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Detection and quantification of capillary electrophoresis zones by fluorescence microscopy[a]

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SUMMARY

A fluorescence detector for capillary electrophoresis was built using an epillumination fluorescence microscope equipped with a 50-W mercury lamp and a photodiode. The performance of the detector was tested with riboflavin and fluorescamine-derivatized amino acids and amphetamine. We compared fluorite and conventional glass objectives and found that, by using a 25-μm I.D. capillary and a fluorite objective, an improvement in fluorescence detection by a factor of 56 can be achieved. A detection level of 500 amol for riboflavin was reached (using on-column detection). This sensitivity was at least comparable to that reported for other capillary electrophoresis systems using on-column non-coherent light fluorescence detectors. The potential and advantages of using a fluorescence microscope for capillary electrophoresis applications are discussed.

INTRODUCTION

Capillary electrophoresis (CE) is a powerful separation technique that resolves analytes into zones of a few nanoliters[1–5]. These features require the ability to detect subpicomole masses, usually dissolved in nanoliter volumes. Several types of detection mode have been used with CE, but fluorescence detection has emerged as one of the most sensitive[1–5]. Laboratory-made on-column fluorescence detectors, equipped with non-coherent light sources, have been specially designed for CE[6–8]. In these detectors, either a mercury or a mercury–xenon arc lamp provides the source of excitation.

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Lenses located at a distance of 1 cm from the capillary are used to focus the excitation radiation on the capillary and to focus the emitted light on the photodetector. Similar designs have been incorporated into commercially available fluorescence detectors for CE[9]. In all these instruments, the fluorescence is collected at right-angles to the main axis of the capillary and the excitation beam. The detection limit for fluorescamine derivatized compounds is about 10 pg of fluorophore for these kinds of detectors[7].

On the other hand, fluorescence microscopes have been optimized to detect fluorescence in small samples. This is due to their large numerical aperture (NA) objectives and high-quality optical components. For this reason, the fluorescence microscope might be a good CE fluorescence detector. Particularly interesting in this regard is the epillumination fluorescence microscope. In this type of instrument, the excitation and the detection of fluorescence occur at the same side of the capillary (colinear arrangement), and the focusing and alignment of the capillary can be guided visually to obtain the optimum response. This paper describes a procedure for adapting a fluorescence microscope to CE, and presents the results of several tests that were conducted to elucidate the optimum optical conditions needed to detect fluorescence in a small capillary.

EXPERIMENTAL

Instrumentation

The microscope was a Zeiss Standard 14 IFD epillumination fluorescence microscope (Carl Zeiss, Thornwood, NY, U.S.A.). The excitation lamp was a 50-W, high-pressure mercury lamp. The following combination of filters was adapted to the microscope: a band-pass excitation filter to cut radiation above 405 nm; a chromatic beam splitter to reflect radiation below 420 nm and to refract radiation above 420 nm; and a long-pass filter (located between the eyepiece and the dichroic mirror) to suppress radiation under 418 nm. A direct current high-voltage power supply was used (Spellman High Voltage Electronics, Plainview, NY, U.S.A.). High-voltage was applied through platinum–iridium electrodes for both electrokinetic injections and sample separations. For recording the signals, a Model L-6512 strip-chart recorder was used (Linseis, Princeton Junction, NJ, U.S.A.) at 20 cm/h and 1 mV output.

Reagents

Sodium tetraborate was obtained from EM Science (Gibbtown, NJ, U.S.A.). Riboflavin, leucine, isoleucine, threonine, valine, serine and amphetamine were purchased from Sigma (St. Louis, MO, U.S.A.). Fluorescamine was obtained from Roche Diagnostic Systems (Nutley, NJ, U.S.A.). Hydrochloric acid and acetone were of HPLC grade from J. T. Baker (Phillipsburg, NJ, U.S.A.). All buffer and sample solutions were prepared with deionized water with 18 MΩ resistance from a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Procedure

The principles of adaptation of fluorescence microscopy to CE are shown in Figs. 1 and 2. Fig. 1 shows the excitation of the sample and Fig. 2 shows the emission and measurement of fluorescence.

