Journal of Chromatography, 352 (1986) 337–343
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 728

VARIABLE-WAVELENGTH ON-COLUMN FLUORESCENCE DETECTOR FOR OPEN-TUBULAR ZONE ELECTROPHORESIS

JONATHAN S. GREEN and JAMES W. JORGENSON*
Department of Chemistry, University of North Carolina, Chapel Hill, NC 27514 (U.S.A.)

SUMMARY

A variable-wavelength on-column fluorescence detector employing a double monochromator has been developed. This detector is well suited for use with both open-tubular capillary electrophoresis and open-tubular liquid chromatography. Electrophoretically determined detection limits range from 4.6 pg for doubly labeled dansyl-lysine to 91 pg for bovine pancreatic trypsinogen. Detector linearity is demonstrated over 3 orders of magnitude for lysozyme, and 3.5 orders of magnitude for dansyl-leucine.

INTRODUCTION

Capillary electrophoresis has been shown to be a technique of high separation efficiency[1–4,7]. For small peptides, separation efficiencies of approximately one million theoretical plates have been demonstrated[1]. For capillaries of 1 m in length and 75 μm I.D., such efficiencies translate to zone widths (4σ) of only 4 mm in length or 18 nl in volume. If the detection system is to record the passage of a zone faithfully, without introducing distortion and broadening, an extremely small and cleanly swept cell and connecting tubing must be used. It is possible to use a post capillary "make-up flow" to sweep solutes migrating out of the end of the capillary into a conventional high-performance liquid chromatographic (HPLC) detector. This approach has the advantage that commercially available equipment is used, and it permits the use of cells of long optical path (10 mm) and wide aperture (1 mm). However, the volume of such a cell is approximately 10 000 times larger than the 1-nl cell volume required for the capillary just described. When the dispersion effects of the cell and connecting tubing are considered, a huge dilution of solute in the make-up flow is required in order to maintain the zone shape effectively.

A more direct approach to detection is "on-column" detection, where a portion of the capillary is itself used as the detector "cell". Yang[5] has demonstrated the advantages of this approach in microcolumn liquid chromatography (LC). On-column detection eliminates the need for any make-up flows, low dead-volume fittings, and connecting tubes. In early work on capillary zone electrophoresis, Mikkers *et al.*[6] utilized equipment devised for capillary isotachophoresis. Zone detection was accomplished on-column, by both conductimetric detection and UV-absorption de-

0021-9673/86/$03.50

tection. Although the sensitivity of this system was low, the feasibility of on-column detection was clearly demonstrated. Very sensitive on-column detectors are needed in order to work at low solute concentrations and thereby avoid sample "overloading" and its attendant zone broadening and skewing[3,6]. On-column UV-absorption detection in capillary electrophoresis was used with more success by Tsuda *et al.*[7] and by Jorgenson and Lukacs[1].

An on-column fluorescence detector for use in capillary electrophoresis, as well as capillary LC, has been described by Guthrie and Jorgenson[8]. This detector made use of filters for selection of excitation and emission wavelengths. Detection of proteins by their intrinsic tryptophan fluorescence requires excitation at 280 nm and detection of emission at approximately 330–340 nm. When interference filters were used to isolate the excitation and emission wavelengths, significant spectral overlap in the transmittance of these filters resulted in a large background signal and thus poor detection limits. In this paper, a system in which a double monochromator is used to select the excitation wavelength, and filters for the emission wavelength, will be described. Use of an arc lamp source and double monochromator allows selection of the excitation wavelength over a range of 200–800 nm. The double monochromator, in particular, provides extremely low levels of stray light (light outside the bandwidth), and this leads to lower background levels. Although this detector will be described for use in capillary electrophoresis, it is also of use in capillary LC.

