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Collinear laser-induced fluorescence detector for capillary electrophoresis

Analysis of glutamic acid in brain dialysates☆

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ABSTRACT

Experiments with capillary electrophoresis using a laser-induced fluorescence detector with a collinear optical arrangement demonstrated several important points. First, increasing the numerical aperture of the microscope objective that is used simultaneously for focusing the excitation laser light as well as collection of emitted fluorescence enhances the signal used for the measurement of the emitted fluorescence and at the same time decreases the noise of interfering light. Second, detection of fluorescein-labelled amphetamine was performed at high-picomolar (10^{-10} *M*) levels. Third, the signal-to-noise ratio of 280 found at the above-mentioned picomolar concentrations indicates that the measurement of low-picomolar concentrations (10^{-12} *M*) of this compound in biological samples should be possible. Fourth, narrow-bore capillaries (5–10 μm internal diameter) were used to detect the neurotransmitters glutamic acid and aspartic acid as their naphthalene-2,3-dicarboxaldehyde derivatives in brain dialysates obtained from a freely moving rat. A mathematical model was developed to explain the relationship between numerical aperture, working distance, magnification of the lens, noise due to laser scattering and signal due to fluorescence. The model correctly predicted the observed values of photomultiplier tube current due to both laser scattering and fluorescence. The potential of the application of capillary electrophoresis with laser-induced fluorescence detection in the neurosciences is discussed.

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INTRODUCTION

Laser-induced fluorescence detection (LIF) is the most sensitive detection method for capillary electrophoresis (CE). The detection limits of CE–LIF are approaching the molecular counting level [1]. New denominations (zepto or 10^{-21} and yocto or 10^{-24}) had to be created to refer to the small amounts of substance that CE–LIF is capable of detecting [2]. Because of this extremely high sensitivity CE–LIF has great potential to become a standard analytical tool in areas of biomedical research requiring better sensitivity than that provided by other currently available analytical techniques.

The development of CE–LIF has been gradual but sustained. Since the seminal article by Gasman *et al.* [3] showing the feasibility of CE–LIF, improvements in this technique have evolved in several directions.

The main axes developed thus far have been: (1) The introduction of new laser lines for excitation to expand the range of detectable compounds. To date, the principal lines which have been used are: 257 nm [4], 325 nm [3,5], 442 nm [6], 476 nm [7], 488 nm [8] and 514 nm [2]. (2) The introduction of charge-coupled devices to perform quick spectral analysis of the sample to distinguish between scattered laser radiation (Raman and Rayleigh scatters) and the emitted fluroescence [2,9]. (3) Post-column detection rather than on-column detection to reduce laser scattering from the walls of the capillary and reach very high sensitivity [1,10,11]. (4) Indirect LIF for the detection of non-fluorescent compounds [12,13]. (5) New fluorescent tags to overcome the poor tagging at low concentrations of analytes [14]. (6) Introduction of collinear rather than orthogonal optical geometrical arrangement to take advantage of very high numerical aperture lenses to improve sensitivity in the on-column detection mode and facilitate the industrial production of CE–LIF instruments [15].

In addition, LIF, along with electrochemical detection, is one of the two methods that enables the experimenter to work with very narrow-bore capillaries [16,17]. These capillaries create advantageous conditions for CE. In very narrow-bore capillaries, less than 20 μm internal diameter (I.D.), the surface-to-volume ratio (S/V) is greater than in 50 μm or 75 μm I.D. capillaries. For instance, the S/V for 15 μm I.D. capillaries is 267 and for 75 μm I.D. capillaries, the S/V is 53, *i.e.*, five times smaller. As a consequence, the heat dissipation in very narrow-bore capillaries is superior. Moreover, the reduction in the cross-sectional area increases the electrical resistance. This in turn reduces the current flowing through them and less Joule heat is generated. This facilitates the use of short capillaries (30 cm or less) and the application of very high voltages (25–30 kV). The length reduction shortens the retention time of the analytes and the very high voltage increases the separation power and the resolution of the capillary. However, there are also disadvantages when very narrow-bore capillaries are used. Some loss of concentration sensitivity occurs. Typically, the minimum detectable concentration (MDC) increased by between two and four orders of magnitude.