Excitation (Fig. 1). The excitation lamp emits a mixed beam of visible and

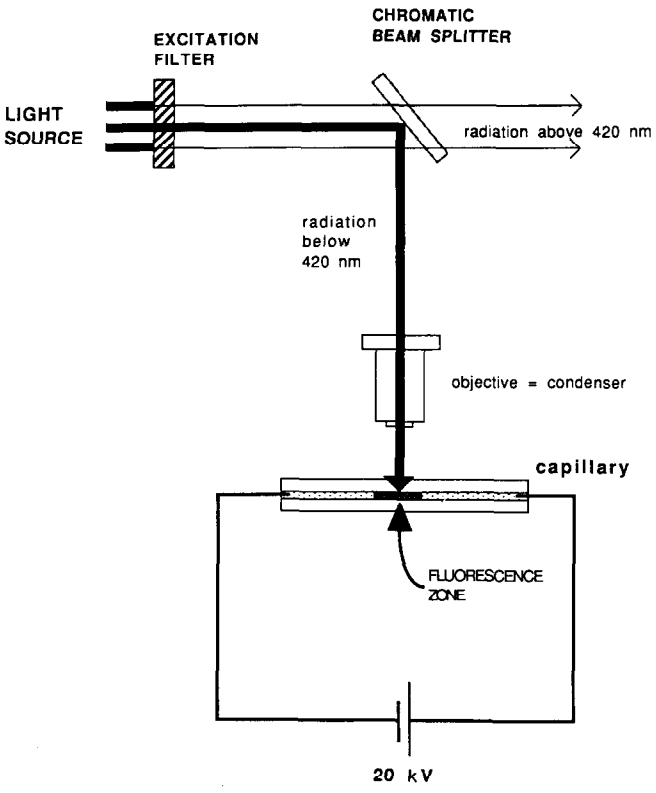

Fig. 1. Excitation of the sample with an epillumination fluorescence microscope. The UV beam is shown as the thick black line reflected at right-angles by the chromatic beam splitter. The fluorescence zone is indicated by the dark dotted area in the center of the capillary.

ultraviolet (UV) light. The excitation filter suppresses a substantial amount of radiation above 405 nm. The beam then reaches a chromatic beam splitter which reflects radiation below 420 nm. Therefore, only the UV is reflected toward the objective of the microscope. Three different fluorite objectives (Neofluar, Carl Zeiss) were used to determine the optimum magnification and numerical aperture: (1) 0.16 NA, 6.3×; (2) 0.32 NA, 16×; and (3) 0.75 NA, 40×. They were compared by electrokinetically injecting the same amount of riboflavin and measuring the relative fluorescence emitted. The same procedure was repeated for ordinary glass objectives using the same combination of magnification and numerical aperture. The generated data were fitted to polynomial equations of the second order. The statistical significance of the fittings was tested by *t*-tests on the correlation coefficients.

Emission and measurement of fluorescence (Fig. 2). The emitted light crosses the objective, the chromatic beam splitter and the long-pass filter that suppresses radiation under 418 nm. The beam is finally focused on a photodiode that transforms the light into an electrical signal which is recorded on a strip-chart recorder. In order to set the capillary on the microscope correctly, a carrier was made of Plexiglas, stainless-steel tubing and Teflon tubing (see Fig. 3). The carrier with the capillary was placed on the

