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Visible diode laser induced fluorescence detection for capillary electrophoretic analysis of amantadine in human plasma following precolumn derivatization with Cy5.29.OSu[1]

Sriram Nagaraj, Sadayappan V. Rahavendran, H. Thomas Karnes *

Department of Pharmacy and Pharmaceutics, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0533 USA

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Abstract

Visible diode laser induced fluorescence (VDLIF) detection (620–700 nm) has become important in bioanalysis due to the increased sensitivity and selectivity that can be achieved in biological matrices. A selective and sensitive capillary electrophoretic method employing VDLIF detection has been developed for the analysis of amantadine in plasma. Amantadine was extracted from plasma into toluene under alkaline conditions and the residue was derivatized with the far-red label Cy5.29.OSu. The reaction mixture was dried under nitrogen, reconstituted and then injected onto a laboratory constructed capillary electrophoresis system equipped with a laboratory constructed visible diode laser detector temperature tuned to oscillate at 647.8 nm. The selectivity of the technique was evaluated by demonstrating a lack of interfering peaks in extracts of blank plasma. A calibration curve ranging from 1.8 to 461.1 ng ml^{-1} was shown to be linear. The precision and accuracy of the assay ($n = 6$) were determined to be within 17% R.S.D. and 15% difference from the nominal concentration respectively. The limits of detection for unextracted amantadine and for amantadine from the extracted concentrate from plasma were determined to be 9.5 fmol and 115 amol respectively.

Keywords: Derivatization; Far-red label; Diode laser; Fluorescence detection; Capillary electrophoresis; Amantadine

1. Introduction

Visible diode laser induced fluorescence (VDLIF) detection (620–700 nm) has become important in bioanalysis due to increased sensitivity and selectivity [1–4]. The increase in sensitivity can be attributed, in part, to the high excitation intensity of the laser beam and partly due to the increased selectivity of the technique. Selectivity is enhanced because biological matrices such as plasma demonstrate minimal background fluorescence in the far-red region (> 620 nm) of the spectrum. In addition, the intensity of the Raman scatter which is strongly wavelength dependent

* Corresponding author. Tel.: +1 804 8288350; fax: +1 804 8288359.

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(proportional to λ^{-4}) is significantly lower at long wavelengths [5]. The output of diode lasers is optimal in this region and analytical methods that are instrument, rather than, matrix limited are possible. Diode lasers also provide attractive alternatives to conventional gas discharge light sources and laser sources (eg: argon ion, helium–cadmium) by virtue of their long lifetimes ($>$ 80000 h), low noise characteristics, minimal power consumption, compact size, low cost and excellent spectral characteristics [6]. Few analytes possess native fluorescence in the far-red region of the spectrum, however, and derivatization using labels that absorb and fluoresce in the far-red region is usually necessary [7].

Amantadine (1-adamantanamine), a low molecular weight primary amine, has been used in the treatment of influenza A virus infections [8] and Parkinson's disease [9]. Amantadine is present in plasma at relatively low concentrations after oral administration ($\sim$ 100–600 ng ml^{-1}) and a sensitive, specific method is required to study the pharmacokinetics of the drug. Amantadine however lacks a chromophore and therefore cannot be easily detected spectroscopically without derivatization. Traditionally, gas chromatographic methods employing flame ionization and electron capture detection, have been used for the quantitation of amantadine in biological matrices [10–15]. Mank and colleagues [3,16,17] investigated the use of cyanine labels as precolumn derivatization reagents for several compounds including primary amines for VDLIF detection in liquid chromatography. Amantadine was analyzed by HPLC using VDLIF detection after extraction from urine and derivatization with CY5.11.OSuc [16]. This paper describes a capillary electrophoretic method using VDLIF detection for the analysis of amantadine in plasma following precolumn derivatization with Cy5.29.OSu, a far-red dicarbocyanine dye [18] with spectral properties ($\lambda_{ex} = 650$ nm, $\lambda_{em} = 667$ nm, $\epsilon = 250000$ M^{-1} cm^{-1}, $\Phi_f = 0.28$) that are well suited for use with diode laser spectroscopy. The dye has a succinimidyl ester functionality suitable for precolumn derivatization of aliphatic primary amine containing analytes. Formation of the derivative is made possible by the succinimidyl ester undergoing nucleophilic attack by the primary amine functionality of amantadine. The proposed derivatization scheme is shown in Fig. 1. Cy5.29.OSu contains sulfonic acid groups, which make it highly water soluble and therefore suitable for use with capillary electrophoresis.

