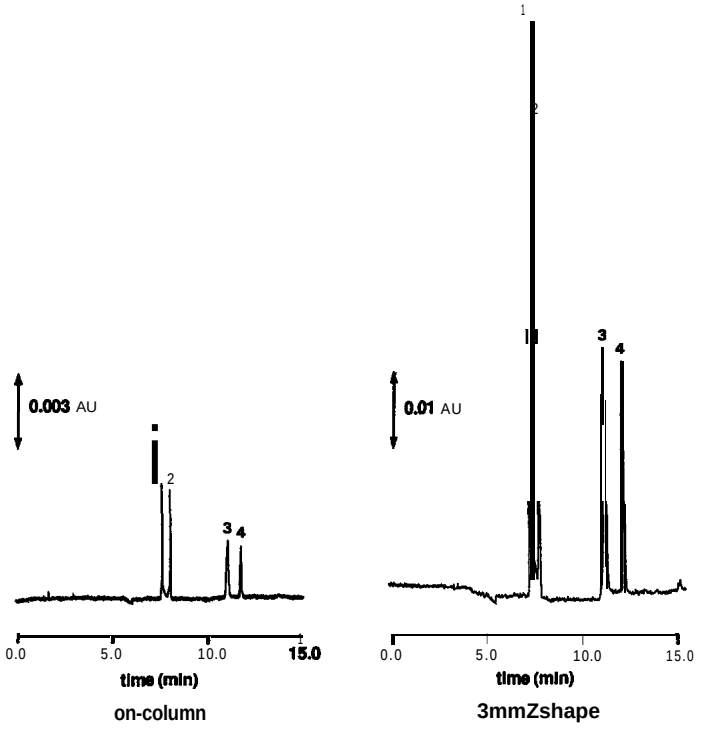


Fig. 6. Separation of peptides by MEC: 60 cm (40 cm to detector) × 75 μm I.D. fused-silica capillary; 10 m*M* borate50 m*M* SDS (pH 8.5) buffer; 11 kV, 20 μA; hydrostatic injection 10 cm, 5 s; detection at 210 nm, rise time 2 s; 50 μg/ml (0.35 ng absolute) each, in water. Peaks: 1 = met-enkephalin; 2 = leu-enkephalin; 3 = val-5-angiotensin II; 4 = angiotensin II.

At 254 nm enhancements in signal of up to 10.2-fold could be realized (see Table II). At a wavelength of 210 nm, a 14.1-fold improvement in the signal could be measured for val-5-angiotensin II.

Considering the noise levels listed above, an overall improvement in S/N of 6.0-fold at 254 nm and of 5.4-fold at 210 nm result when using the 3-mm Z-shaped flow cell.

Using a Z-shaped flow cell of cu. 4 mm path length, enhancements of more than lo-fold in S/N could be realised (see Fig. 7). Capillary columns of smaller I.D. (e.g., 50 μm) yields lower S/N. For 2'-deoxyguanosine an S/N of 3.7 was measured, in contrast to 6.0 with the 75 μm I.D. capillary column. Reducing the I.D. of the capillary column while maintaining the O.D. constant (280 μm) results in a thicker glass wall of the tubing, which reduces the proportion of light passing through the sample.

Maximum tolerable path length and observed column efficiency

Several workers [21,22] have shown that for on-column detection with a slit aperture, the maximum aperture length should not exceed 0.8 mm. Thus, for an optimum longitudinal flow cell the maximum acceptable path length should be $\leqslant 0.8$ mm to prevent distortion of the zone. In practice, however, a compromise must be made between maximum achievable sensitivity and minimum peak distortion. In Table III the loss in efficiency is given, for the electropherograms shown in Fig. 5. With a decrease of more than 30% for the 60-cm capillary column (40 cm to the detector), a considerable loss in efficiency results when using the 3-mm longitudinal flow cell. On the other hand, the loss in resolution is less pronounced, owing to its square root dependence on the efficiency. This is illustrated by comparing the resolution of the two electropherograms in Fig. 5. Alternatively, Z-shaped flow cells can be used in longer capillary columns (e.g., 120 cm, 100 cm to the detector) to minimize the loss in efficiency to an acceptable value (see Table III). The observed loss in efficiency shows good agreement with the theoretical values calculated with eqn. 3, and confirms its validity.