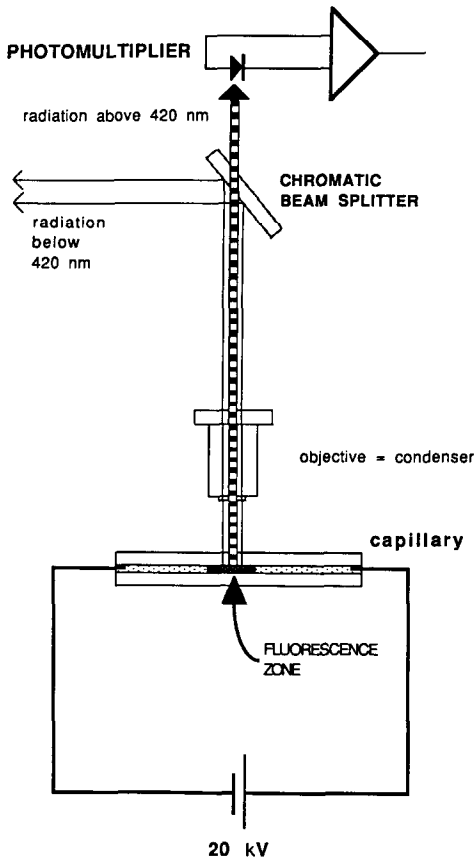

Fig. 2. Emission and measurement of fluorescence. The emitted light is indicated by the thick dashed line that crosses the chromatic beam splitter and is detected by the photodiode.

platina of the microscope and centered. The objective combined with a 10 × ocular was focused to obtain a sharp image of the capillary. The capillary used was fused silica (Scientific Glass Engineering, Austin, TX, U.S.A.), 25 μm I.D., 100 cm long, and primed with 0.05 *M* sodium tetraborate buffer (pH 6.0). The length of the capillary between the injection end and the objective of the microscope was 40 cm. The internal volume of this segment was 196 nl. Each end of the capillary was immersed in a 400-μl conical microcentrifuge vial containing the same buffer solution.

The electroosmotic flow was measured by electrokinetically injecting a $0.5 \cdot 10^{-3}$ *M* solution of riboflavin in borate buffer solution (pH 6.0) ten times. The injection parameters were 10 kV over a 7.5-s period. The ten different injections were run at 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 kV and the flow was calculated for each voltage. For this purpose, 196 nl were divided by the migration time (expressed in minutes) of the riboflavin peak. The data were fitted to a straight line. The statistical significance of the fitting was tested by a *t*-test on the correlation coefficient.

The detection limit for riboflavin was measured by injecting solutions of

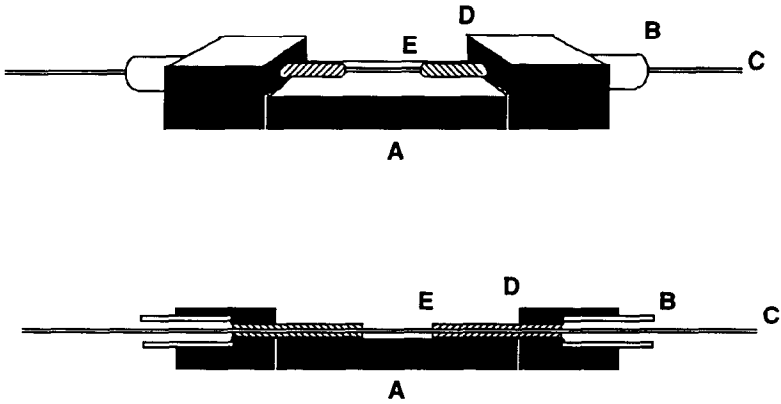

Fig. 3. Top, capillary carrier; bottom, cross section. A 50 × 25 × 5 mm parallelepiped (A) is the basis of the carrier. Two other 20 × 14 × 9 mm parallelepipeds (D) make the support for the capillary holders (E). Each of these holders is made of a 30 mm × 0.45 mm O.D. × 0.25 mm I.D. stainless-steel tube inside a 30 mm × 0.57 mm O.D. × 0.50 mm I.D. stainless-steel tube soldered together. Two holes are drilled at the main axis of D. One is of 9.5 mm bore and receives a 30 mm long piece of Teflon tubing (B). The other is of 0.60 mm bore for the capillary holder. Pieces D and A are glued with epoxy. All the Plexiglas pieces are coated with black paint to enhance the contrast between the fluorescence and the background. A section of 10 mm of the cover of the capillary (C) is burned to clear the glass surface of the capillary. Then the capillary is inserted until the uncovered section is located between the two capillary holders. The whole piece is mounted on the platina of the microscope and the uncovered section of the capillary is centered and focused.