2. Experimental

2.1. Chemicals

Amantadine was purchased from Aldrich (Milwaukee, WI). Cy5.29.OSu was procured from Amersham Life Sciences (Pittsburgh, PA). Toluene was purchased from J.T. Baker (Phillipsburgh, NJ). Blank plasma was purchased from Biological Specialties (Colmar, PA). Sodium hydroxide, sodium phosphate monobasic, sodium phosphate dibasic and tetramethyl ammonium hydroxide were purchased from Sigma (St. Louis, MO). Sodium dodecyl sulfate was obtained from Eastman Kodak (Rochester, NY). Methanol was purchased from Burdick and Jackson (Muskegon, MI).

2.2. Derivatization procedure

The derivatization procedure was optimized using a sequential single factor approach with respect to time, temperature and excess label. The optimization studies were carried out in two stages:

(a) Optimization of time and temperature: 12.5 μl amantadine (250 nmol ml^{-1}) in methanol was added to 3 ml reaction vials and evaporated to dryness. 12.5 μl Cy5.29.OSu (250 nmol ml^{-1}) in acetonitrile–DMF (97:3%) was added to the residues and the volume was made up to 50 μl with acetonitrile. The reaction vials were then heated at 60 and 70°C for 30, 60, 90 and 120 min. The reaction mixtures were evaporated to dryness and the residues were reconstituted in 10 μl distilled deionized water. The reaction mixtures were then injected onto a capillary (50 μm i.d. $\times$ 355 μm o.d. $\times$ 58 cm length, fused silica capillary) by gravity injection (i.e by raising the injection end of the capillary by 100 mm for 10 s relative to the

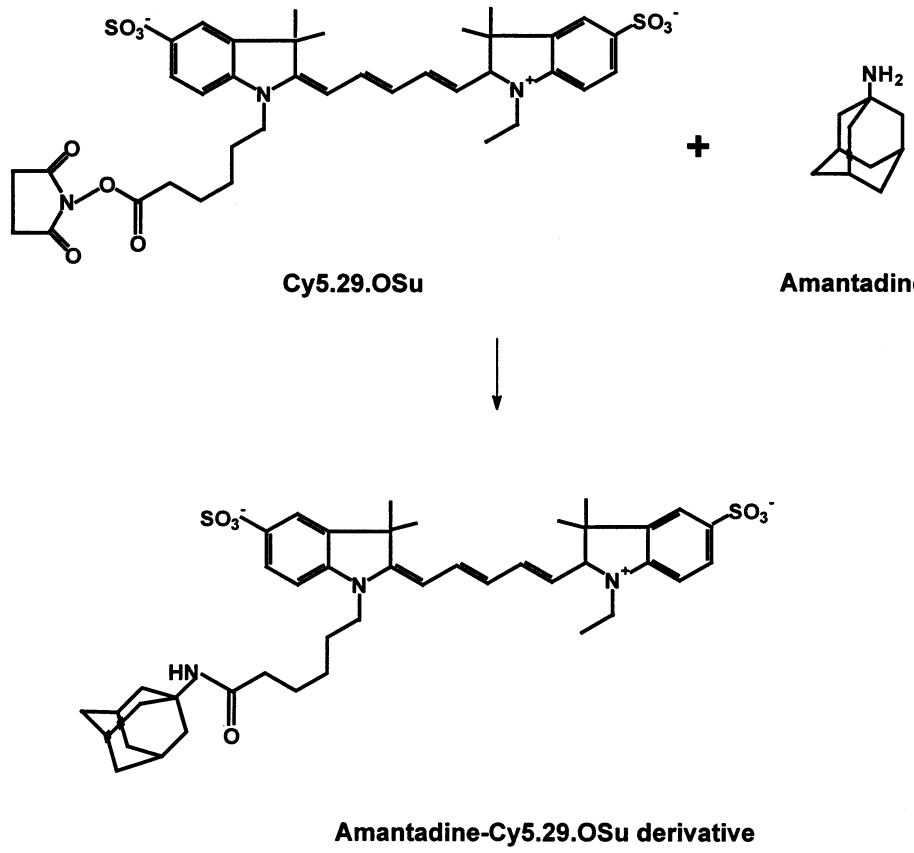

Fig. 1. Derivatization scheme of amantadine with Cy5.29.OSu.