In our previous work, we suggested that the loss in the MDC, in very narrow-bore capillaries, might be counteracted by raising the numerical aperture (NA) of the collecting lenses [15,18]. In the capillary diameter *vs.* fluorescence curve, the fluorescence collected shows a sharp rise between 5 μm diameter I.D. and 10 μm I.D. capillaries when a 0.75-NA objective is used. Therefore, we speculated that the high NA permitted by the collinear geometry could enhance the signal and raise the MDC while preserving the excellent resolution and the separation efficiency of very narrow-bore capillaries. We also found that increasing the laser power increases the signal due to the fluorescence and the background light due to laser scattering. However, when NA increases, the signal is enhanced at a higher rate than the background light, improving the signal-to-noise ratio (S/N). These advantageous but disparate changes of signal and noise were unexpected and intuitively contradictory. Therefore, we decided to investigate further the advantages of high-NA lenses and very narrow-bore capillaries.

Then, we decided to use CE–LIF in narrow-bore capillaries to solve a biologically relevant analytical problem. Currently, by using brain

microdialysis, it is possible to monitor changes in the chemical composition of the brain *in vivo* [19]. So far, the techniques that have been used for chemical analysis of the brain dialysates have a good concentration sensitivity and a poor mass sensitivity. Therefore, large amounts of sample (greater than 5 μl) have to be collected in order to obtain enough quantity of analytes. Typically, the sampling times of 5–30 min are required. However, certain amino acid neurotransmitters, such as glutamic acid and aspartic acid, are released and taken up in fractions of a second. An analytical technique to study the *in vivo* release of these neurotransmitters has to be fast and sensitive enough to allow the analysis of these very small volumes of sample. CE–LIF in very narrow-bore capillaries seems to fulfil these requirements. Volumes smaller than 1 nl, can be used and the analysis time is reduced to less than 2 min.

In the present article, we report experiments carried out to clarify why the S/N ratio improves as a function of NA in a collinear detection system. Then, we present data showing the efficiency of the detector to make fast measurements of excitatory amino acids in brain dialysates when 15 μm I.D. capillaries are used.

MATERIALS AND METHODS

Instrument

The general design of the instrument is described elsewhere [15]. Briefly, the detector was made of a 141-FD standard model epiillumination fluorescence microscope (Zeiss, Caracas, Venezuela). The lasers used for excitation were either air-cooled argon ion lasers (Ion Laser Technology, Salt Lake City, UT, USA; Model 5425) or an air-cooled helium–cadmium ion laser (He–Cd) from Liconix, Santa Clara, CA, USA; Model 4214). The work was done either with the 476-nm line of the argon ion laser at a power of 3 mW or with the 442-nm line of the He–Cd at a power of 12 mW. For all the experiments, the detector was equipped with a dichroic mirror. The objective used had one of the following numerical apertures: 0.20, 0.40, 0.75 or 0.85. All of the objectives were of non-immersion type and lenses were constructed of calcium fluoride (Zeiss, Caracas, Venezuela). The fluorescence was filtered through a long-pass filter and a notch filter to attenuate background radiation. Optical properties of the filters were varied according to the laser line being used and the emitted fluorescence. For the 476-nm line, the dichroic mirror reflected below 490 nm, no notch filter was used and the high-pass filter was centred at 490 nm with a bandwidth of 30 nm. For the 442-nm line, a band-pass filter centred at 440 nm, followed by a dichroic mirror centred at 470 nm, and a notch filter centred at 442 nm with a 20 nm bandwidth (Andover Corporation, Salem, NH, USA). No high-pass filter was used. The light was detected by a photomultiplier tube (PMT) from Hamamatsu (Bridgewater, NJ, USA; Model R928 Multialkali), operated a 700 V, and connected to a microammeter for current-to-voltage conversion (Keithley, Cleveland, OH, USA; Model 485). The picoammeter was connected to a Model L-6512 Linseis strip-chart recorder (Lanseis, Princeton Junction, USA).

The CE separations were performed in fused-silica capillaries obtained from Polymicro Technologies (Phoenix, AZ, USA). The length of the capillaries were 30 cm and 80 cm, and the I.D.s were 15 μm and 75 μm, respectively. The power supply was a Bertan Model 30R high-voltage power supply (Hicksville, NY, USA), and the injection electrodes were platinum–iridium wires. The capillary was set on the instrument as described elsewhere [15,18].

Reagents

Sodium borate, sodium cyanide, fluorescein isothiocyanate (FITC), amphetamine sulphate, glutamic acid, aspartic acid and methanol were obtained from Sigma (Saint Quentin Fallavier, France). Naphthalene-2,3-dicarboxaldehyde (NDA) was obtained from Molecular Probes (Eugene, OR, USA), and 18 MΩ water from a Millipore system.