EXPERIMENTAL

Fluorescence detector

Fig. 1 shows a schematic drawing of the optical layout of the fluorescence detector. The detector is equipped with a 200-W, high-pressure, short-arc, mercury–xenon lamp (HVF 200-1, Optical Radiation, Azusa, CA, U.S.A.) as a source. The source is housed in a Kratos LH 150 lamp housing (Kratos, Ramsey, NJ, U.S.A.). The lamp output is collimated by lens L1, a 38-mm diameter, 53-mm focal length, plano-convex, fused-silica lens (Kratos), and passed through a water-filled liquid filter (LHA 162, Kratos) to absorb a portion of the infrared radiation and thereby prevent overheating of the monochromator. The light is then focused onto the double monochromator (GM 200, Kratos) entrance slit with lens L2, a 30-mm diameter, 76-mm focal length, plano-convex, fused-silica lens (Kratos). The light leaving the monochromator is collimated by lens L3, a 25-mm diameter, 50-mm focal length, "Suprasil" synthetic fused-silica plano-convex lens (01LQP001, Melles Griot, Irvine, CA, U.S.A.), and then focused onto the capillary by an identical lens,

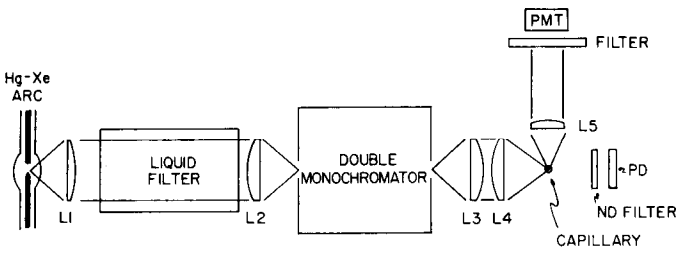

Fig. 1. Schematic diagram of the optical layout of the on-column fluorescence detector.

L4. Just past the capillary is a neutral density filter (10% transmittance for work at 280 nm excitation; 1% transmittance for work at 365 nm excitation; 03FNQ057 or 03FNQ065, Melles Griot), followed by a reference photodiode (SC39B), Hamamatsu, Middlesex, NJ, U.S.A.). The neutral density filter prevents saturation of the photodiode. At a right angle to both the capillary and the excitation light path, the fluorescence emission is collected by L5, a movable, 15-mm diameter, 25-mm focal length, Suprasil plano-convex lens (01LQP177, Melles Griot), and passed through an emission filter (470 nm cut-on filter, No. 5219, Oriel, Stratford, CT, U.S.A., for use with dansyl- and fluorescamine-labeled substances; 340 nm interference filter, Esco, Oak Ridge, NJ, U.S.A., for use with proteins) to the photomultiplier tube (PMT) (R-212, Hamamatsu). The PMT is housed in a Pacific Model 3150 housing (Pacific Precision Instruments, Concord, CA, U.S.A.) and connected to a Model 110 Photometer (Pacific Precision Instruments) for signal amplification. The photometer output and the reference photodiode signal are fed into an analog subtraction circuit, which has been described elsewhere[8]. This circuit permits some compensation for noise introduced by source intensity fluctuations.

The detector block (constructed in our laboratory) consists of a 7.6 × 7.6 × 4.6 cm aluminum block with a 4.6-cm diameter hole, bored 2.3 cm into the center, to allow for excitation. On the opposite face is a 3.0-cm diameter hole to allow for placement of the neutral density filters and the photodiode. As indicated, the collection optics are mounted at a right angle to this. At right angles to both the excitation and collection optics, there is a horizontal v-shaped groove, into which the capillary is secured with two nylon screws. The entire block is mounted on x–y–z positioners (Nos. 1620 and 1632, Oriel) to allow positioning of the capillary at the focus of the excitation beam.

Columns

Bare fused silica, 50 cm × 75 μm I.D. [Scientific Glass Engineering (SGE), Austin, TX, U.S.A.], was used for linearity and detection limit studies of dansyl- and fluorescamine-labeled solutes, and surface-modified fused silica, either 50 cm or 1 m × 75 μm I.D. (SGE), was used for protein work. The surface modification involved reaction of the surface silanols with a 5% aq. solution of 3-(glycidoxypropyl)-trimethoxysilane, using a procedure described by Chang *et al.*[9].

Electrophoresis apparatus

The basic electrophoresis apparatus has been described elsewhere[1]. The high-voltage supply (RHR30PN30, Spellman High-Voltage Electronics, Plainview, NY, U.S.A.) is capable of delivering up to ±30 kV. Sample "injections" are carried out by the electromigration technique previously described[1–4]. However, injections are now timed by a digital timing circuit (constructed in our laboratory) to allow for a higher degree of precision. Protein electrophoresis was done at +10 kV, while electrophoresis of dansyl- and fluorescamine-labeled solutes was done at +20 or +30 kV (see individual tables and figure legends for conditions).