outlet end of the capillary) and separation was carried out at 17.5 kV in 50 mM sodium borate buffer (pH 9.1) on a Dionex CES I system (Dionex, Sunnyvale, CA) using absorbance detection at 648 nm.

(b) Optimization of label concentration: 12.5 μl amantadine (25 nmol ml^{-1}) in methanol was added to several reaction vials and evaporated to dryness. The residues were reacted with the label as follows: 12.5 μl 1.25×10^{-4} M; 12.5, 25, and 75 μl 2.5×10^{-4} M Cy5.29.OSu (which represent 125, 250, 500, and 1500 nmol ml^{-1} respectively) at the optimized time and temperature conditions (90 min and 70°C). The reaction mixtures were dried and reconstituted in 100 ml distilled deionized water. The samples were analyzed using the laboratory constructed capillary electrophoresis-VDLIF detection system described in Section 2.5.

2.3. Thin layer chromatography

Reverse phase thin layer chromatography (RP-TLC) was performed to confirm the formation of the derivative. The reaction mixture (2 μl) in acetonitrile was spotted on a C-18 plate along with 2 μl Cy5.29.OSu as the control. TLC was performed using methanol–water (70:30%) as the mobile phase. In addition another TLC plate was spotted with Cy5.29.OSu and Cy5.29.COOH and TLC was performed as noted above.

2.4. Spectral scan of the derivative

The spot corresponding to the derivative was scraped from the TLC plate into a 1.5 ml microcentrifuge vial. Methanol (200 μl) was added to the microcentrifuge vial and the vial was cen-

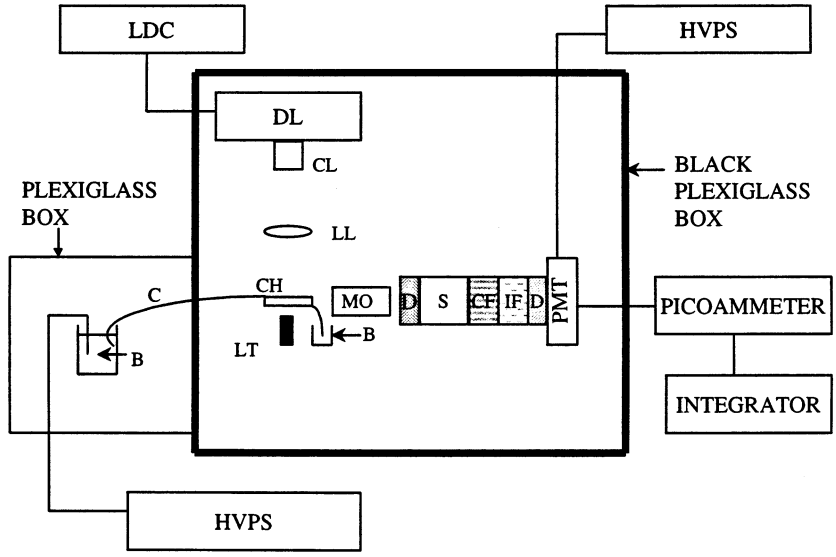

Fig. 2. Schematic diagram of laboratory constructed CE-VDLIF detection system. LDC, laser diode controller; DL, diode laser; CL, collimating lens; LL, laser lens; C, capillary; CH, capillary holder; LT, light trap; MO, microscope objective; D, iris diaphragm; S, spacer tube; CF, cut off filter; IF, interference filter; PMT, photomultiplier tube; B, buffer vials; HVPS, high voltage power supply.

trifuged at 10000 rpm for 5 min in a micro ultracentrifuge (Abbott Laboratories, Abbott Park, IL). The supernatant was pipetted into another microcentrifuge tube and was dried under nitrogen. The residue was reconstituted in approximately 3 ml of distilled deionized water. The solution was pipetted into a cuvette and placed in a luminescence spectrometer (Perkin Elmer model LS-50) equipped with a pulsed xenon excitation source and a red sensitive R928 photomultiplier tube. The excitation maxima of the derivative sample was determined by scanning between 300 and 800 nm with the emission monochromator set to zero. The emission maxima was then determined by setting the excitation monochromator to the excitation maximum wavelength.