Amphetamine sample was prepared by dissolving 1 mg in 1 ml of 0.05 *M* borate buffer at pH 9.5 to obtain a $2.7 \cdot 10^{-4}$ *M* solution, and FITC was prepared by dissolving 1 mg in 5 ml of acetone to obtain a $5 \cdot 10^{-4}$ *M* solution. Then 1 ml of each solution was mixed to obtain a $2.5 \cdot 10^{-4}$ *M* solution of fluorescein thiocarbamyl–

amphetamine (FTC–amphetamine), assuming full reaction and amphetamine as the limiting reagent. A blank solution was similarly prepared by mixing $5 \cdot 10^{-4}$ *M* FITC with 1 ml of borate buffer. These solutions were allowed to react for 4 h in the dark and afterwards they were diluted with borate buffer to obtain $2.5 \cdot 10^{-6}$ *M*, $2.5 \cdot 10^{-7}$ *M*, $2.5 \cdot 10^{-8}$ *M*, $2.5 \cdot 10^{-9}$ *M*, $2.5 \cdot 10^{-10}$ *M* and $2.5 \cdot 10^{-11}$ *M* solutions of FTC–amphetamine in one series and blank in another series.

In the first experiment, the effect of numerical aperture on fluorescence and background illumination was tested. Either the $2.5 \cdot 10^{-10}$ *M* solution of FTC–amphetamine or the buffer was continuously drawn through a 75 μm I.D. capillary by vacuum. The 476-nm line of the argon ion laser was used. The PMT current was measured for both solutions using different objectives. The PMT current due to FTC–amphetamine was obtained by subtracting the PMT current due to FTC–amphetamine minus the PMT current due to the buffer. The results were plotted on a NA *vs.* PMT current graph.

In the second experiment, FTC–amphetamine or the corresponding blank was injected by gravity (siphon effect) into a 75 μm I.D. capillary and electrophoresed at 25 kV. For the injection, the anodic end of the capillary was raised 5 cm above the grounded end for 5 s.

In the third experiment, glutamic acid and aspartic acid were measured in the brain dialysate of a rat. A brain microdialysis experiment was peformed as described elsewhere [19]. The microdialysis probe was inserted into the striatum of a rat and perfused with artificial cerebrospinal fluid (ASCF) at 0.1 μl/min. Samples were collected every 10 min and reacted with 5 μl of 0.1 m*M* NDA in methanol, 5 μl of 10 m*M* sodium cyanide, in water and 10 μl of 20 m*M* borate buffer at pH 9.5. A blank solution containing only 1 μl of ACSF was mixed with the reagents as described above. Standard solutions of glutamic acid and aspartic acid were prepared and derivatized to determine the migration time of these amino acids. After 30 min of reaction in the dark, the sample, blank and standards were electrokinetically injected (6 kV, 3 s) in a 30-cm-long 15 μm I.D. × 150 μm O.D. fused-silica capillary filled with buffer. Then, 30 kV were applied to separate the components of the mixture. The 442-nm line of the He–Cd laser was used for excitation.

RESULTS

The results of the first experiment are shown in Fig. 1. The upper curve corresponds to the measurement of the FTC–amphetamine solution. The middle curve corresponds to the PMT currents produced by the buffer. The lower curve is the difference between these two curves. It is clear that the amount of light generated by the buffer-filled capillary decreases as the NA of the lenses increases and reaches an asymptote at 0.75 NA (middle curve). However, the amount of light due to the fluorescence increases as a quadratic function of the NA. Therefore, for the 0.20-NA objective, the fluorescence of FTC–amphetamine represents less than 2% of the total amount of light that the objective is collecting and the buffer-filled capillary is producing 50 times more light than the fluorescence. For the 0.85-NA objective the situation changes. The fluorescence is 50% of the total amount of light collected by the objective and the buffer-filled

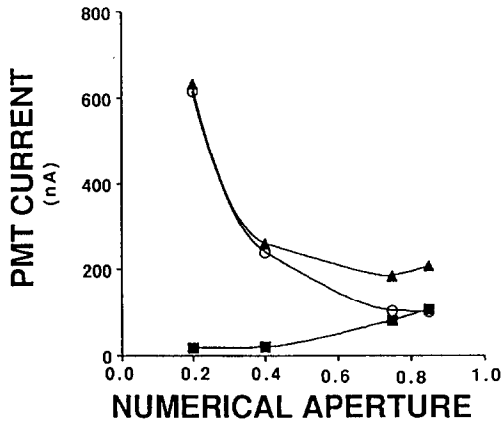

Fig. 1. The PMT current generated by a buffer-filled capillary (open circles) decreases as the NA of the lenses increases. The PMT current due to a capillary flushed with fluorescent material increases (black squares) as a function of NA. However, it increases between the 0.75-NA and 0.85-NA objectives. As a result, the PMT current due to fluorescence (black squares) increases as a function of NA. The final result is an enhancement of S/N ratio as a function of NA.