decreasing concentration. The injection parameters were again 10 kV over a 7.5-s period. The detection limit was defined as the concentration that yields a 3:1 signal-to-noise ratio. The samples used were fluorescamine-derivatized amino acids and the drug amphetamine. To accomplish derivatization, 1 mg of fluorescamine was totally dissolved in 1 ml of acetone. This solution was then mixed with 1 ml of the sample which consisted of several amino acids or amphetamine dissolved in borate buffer (pH 8.3). The capillary column was primed with this borate buffer. The samples were electrokinetically loaded into the capillary by applying 10 kV for a period of 15 s. The samples were then separated by applying 20 kV for 40 min.

RESULTS

As shown in Fig. 4, a linear regression analysis for the ten riboflavin injections was obtained. The equation that best fitted the data was $y = 0.831x - 0.067$, and the correlation coefficient squared was 0.984. This fit was statistically significant ($t = 22.76, p < 0.001$). Based on this equation, injection volumes of 2.07 nl and 1.014 nl at 10 kV during 15 s and 7.5 s, respectively, were calculated.

Fig. 5 shows the impact of magnification and numerical aperture on the relative fluorescence of the same amount of riboflavin injected into the capillary. For the fluorite objectives, both variables (magnification and numerical aperture) were quadratically related to the relative fluorescence. These relationships were statistically significant [magnification (x) *vs.* relative fluorescence (y), $t = 31.8, p < 0.001$, best fit equation $y = 3.2 - 0.3x + 0.3x^2$; and numerical aperture (x) *vs.* relative fluorescence

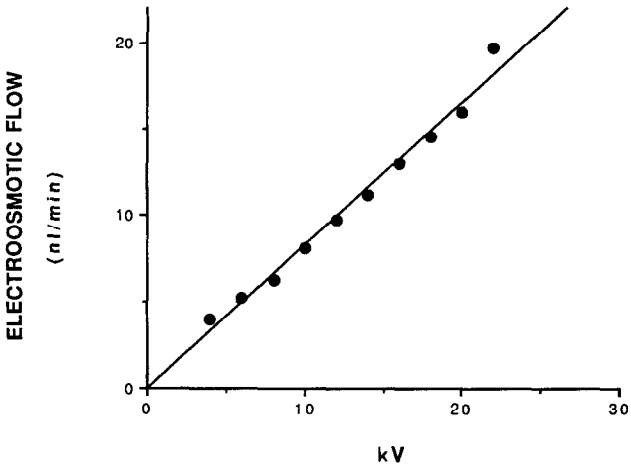

Fig. 4. Relationship between the applied voltage and the electroosmotic flow as measured by electrokinetically injecting riboflavin at 10 kV in 7.5 s and running at ten different voltages. $y = -0.064 + 0.831x$; $R^2 = 0.984$.