2.5. Capillary electrophoresis system

Capillary electrophoresis was performed on a laboratory constructed CE system using a fused silica capillary (60 cm × 350 μm o.d. × 50 μm i.d.) (Polymicro Technologies, Phoenix, AZ) except where otherwise indicated. Voltage was provided by a CZE 1000R high voltage power supply (Spellman High Voltage Electronics, Plainview, NY). The inlet end of the capillary was housed in a plexiglass box equipped with a safety interlock system. The capillary outlet was housed inside the diode laser detection system. The length of the capillary from the inlet to the detection window was 48 cm. Samples were introduced by electrokinetic injection for 5 s at 17.5 kV. Electrophoresis was carried out optimally at 17.5 kV using a buffer which consisted of 20 mM sodium phosphate buffer (pH 6), 10 mM sodium dodecyl sulfate (SDS), 40 mM tetramethyl ammonium hydroxide (TMAH) and methanol (10% v/v).

2.6. VDLIF detection system instrumentation

The instrumentation for the VDLIF detection system is shown in Fig. 2. The system was constructed by modification of an earlier design of an HPLC-VDLIF system [19]. The modifications were primarily comprised of appropriate optics, filters and a capillary holder that were needed to focus the laser beam into and collect fluorescence from the capillary detection window. The system consists of a visible solid state diode laser (Toshiba model TOLD 9421(S)) housed in a laser diode mount (ILX-Lightwave model 4412)

equipped with a collimating lens (5.0 mm focal length, 0.5 N.A., ILX-Lightwave model 4014). The diode laser was tuned to 647.8 nm (output power 4.1 mW) with a laser diode controller (ILX-Lightwave model LDC-3722) equipped with a current source and thermoelectric cooling unit (53.07 mA, 0°C). The laser beam was passed through a laser lens (Oriel model 45272) and focused onto the detection window of the capillary, which was held in a specially designed capillary holder. The detection window was formed by burning off ~ 1 cm length of capillary coating. The capillary holder held the capillary fixed in space in order to facilitate alignment of the laser beam onto the detection window. Fluorescence from the detection window was collected at a 90° angle through a $45\times$ microscope objective (0.65 N.A., E. Leitz Wetzlar, Germany), then passed through an iris diaphragm (Oriel model 62030) and an Optometrics model 2-2680 nm long pass filter ($\lambda_c = 680 \pm 5$ nm, transmittance $\geq 85\%$) which was placed in series with an Optometrics model 2-6712 interference filter ($\lambda = 671 \pm 2$ nm, FWHM $= 10 \pm 2$ nm, peak transmittance $\geq$ 40%). Another iris diaphragm was placed in the emission light path and the light was finally directed onto a side-on red-sensitive photomultiplier tube (PMT) (Hamamatsu model R928). The PMT was placed in an Oriel model 70680 side-on housing. The PMT voltage was adjusted to 1100 V with an Oriel model 70705 high voltage power supply and the signal was collected using a Keithely model 485 autoranging picoammeter. Data were recorded using a Hewlett Packard model 3396A integrator. The diode laser mount, optics and PMT were mounted on an Oriel model 10988 honeycomb optical base plate (36 in. $\times$ 48 in.) to reduce noise due to vibrational shock. The diode laser mount, laser lens and the capillary holder were additionally protected from vibrational shock by mounting them on an Oriel model 11190 low-profiled optical rail. The capillary holder, microscope objective and PMT were mounted on Oriel model 16021 precision translators. The entire instrument was housed in a black plexiglass box in order to shield it from ambient light.