Reagents

The fused-silica surface modification reagent, 3-(glycidoxypropyl)trimethoxysilane, was obtained from Petrarch Systems, Bristol, PA, U.S.A. The buffer for elec-

trophoresis was 0.0125 *M* (pH 6.86) phosphate buffer (J. T. Baker, Phillipsburg, PA, U.S.A.). Dansyl-amino acids, proteins, amylamine and fluorescamine were purchased from Sigma, St. Louis, MO, U.S.A.

RESULTS AND DISCUSSION

The detector linearity was studied using an adaptation of an ASTM method[10]. This method allows evaluation of detector linearity, independent of electrophoretic and sample injection effects. To demonstrate linearity at the two excitation wavelengths of primary interest, 280 and 365 nm, stock solutions of egg-white lysozyme and dansyl-leucine were prepared, and dilutions were made. The resulting standard solutions spanned 3.5 orders of magnitude for the dansyl-leucine, and 3 orders of magnitude for the lysozyme. The lysozyme concentration was limited by its insolubility at higher concentrations.

A bare fused-silica capillary (75 μm I.D.) was inserted into the detector and, at an excitation wavelength of 365 nm, the standard solutions of dansyl-leucine were drawn through the column, beginning with the least concentrated solution. The same procedure was followed for lysozyme at 280 nm, using a surface-modified capillary. A linear least-squares algorithm was then applied to the log–log data of response *vs.* concentration.

Scott[11] has defined a power function, which describes detector response for detectors that are approximately linear. The function is

$$y = Ac^r \tag{1}$$

where y = detector response in appropriate units, c = solute concentration, A = a constant, r = the "Response Index". For a detector that is linear, $r = 1$. If $r \neq 1$, the extent that it deviates from unity can be used as a measure of the deviation of the detector from linearity. Scott has arbitrarily stated that, for reasonable linearity, r should fall between 0.98 and 1.02. However, as long as r is known, calibration curves can be used with good success. The Response Index can be found quite simply by taking the logarithm of eqn. 1, yielding the following line equation:

$$\log y = \log A + r\log c \tag{2}$$

The slope of the resultant line is now r.

For our work, the slope (r) of the log–log plot was 1.00 for the dansyl-leucine,

TABLE I

STANDARDS: EXCITATION WAVELENGTH (λ_{ex}), EMISSION WAVELENGTH (λ_{em}), LINEAR RANGE (M), SLOPE BY LINEAR LEAST-SQARES ALGORITHM (r), STANDARD DEVIATION OF SLOPE (σ_r)

Solute	λ_{ex}(nm)	λ_{em}(nm)	*Linear range (M)*	r	σ_r
Lysozyme	280	340	$1 \cdot 10^{-6}$–$1 \cdot 10^{-3}$	0.995	$3.79 \cdot 10^{-5}$
Dansyl-leucine	365	490	$1 \cdot 10^{-6}$–$5 \cdot 10^{-3}$	1.00	$1.75 \cdot 10^{-5}$

TABLE II

DETECTION LIMITS

Solute	λ_{ex}	λ_{em}	*Concn. (M)*	*Concn. (ng/ml)*	*Mass (g)*	*Moles*
Trypsinogen	280	340	$2.0 \cdot 10^{-7}$	4800	$9.1 \cdot 10^{-11}$	$3.8 \cdot 10^{-15}$
α-Chymotrypsinogen	280	340	$1.0 \cdot 10^{-7}$	2500	$5.0 \cdot 10^{-11}$	$2.0 \cdot 10^{-15}$
Dansyl-ε-lysine	365	490	$2.4 \cdot 10^{-7}$	91	$5.2 \cdot 10^{-12}$	$1.4 \cdot 10^{-14}$
Di-dansyl-lysine	365	490	$1.8 \cdot 10^{-7}$	110	$4.6 \cdot 10^{-12}$	$7.5 \cdot 10^{-15}$
Fluor*-amylamine	365	490	$5.8 \cdot 10^{-7}$	210	$8.1 \cdot 10^{-12}$	$2.2 \cdot 10^{-14}$

* Fluorescamine-labeled.

and 0.995 for the lysozyme, indicating excellent detector linearity at two separate wavelengths (see Table I).