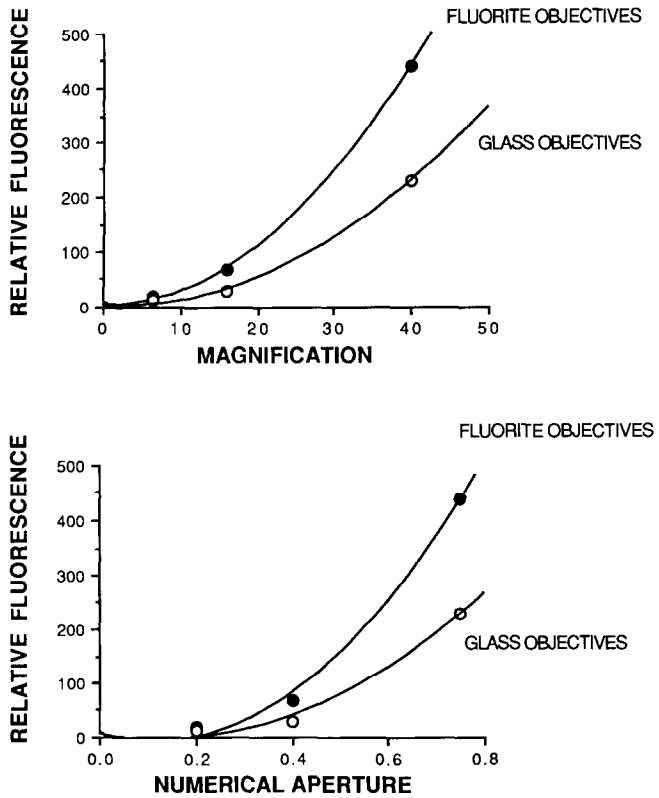

Fig. 5. Relationship between magnification and relative fluorescence (top) and numerical aperture and relative fluorescence (bottom) for fluorite objectives (●) and conventional glass objectives (○). The best fitting equations were quadratics. The fluorite objectives yielded more fluorescence particularly at the highest magnification and numerical aperture. Equations: top, ●: $y = 3.2 - 0.3x + 0.3x^2$; ○: $y = 3.4 - 0.7x + 0.16x^2$; bottom, ●: $y = 6.9 - 254.4x + 1104.1x^2$; ○: $y = 5.0 - 153.6x + 601.1x^2$.

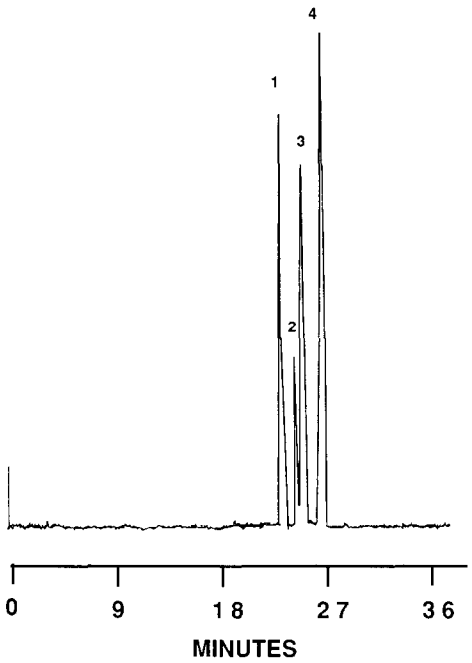

Fig. 6. Electropherogram of a 1 m*M* solution of fluorescamine-derivatized leucine and isoleucine (1), valine (2), threonine (3) and serine (4).

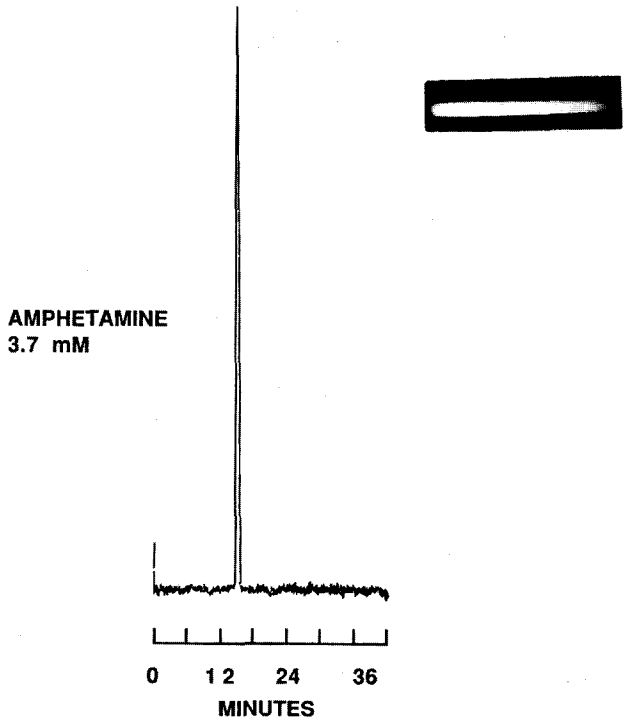

Fig. 7. Electropherogram of a 3.7 m*M* solution of fluorescamine-derivatized amphetamine. The inset shows the actual band of amphetamine detected by the microscope.