2.7. Diode laser wavelength, power output and alignment

The output wavelength and power of the diode laser were measured with the use of an ILX-Lightwave model OMH-6720B silicon power/wavehead coupled to an ILX-Lightwave model OMM-6810B optical multimeter. The appropriate wavelength (647.8 nm) was achieved by varying the temperature of the diode laser at a constant power output of 4.1 mW.

The capillary detection window was aligned with the diode laser beam using horizontal precision translators in the X and Y directions. The laser beam was deemed to be aligned with the detection window at the position which provided the highest signal (Cy5.29.COOH) to background ratio.

2.8. Plasma analysis

Amantadine working stock solutions were prepared in methanol at concentrations of 2.5×10^{-4}, 1×10^{-4}, 5×10^{-5}, 2.5×10^{-5}, 1×10^{-5}, 5×10^{-6}, and 1×10^{-6} M through serial dilution. A rimantadine (internal standard) working stock solution was prepared at 3.2×10^{-5} M. Plasma standards were prepared by the addition of 37.5 μl of each amantadine working stock solution and 37.5 μl of the rimantadine working stock solution to a 15 ml polypropylene centrifuge tube containing 3 ml of blank plasma. The final concentrations of amantadine were 1.8, 9.2, 18.4, 46.1, 92.2, 184.4, and 461.1 ng ml^{-1}. Each plasma standard contained 70 ng ml^{-1} rimantadine. A set of seven duplicate standards was prepared by aliquoting 1 ml of each plasma standard into a 15 ml polypropylene centrifuge tube.

The plasma extraction procedure was a modification of the method of Sioufi et al. [14]. 1 N NaOH (100 μl) was added to a 15 ml polypropylene centrifuge tube containing 1 ml of blank/spiked plasma and the tube was vortex mixed for 10 s. Toluene (2 ml) was then added to each tube. The tubes were capped and shaken for 1 h on a longitudinal shaker. The tubes were then centrifuged at 3000 rpm for 10 min. An aliquot (1.5 ml) of the toluene layer was pipetted into a 13

mm × 100 mm screw cap borosilicate glass test tube and evaporated to dryness under nitrogen. Cy5.29.OSu (2.5×10^{-4} M) (25 μl) in acetonitrile–DMF (97:3%) was added to the residue and the volume was adjusted to 100 μl with acetonitrile. The derivatization reaction was carried out optimally at 70°C for 90 min. The reaction mixture was reduced to dryness and the residue was reconstituted in 100 μl deionized distilled water. The sample was then injected onto the laboratory constructed capillary electrophoresis system.

3. Results and discussion

3.1. Confirmation of the derivative

The formation of the derivative was confirmed by RP-TLC and CE-absorbance detection at 648 nm in preliminary studies. TLC of the reaction mixture resulted in a spot ($r_f = 0.76$) that was distinct from unreacted Cy5.29.OSu ($r_f = 0.86$ and 0.91). When the unreacted Cy5.29.OSu was spotted on the TLC plate along with Cy5.29.COOH and developed, it was observed that the spot with $r_f = 0.91$ corresponded to the acid form of the dye. The excitation and emission maxima of the isolated derivative were determined to be 646.7 and 664.9 nm, respectively, which was not significantly different from the excitation and emission maxima of the unreacted dye (650 and 666 nm, respectively). Capillary electrophoresis of the reaction mixture resulted in a peak that was distinct from unreacted Cy5.29.OSu.

3.2. Optimization studies

The derivatization reaction was optimized using a sequential single factor approach. This approach does not account for interactions between variables but is more practical than a simplex optimization approach since fewer experiments are necessary. The sequential single factor approach results in near optimal conditions. The studies for the optimal time and temperature for the derivatization reaction were performed in triplicate and the optimum time and temperature were determined to be 90 min and 70°C, respectively. The derivatization reaction was optimized with respect to label concentration in order to increase the yield of the derivative, which would enhance detection sensitivity. It was observed that the yield of the derivative increased as the concentration of Cy5.29.OSu was increased from 125 to 1500 nmol ml^{-1} and when the amantadine concentration was kept constant at 25 nmol ml^{-1}. Although no loss in resolution of the derivative from the excess label peaks occurred even when 1500 nmol ml^{-1} label was used, there was a large variation in the yield of the derivative at that concentration. A 500 nmol ml^{-1} concentration of Cy5.29.OSu in the reaction mixture was therefore established for subsequent analysis. This concentration was chosen in part due to the variability in the yield of the derivative at higher concentrations and partly due to cost considerations.