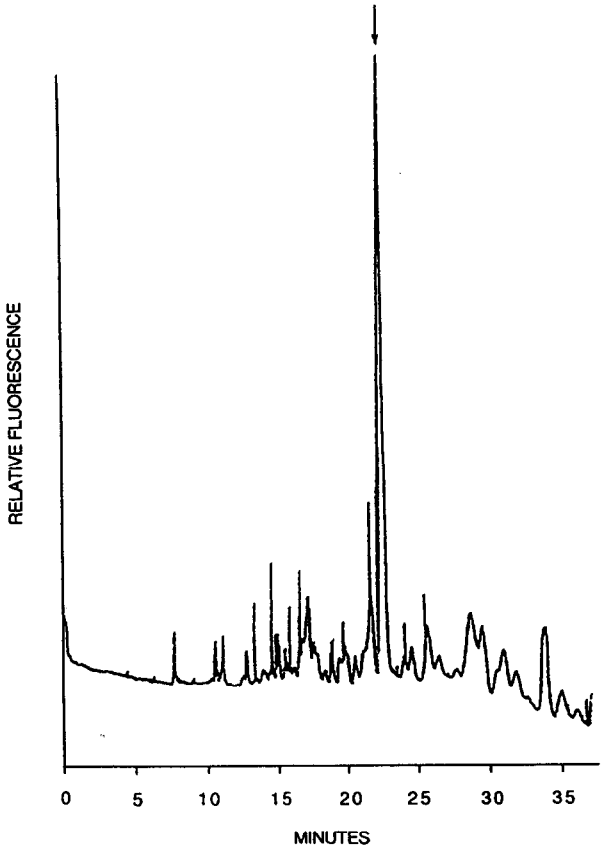

Fig. 2. FTC–amphetamine at 10^{-10} *M* concentration.

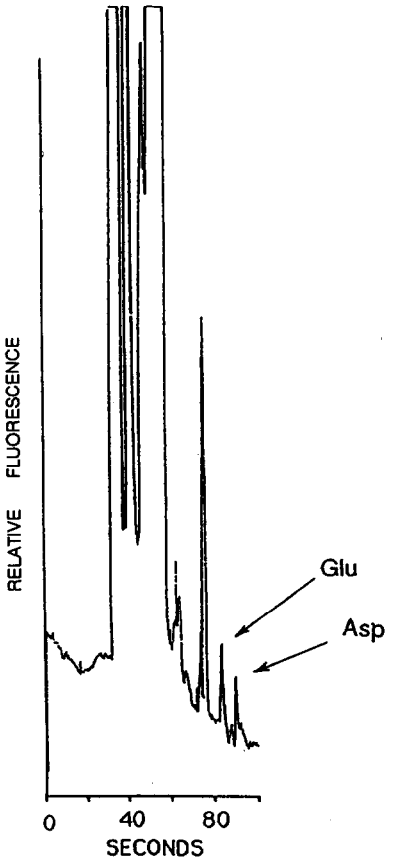

Fig. 3. Glutamic acid and aspartic acid are detected in 23 pl of brain dialysate from a freely moving rat.

capillary produces about the same amount of light as the fluorescent material.

The results of the second experiment are shown in Fig. 2. The $2.5 \cdot 10^{-10}$ *M* FTC–amphetamine solution produces a peak with a *S/N* of 280 and 21.6 min migration time.

The electropherogram of Fig. 3 shows the results of the third experiment. In less than 85 s the peaks corresponding to glutamic acid and aspartic acid were observed.

DISCUSSION

The results presented in Fig. 1 may seem contradictory at the first glance. The effects of increasing the NA on the PMT current are twofold. First, the collection efficiency of the photoluminescence radiation increases. This behaviour can be understood by examining the details of the geometry and the prominent scattering mechanism of the system.

Frequently, the increase in the collection efficiency with the increase in the NA is described by the equation:

$$\text{Collection efficiency} = \sin^2\left(\frac{\arcsin\left(\frac{NA}{\eta}\right)}{2}\right) \quad (1)$$

where NA is the numerical aperture and η is the refractive index of the lens material. The use of this formula in the present context is not adequate as it does not take into account the area of the illuminating surface, which in the present case is not a point source. In fact, the illuminating area varies inversely with the NA and this environment demands a different treatment.

The power, $\Delta\Phi$, incident on the objective is given by:

$$\Delta\Phi = \frac{L\Delta A_1 \Delta A_2}{D^2} \quad (2)$$

where ΔA_1 is the illuminating area, ΔA_2 is the area of the objective, L is the irradiance of the photoilluminating area and D is the distance between illuminating area and the objective.