(y), $t = 22.68$, $p < 0.02$, best fit equation $y = 6.9 - 254.4 + 1104.1x^2$]. The glass objectives also showed a quadratic relationship between magnification, numerical aperture and relative fluorescence. However, the parameters for the best fit equations were different (see equations in Fig. 5). The correlation coefficients squared for the fluorite objective were double those for the glass objectives. The detection limit for riboflavin was $0.5 \cdot 10^{-6}$ *M*.

Fig. 6 shows the electropherogram of a mixture of five derivatized amino acids. Two of them, leucine and isoleucine, coeluted at the same migration time. Fig. 7 shows the electropherogram of derivatized *d*-amphetamine and the actual fluorescent zone running under the objective of the microscope.

DISCUSSION

The results show that a conventional fluorescence microscope can easily be converted into a fluorescence detector for capillary zone electrophoresis. As the pH of the buffer in the riboflavin experiment was 6.0, the riboflavin molecules were at their isoelectric point and therefore neutral[10]. In the electroosmotic flow measurement experiment, the straight line that best fitted the ten riboflavin injections did not intersect the coordinate system at the origin, but at -0.06, which represents a reasonable deviation. Among the fluorite objectives tested, the 0.75 NA and 40 × objective gave the best results. In the tested range, the fluorescence increased quadratically with the numerical aperture and the magnification. This result shows that fluorescence on-column detection (using fused-silica capillaries) can be improved by a factor of 56 just by using a 40 ×, 0.75 NA fluorite objective instead of the conventional 6 ×, 0.16 NA glass objective. The minimum riboflavin concentration detected on-column was $0.5 \cdot 10^{-6}$ *M*. Considering that bias during electrokinetic injection was minimized by the pH of the buffer, and that a volume of 1 nl was injected, this indicates that 0.5 fmol of riboflavin (injected) was detected. For other CE detectors, lower detectable concentrations have been reported[7]. This point deserves further consideration.

In other experiments, 65 and 75 μm I.D. capillaries were used. Therefore, the injected amount was larger. In addition, a 100-W lamp, photomultiplier tubes and electronic circuits for noise supression were used. These devices improve the sensitivity of the detection system by 2–3 orders of magnitude in comparison with a 50-W lamp, photodiodes and no noise supression. In this work the concentration detected was not as low as those with other detectors[7,8]. Nevertheless, the total amount of analyte injected was smaller than that reported for other detectors. This suggests that the combination of fluorescence microscopy, a high-power UV lamp, a photomultiplier tube, and noise supression should improve the detection limit for the detector reported here by 2–3 orders of magnitude. As the microscope magnifies the capillary, only a 100-μm section (equivalent to 0.05 nl) is available for measurement. This indicates that the minimum amount detected is 0.025 fmol and represents an improvement over fluorimetric on-column detection, using non-coherent excitation, for capillary electrophoresis.

The microscope offers several additional features that make it particularly useful for CE. The epillumination method focuses the UV beam on the capillary, providing a very concentrated excitation of the sample. The colinear arrangement prevents loss

of fluorescence by self-absorption, especially at high analyte concentrations[11]. The objective and the ocular of the microscope concentrate the emitted light on the photodetector, thereby improving the efficiency of the measuring device. The objective is kept at 0.33 mm from the capillary, compared with 1 cm or more for other detectors. This feature allows the recovery of more light by reducing the wastage due to light scattering. The platina of the microscope is displaced by precision micrometer screws. Therefore, alignment of the capillaty and the UV beam is very simple. This is an advantage when changing one capillary for another of different outside diameter.

Dramatic improvements in sensitivity for CE fluorescence detection have been obtained by laser-induced fluorescence[12–15]. We have conducted preliminary tests with the fluorescence miroscope, an air-cooled argon-ion laser, a water-cooled gallium arsenide photomultiplier tube and a photon counter for statistical signal processing. Low milliattomole amounts of fluorescein-derivatized amino acids have been detected using on-column fluorescence detection[16]. We conclude that a fluorescence microscope can be used as an optical fluorescence device for CE.

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