3.3. Capillary electrophoresis

When the reaction mixture was injected into the laboratory constructed CE-VDLIF system and run according to the same conditions that were found to be optimal for the Dionex system, it was observed that baseline resolution between the derivative peak and other peaks in the reaction mixture was not achieved. Experiments were conducted to determine whether the unresolved peaks were due to unreacted Cy5.29.OSu. Cy5.29.OSu can be hydrolized rapidly under alkaline conditions to form Cy5.29.COOH, which elutes long after the derivative peak. An increase in the concentration of amantadine in the reaction mixture, whilst keeping the amount of label fixed, did not result in a decrease in the height of the unresolved peak. In addition, hydrolysis of the label with 100 mM borate buffer (pH 9.1) did not effect the size of the unresolved peak. These results suggest that the unresolved peak was either a contaminant or a reaction by-product, rather than unreacted Cy5.29.OSu.

Studies were performed in order to separate the derivative from the unresolved peak. Improved separation of the derivative from the peak occurred when the pH of the buffer was reduced to 6.0, due to a decrease in the electroosmotic flow (EOF). This increased separation time for the

various components of the reaction mixture and improved the resolution to 1.23. The addition of methanol to the run buffer resulted in a decrease in the EOF due to an increase in viscosity and a decrease in the dielectric constant and zeta potential of the buffer. Hence the addition of methanol to the run buffer was investigated. The addition of up to 40% methanol to the run buffer further improved the separation relative to the peak showing a resolution of 1.27. Baseline resolution however was not yet achieved.

It was decided to exploit the micellar partitioning characteristics of the derivative to separate it from the unresolved peak. For this purpose micellar electrokinetic capillary chromatography (MEKC) was investigated [20]. The micellar phase corresponds to the stationary phase in conventional chromatography and moves with a velocity that is different from the surrounding aqueous phase during electrophoresis. Solutes which partition into the micellar phase can therefore be separated from more hydrophyllic solutes. Nishi et al. [21] have reported the use of tetraalkylammonium salts in MEKC to increase the interaction between anionic solutes and SDS micelles. The addition of SDS and tetramethylammonium hydroxide (TMAH) to the phosphate buffer (pH 6) resulted in a change in the elution order of the peaks due to the incorporation of the derivative into the SDS micelle. A 20 mM phosphate buffer (pH 6) containing 40 mM TMAH and 10 mM SDS resulted in near baseline resolution (Rs = 1.5) and the addition of methanol (10% v/v) to the buffer resulted in baseline resolution of the derivative peak (Fig. 3).

3.4. Limit of detection

The limit of detection (LOD) for unextracted amantadine was determined to be 9.5 fmol (21.1 ng ml^{-1}). The LOD was calculated as the amount that provided a signal 3 times the mean peak-to-peak noise (3 $S_{p\text{-}p}$). The peak-to-peak noise was determined across the elution window of the analyte peak, after injection of a blank into the capillary column. The limit of detection for amantadine extracted and concentrated from 1 ml of plasma was determined to be 115 amol.

3.5. Validation of the assay in plasma

The analysis of amantadine in plasma was validated by the establishment and evaluation of a selected linear range, examination of the limit of quantitation (LOQ), accuracy and precision as well as an evaluation of selectivity. A linear range was established by constructing a calibration curve using seven duplicate standards ranging from 1.8 to 461.1 ng ml^{-1}. When the standards were injected onto the capillary electrophoresis system it was observed that the internal standard peak was not well resolved from other interfering peaks which resulted in a large variability in the peak height of the internal standard. The Hafelfinger parameter $s_{brel} - 2rs_{arel}$ was calculated to be a positive number (24.05) for the control represent-