Table II shows electrophoretically determined detection limits for two dansylated amino acids (one of them doubly labeled). As expected, the mass detection limits are almost identical, while the molar detection limit for the doubly labeled amino acid is approximately half that of the singly labeled amino acid. In terms of molar concentrations, all solutes had detection limits within the same order of magnitude. Detection of solutes excited at 365 nm is limited by a noise level that is higher than expected. Although the source of the noise has not been identified, it is believed to originate, at least in part, in the arc lamp. Arc lamps are known to have problems with spatial stability of the arc. For conventional purposes, this does not interfere with measurements, because usual applications use 1-cm cuvettes, and this does not involve critical focusing. However, when the arc image is focused onto a monochromator slit, then refocused as a small spot onto a 75-μm capillary, the effect of arc wander can become substantial.

Fig. 2 shows the separation of a group of dansylated amino acids near the

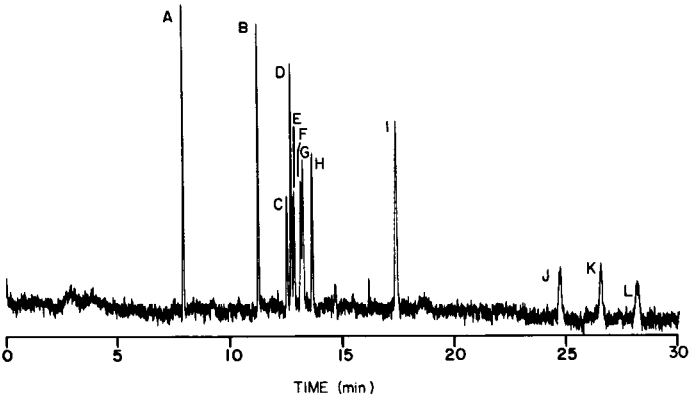

Fig. 2. Zone electrophoretic separation of 12 dansyl-amino acids on 100 cm × 75 μm I.D. fused silica: A = ε-labeled lysine; B = doubly labeled lysine; C = isoleucine; D = methionine; E = asparagine; F = serine; G = alanine; H = glycine; I = doubly labeled cystine; J = glutamic acid; K = aspartic acid; L = cysteic acid. The concentration of each solute is $5 \cdot 10^{-6}$ *M*, in 0.0125 *M* phosphate buffer (pH 7). Injection time, 1 s at 30 kV; electrophoresis at 30 kV.

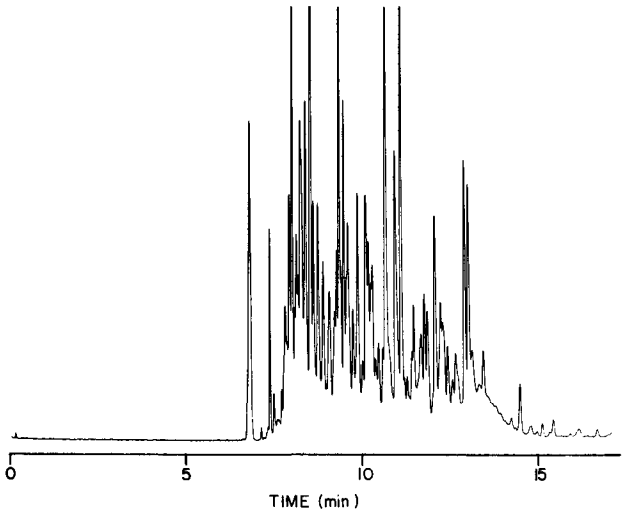

Fig. 3. Zone electrophoretic separation of fluorescamine-labeled peptides from a tryptic digest of chicken ovalbumin on 100 cm × 75 μm I.D. fused silica. The sample was dissolved in 0.0125 *M* phosphate buffer. Applied voltage, 30 kV. No attempt was made to identify the components.