Even though the illuminating area decreases with the increase in NA, the brightness or irradiance of the spot increases and the product $L\Delta A_1$ remains constant as long as the power of the exciting laser is unaltered. Thus, in the present analysis, the product $L\Delta A_1$ is considered constant in eqn. 2.

For objectives of higher NA, the working distance is smaller than those of lower NA and, therefore, higher collection efficiency is expected. In order to evaluate the experimental results numerically, the intensity of photoluminescence was plotted against distance, D. Pertinent data of the objectives needed in the present investigation, such as working distance and amplification factors, are given in Table I.

Curve-fitting procedures by using the program Mathematica [22] were carried out and indeed the best fit was obtained with the expected expression given by:

$$\text{PMT current} = K + \frac{14}{(D+0.2)^2} \quad (3)$$

For the present detecting system, the value of K was found to be 15.7. This constant is related to the dark current of the PMT and hence depends upon the applied voltage, type of PMT, radiation leakage and details of the experiment set-up.

It was found that the effective distance is slightly increased as compared with the working distance of the objective provided by the manufacturer. This is also understandable, since the design of the instrument (optical position of the PMT with respect to the eyepiece) always affects the working distance.

Fig. 1 also shows the PMT current induced by the scattering process. It can be seen that the scattered intensity decreases as the NA of the objectives increases. This observation is apparently contradictory as collection efficiency increases with NA. This behaviour can be explained on the basis of the area of the scattering region.

In the present experimental technique, major contributions to the scattering originate from the cylindrical surface of the capillary rather than the photoluminescent material. The area of the spot illuminated on the capillary decreases substantially as the magnification increases, which occurs as the NA of the lenses increases (see Table I). In other words, the spot area is inversely proportional to the magnification factor of the lens. We have, therefore, plotted PMT current against magnification of the objectives (not of the total system). Curve fitting was carried out with the above-mentioned program. In this case, the best fit was obtained, as expected, for an inverse relation. The analytical expression of PMT current due to scattering is given by:

$$\text{PMT current} = 14.7 + \frac{3744.5}{M} \quad (4)$$

where M is the magnification factor. Here also, 14.7 represents PMT dark current.

It is clear from the above discussion that objectives with higher NA provide two advantages. First, the light-gathering power of the system is enhanced and, second, scattering is reduced because of the smaller size of the focal spot on the capillary.

The values estimated with eqns. 3 and 4 for four different values of NA of the objectives are shown in Table II along with the experimental values. Note that the predicted values are in accordance with the experimental values. This

TABLE I

CHARACTERISTICS OF THE LENS USED IN EXPERIMENT 1

NA	Working distance (mm)	Magnification
0.2	10.8	6.3
0.4	0.9	16.0
0.75	0.33	40.0
0.85	0.18	50.0

TABLE II

PREDICTED AND OBSERVED VALUES OF PMT CURRENT (nA) DUE TO SCATTERING AND FLUORESCENCE MECHANISMS

NA	Predicted fluorescence	Observed fluorescence	Predicted scattering	Observed scattering
0.2	15.8	16	613	630
0.4	27.0	19	248	240
0.75	66.0	80	104	108
0.85	112.0	106	100	90

strengthens our views about the importance of high NA in collinear detection systems.

The detection of FTC–amphetamine at 10^{-10} *M* concentration improves the MDC that we obtained for fluorescamine–amphetamine in our first collinear fluorescence detector [18]. Furthermore, it opens up the possibility of using CE–LIF for amphetamine detection in blood.

Glutamic acid and aspartic acid were measured in brain dialysates. According to the standard measurements, the concentration of glutamate was $0.6 \cdot 10^{-6}$ *M*, which fits with the concentrations reported by other authors using liquid chromatographic techniques [20]. Several characteristics of the present analysis make it interesting. The amount of sample injected into the capillary was estimated to be about 0.5 nl. Since the actual dialysate represented 4.7% of the whole injection, the volume of dialysate injected was 23 pl. The flow-rate of perfusion was 100 nl/min. The 23 pl injected correspond to the dialysate collected during 72 ms. Thus, at least in theory, it should be possible to follow the release of glutamic acid in the brain on the scale of milliseconds. In practice, several technical problems will have to be solved before reaching that point. For instance, extremely low volumes of dialysate have to be derivatized at the nanoliter level. The technology to manipulate nanoliter volumes already exists [21]. Moreover, the rapidity of the analysis itself can be increased to reach a few seconds. If that turns out to be achievable in practice, then CE–LIF will be a very attractive technique for the *in vivo* monitoring of substances in the brain, such as neurotransmitters.

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