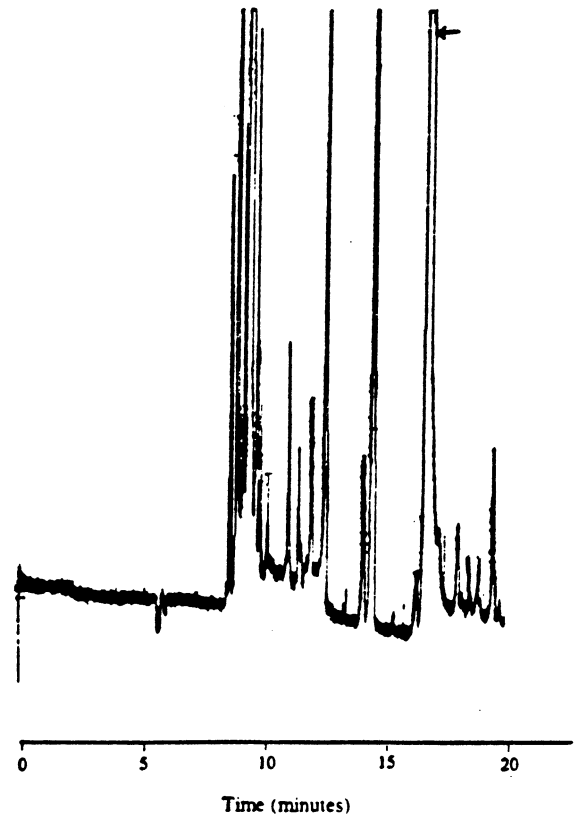

Fig. 3. Electropherogram showing baseline resolution of the derivative peak. Run buffer 20 mM sodium phosphate (pH 6), 10 mM SDS, 40 mM TMAH–methanol (90/10). The derivative peak is indicated by the arrow.

Table 1
Precision and accuracy results for the analysis of amantadine in plasma

Control concentration (ng ml^{-1})	Measured concentration (ng ml^{-1})	S.D.	R.S.D. (%)	DFN (%)
4.6 ($n=5$)	3.9	0.645	16.4	−14.8
138.3 ($n=6$)	152.4	24.68	16.1	10.2

Calibration range 1.8–461.1 ng ml^{-1}.

ing the low range of the calibration curve [22], therefore the use of an internal standard would be unlikely to improve the precision of the assay. Therefore it was decided to use the response of the amantadine peak alone rather than the response ratio to construct the calibration curve. A weighted linear least squares regression was used to obtain the calibration curve of the extracted plasma standards which demonstrated a correlation coefficient of $r=0.999904$. The back calculated residual standard concentrations were within 20% of the nominal values.

Based on the LOD for unextracted amantadine the theoretical limit of quantitation (LOQ) in plasma calculated as 10 $S_{p\text{-}p}$ would be 860 pg ml^{-1}. This takes into account a concentration factor derived from extracting amantadine from 1 ml of plasma. The effective LOQ of the assay, however, as determined by the lowest concentration point of the calibration curve was 1.8 ng ml^{-1}. The LOQ of amantadine in plasma was found to be lower than the LOD of unextracted amantadine (21.1 ng ml^{-1}) due to the preconcentration during sample preparation.

The precision and accuracy of the assay were determined using control samples ($n=6$) at two concentrations (4.6 and 138.4 ng ml^{-1}) which represented the low and mid to upper ranges of the calibration curve. Precision was represented by the percent R.S.D. of the back-calculated concentrations. Accuracy was calculated as percent difference from nominal (%DFN) of the back-calculated concentrations. The precision and accuracy of the assay were within 17 and 15% respectively (Table 1.) and were deemed to be acceptable. The selectivity of the assay was demonstrated by observing no interfering peaks across the elution window of the amantadine peak in blank plasma extracts ($n=6$ sources). Electropherograms of extracted blank and spiked plasma are shown in Fig. 4a and b, respectively. The labeling efficiency of the derivatization reaction was determined to be between 8–15%. The efficiency was calculated based on the assumptions that the reaction between the drug and the label follows a 1:1 stoichiometry and the absorptivities of all the peaks in the reaction mixture are equal. The labeling efficiency was then calculated by dividing the moles of derivative formed by the moles of amantadine added to the samples.