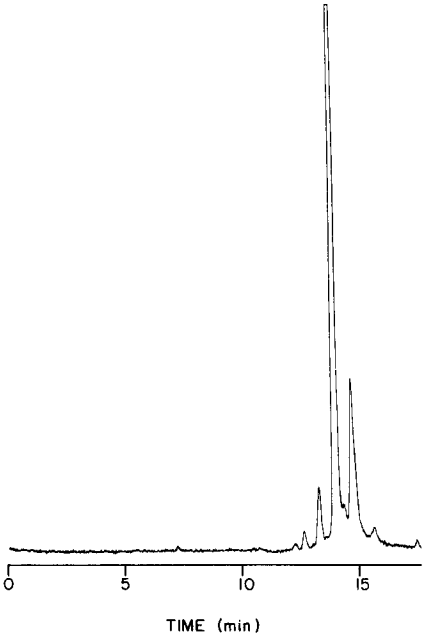

Fig. 4. Zone electrophoretic separation of bovine pancreatic trypsinogen on surface-modified 50 cm × 75 μm I.D. fused silica. The sample concentration was 0.2% (w/v) in 0.05 *M* phosphate buffer. Injection time, 5 s at 10 kV; electrophoresis at 10 kV.

detection limit. The purpose of the figure is to demonstrate the exceptional efficiency of the zone electrophoretic system. The separation efficiency of this system increases as the solute concentration drops. However, as concentration is lowered, detection becomes increasingly difficult. Thus the need for improved sensitivity is apparent. Fig. 3 shows a separation of fluorescamine-labeled peptides from a tryptic digest of chicken ovalbumin. Assuming that trypsin reproducibly cleaves ovalbumin, this method can be used as a "fingerprint" technique for identification of a protein. In other words, a given protein should yield the same number and magnitude of peaks following digestion and labeling, thereby allowing identification by means of the capillary electrophoresis system. Fig. 4 is the electropherogram of trypsinogen. The largest peak in this electropherogram corresponds to trypsinogen, while the smaller peaks are of unknown identity. The excellent sensitivity for detection of this tryptophan-rich protein (seven tryptophan residues) is apparent.

The fluorescence detector described in this paper has several inherent advantages over previously described detectors: (1) it is an on-column detector, and this eliminates the problems associated with post-column detection of minute volumes; (2) although the mercury–xenon arc lamp yields less light intensity than is available from a laser, it provides a choice of continuously variable excitation wavelengths, including several "well-placed" and intense mercury emission lines superimposed on the continuum; (3) the use of a double monochromator in the excitation beam allows the selection of narrower bandwidths than commercially available filters, and thus minimizes spectral stray light that reaches the photomultiplier tube. As a fluorescence detector, this system appears to provide versatility, while maintaining good sensitivity and convenience at relatively low cost.

As a group, proteins are not well suited to fluorescence detection, as detection relies on the intrinsic fluorescence of tryptophan residues, and tryptophan is one of the less common amino acids in proteins. However, a reasonable number of proteins do contain tryptophan, and this provides us with many suitable "model" proteins for studying separation of proteins by capillary electrophoresis.

ACKNOWLEDGEMENTS

Support for this work was provided by Hewlett-Packard Corporation, by the National Science Foundation under Grant CHE-8213771, and by the University Research Council of The University of North Carolina.

REFERENCES

1 J. W. Jorgenson and K. D. Lukacs, *Science*, 222 (1983) 266.
2 J. W. Jorgenson and K. D. Lukacs, *Anal. Chem.*, 53 (1981) 1298.
3 J. W. Jorgenson and K. D. Lukacs, *Clin. Chem.*, 27 (1981) 1551.
4 J. W. Jorgenson and K. DeArman Lukacs, *J. Chromatogr.*, 218 (1981) 209.
5 F. J. Yang, *J. High Resolut. Chromatogr. Chromatgr. Commun.*, 4 (1981) 83.
6 F. E. P. Mikkers, F. M. Everaerts and Th. P. E. M. Verheggen, *J. Chromatogr.*, 169 (1979) 11–20.
7 T. Tsuda, K. Nomura and G. Nakagawa, *J. Chromatogr.*, 264 (1983) 385.
8 E. J. Guthrie and J. W. Jorgenson, *Anal. Chem.*, 56 (1984) 483.
9 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 120 (1976) 321.
10 *Standard Practice for Testing Fixed-Wavelength Photometric Detectors Used in Liquid Chromatography*, ANSI/ASTM E 685-79, American Society for Testing and Materials, Philadelphia, PA, 1979.
11 R. P. W. Scott, *Liquid Chromatography Detectors*, Elsevier, Amsterdam, 1977, Ch. 2.