Despite the observed loss in efficiency of cu. 32% on average for the 60-cm

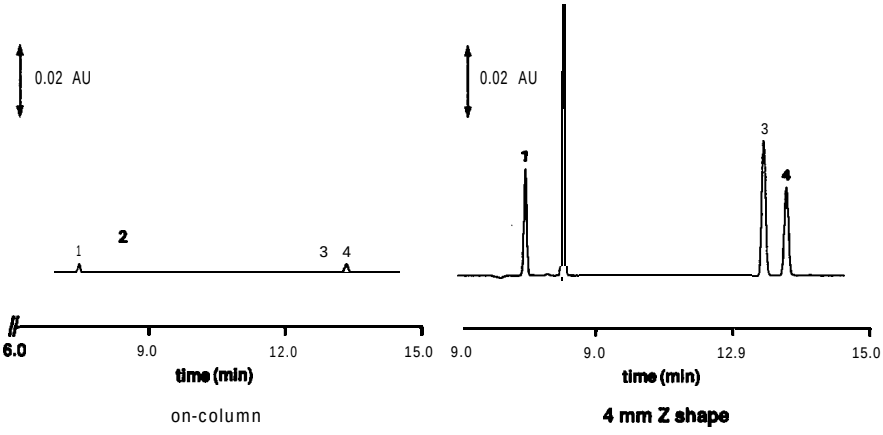


Fig. 7. Separation of nucleosides and nucleotides by MEC. Conditions and peaks as in Fig. 5.

TABLE III

CALCULATED AND OBSERVED LOSS IN EFFICIENCY (N)

Compound	60-cm capillary column (40 cm to detector)			
	On-column ($N \times 10^3$)	Z-shaped ($N \times 10^3$)	Loss (%)	
			Calc.[a]	Obs.
2′-Deoxycytidine	100	68.5	32	32
2′-Deoxyguanosine	145	98.2	40	32
2′-Deoxyguanosine-5′-MP	202	136	49	33
2′-Deoxycytidine-5′-MP	190	133	47	30
	120-cm capillary column (100 cm to detector)			
	On-column ($N \times 10^3$)	Z-shaped ($N \times 10^3$)	Loss (%)	
			Calc.[a]	Obs.
2′-Deoxycytidine	391	293	23	25
2′-Deoxyguanosine	382	322	22	16
2′-Deoxyguanosine-5′-MP	351	314	21	11
2′-Deoxycytidine-5′-MP	317	267	19	16

[a] Calculated with eqn. 3, assuming N = $N_{\text{on-column}}$.

column and of *ca.* 17% on average for the 120-cm column, the resolution is not greatly affected, demonstrating the usefulness of Z-shaped flow cells even for columns as short as 40 cm.

Linearity, detection limits and reproducibility

To evaluate the linearity of the Z-shaped flow cell independent of the electrophoretic process (*i.e.*, injection, temperature, column, etc.), the static linearity test was used [16]. For the solutes tested the linearity ranged from 2.5 to 3 orders of magnitude at both 210 and 254 nm, with response indices of 0.93 and 0.96, respectively.

At the high concentration end the calibration graphs flatten owing to stray light caused by the small aperture ratio. The limits of detection (S/N = 2) for the nucleosides were 0.31-0.47 and 0.51–0.63 μg/ml for the enkephalins, respectively.

In comparison with on-column detection, the detectability was up to six times lower when the 3-mm Z-shaped flow cell was used.

The reproducibility between flow cells was measured with a series of eight capillary columns. The entire series showed identical performance within the limits of our test system. Similar results in flow-cell reproducibility and enhanced detectability have been found for other type of detectors using 3-mm Z-shaped flow cells.

CONCLUSIONS

Capillary columns with Z-shaped flow cells provide higher sensitivities than conventional on-column detection. With a 6-fold improvement in S/N under CE conditions, the gain is less than one would expect just considering the increase in path

length (theoretically up to 80-fold). This is due to the fact that a considerable part of the light is probably guided by the glass wall of the capillary, the presence of scattered light and the increase in noise with the applied voltage. On the other hand, the substantial gain in the signal of up 14.1-fold confirms that Z-shaped flow-cell detection has significant potential when using common UV detectors. The observed loss in efficiency of 17-32% shows good agreement with the theoretical values. Most capillary electroseparations, in particular free solution, semi-preparative and MEC with typical efficiencies of (3-4) $\cdot 10^5$ plates/m, can be accomplished utilizing the Z-shaped flow cell without sacrificing much of the resolution.

To benefit fully from the advantages of the Z-shaped flow cell, however, further improvements are required. This would include synergy between the detector and flow cell. On the detector side, optimization of the light beam (parallel light rather than divergent or convergent) and adaptation of the photodiode (miniaturization) should reduce the noise considerably. On the flow cell side, the influence of the wall thickness, grounding of the flow cell, possible use of refractive index matching fluids, etc., should be further investigated.

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