4. Conclusions

The laboratory constructed CE-VDLIF detection system was found to be suitable for the measurement of amantadine derivatized with Cy5.29.OSu. The assay was developed and validated at clinically relevant concentrations. The assay was demonstrated to be selective for the analysis of amantadine in plasma. The LOD for unextracted amantadine was found to be 9.5 fmol. The experimental factors that may have limited the LOD were impurities present in the dye and the labeling efficiency of the reaction. The LOQ for amantadine in plasma obtained using CE-VDLIF detection (1.8 ng ml^{-1}) was comparable to the quantitation limits reported in the literature. The derivatization procedure developed here can be used for the labeling of other aliphatic primary and secondary amines. We observed that

Fig. 4. Evaluation of selectivity: (a) electropherogram of extracted blank plasma, and (b) electropherogram of extracted spiked plasma; both taken through the derivatization procedure. The peak at 19.17 min represents 1.8 ng ml^{-1} amantadine. The amantadine peak is indicated by the arrow.

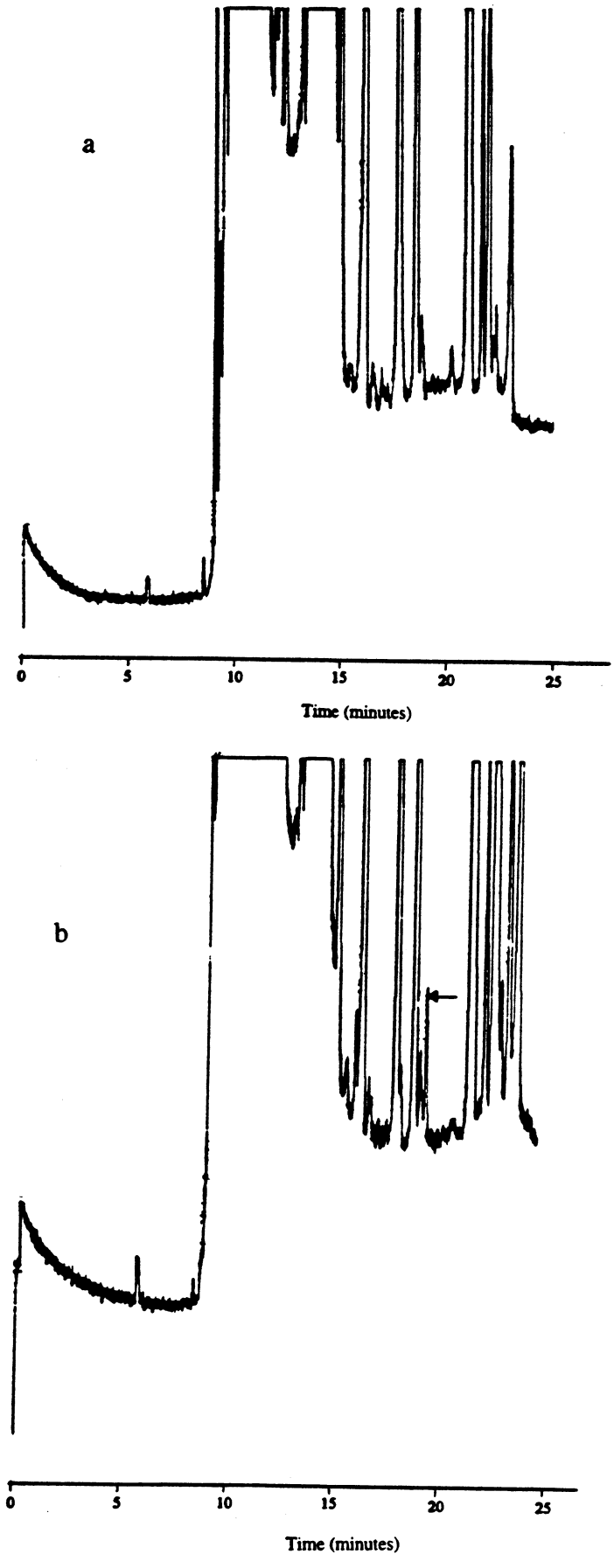

Fig. 4.

VDLIF detection resulted in greater than 2 orders of magnitude (~ 400-fold) improvement in detectability as compared to absorbance detection performed on a commercially available CE system. VDLIF detection certainly appears to be a useful tool to improve concentration sensitivity in capillary electrophoresis.

Acknowledgements

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