BIOMEDICAL CHROMATOGRAPHY, VOL. **12**, 8–12 (1998)

Investigation of Far Red Dyes for use in Peroxyoxalate Chemiluminescence Detection and Analysis of the CY5 Derivative of Amantadine Hydrochloride in Human Plasma

Andy Ellingson and H. Thomas Karnes*

Department of Pharmacy and Pharmaceutics, Medical College of Virginia, Virginia Commonwealth University, P.O. Box 980533, 410N. 12th St., Richmond, VA, 23298–0533, USA

Peroxyloxalate chemiluminescence is well established as a tool for improvement of selectivity and sensitivity for chemiluminophores and their derivations in HPLC eluates. Chemiluminescence in the far-red spectral region was investigated in this work to further enhance the sensitivity of chemiluminescence through more efficient singlet excitation energy transfere and to enhance the selectivity of the approach through a reduction in matrix and scatter interference. A number of fluorescent compounds that can be excited in the UV, visible and far red spectral regions were investigated for chemiluminescence yield using the bis(2,4,6-trichlorphenyl) oxylate reaction. It was found that a trend of increasing chemiluminescence with increasing excitation wavelength could be observed with cy5 providing the most efficient chemiluminescence. The succinate ester of cy5 was used to derivatize amantadine hydrochloride, an antiparkinsons drug, to form the derivative. The derivative was separated from reaction by products by C_{18} reversed phase HPLC and detected using a Soma S-3400 chemiluminescence detector. The detection limit for the diluted derivative was 200 femtomoles on column and sufficient for plasma analysis. Selectivity in plasma was demonstrated through derivatization of extracts of plasma that had been spiked with amantadine hydrochloride. © 1998 John Wiley & Sons, Ltd.

Biomed. Chromatogr. **12**, 8–12, (1998)

Keywords: chemiluminescence; HPLC; cyanine dye; yield; amantadine

INTRODUCTION

Chemiluminescence (CL) is a powerful detection technique that is both highly selective and sensitive. Selectivity is attributed to the relatively small number of compounds which chemiluminesce and improved sensitivity is a result of the absence of background noise from stray light (Givens *et al*., 1990). Few compounds present in biological matrices emit light in the far red spectral region, thus far red chemiluminescent labels may be attractive for further enhancement of selectivity (Kimoto *et al*., 1996). It has been reported that compounds having low singlet excitation energy are more effectively excited by the reactive intermediate (1,2-dioxetanedione) produced in peroxyoxalate chemiluminescence (PO-CL) (Honda *et al*., 1985). Far red dyes may therefore chemiluminesce more intensely than other commonly used chemiluminophores and further enhancement of sensitivity may also be realized. PO-CL has been well established as an effective method to excite a fluorescent compound (Honda *et al*., 1985). In this investigation, fluorescent compounds in the UV, visible, and far red regions of the spectrum were evaluated as chemiluminophores by PO-CL. Chemiluminescent intensity was measured by flow-injection analysis. Fluorescence and absorbance spectra were obtained and a value denoted Ydf was calculated to quantitate the chemiluminescent efficiency of each compound as done by Honda *et al*. (1985). CY5, a dicarbocyanine dye (Fig. 1), was found to be the most efficient chemiluminophore and was selected for further investigation. Amantadine hydrochloride (Fig. 1), a primary amine used to treat both influenza and Parkinsonism (Rakeshaw, 1983), was selected as a model analyte. An amantadine hydrochloride-CY5 derivative was prepared and separated from unreacted CY5 by reverse phase (RP) HPLC and subsequently detected by PO-CL. To show the applicability of PO-CL detection in biological samples, human plasma was spiked with amantadine hydrochloride, extracted, derivatized, and detected by PO-CL.

* Correspondence to: H. T. Karnes, Department of Pharmacy and Pharmaceutics, Medical College of Virginia, Virginia Commonwealth University, P.O. Box 980533, 410N. 12th St., Richmond, VA, 23298–0533, USA.

Contract grant sponsor: APQ Section of AAPS.

EXPERIMENTAL

Instrumentation. Flow-injection analysis was performed using a syringe pump (Harvard Apparatus Syringe Infusion Pump 22, Harvard Apparatus, South Natick, MA, USA) to deliver hydrogen peroxide (1×10^{-2} M in acetonitrile (ACN)) and (bis(2,4,6-trichlorophenyl))oxalte (TCPO) (5×10^{-3} M in ethyl acetate) in a 3:1 ratio at 2 mL/min to a laboratory constructed mixing device based on a previous design (Kobayashi and Imai, 1980). The optimal CL reagent concentrations and proportions were determined by Walters in a previous study (Walters *et al*., 1994). These reagents were mixed with the flow-injection mobile phase (95% ACN in H_2O) pumped at 3 mL/min using a reciprocating head pump (Hewlett Packard Series 1050, Model 79852A, Federal German Republic). Chemiluminescence was measured with an S-

CCC 0269–3879/98/010008–05/$17.50

Received 31 July 1996
Accepted 14 August 1996

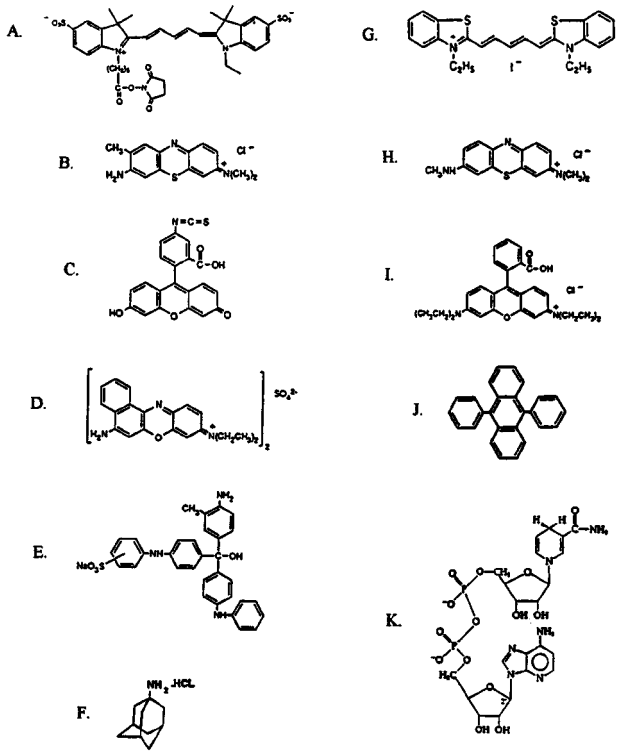

Figure 1. Structures (A) CY5; (B) Toluidine Blue; (C) FITC; (D) Nile Blue A; (E) Alkali Blue; (F) Amantadine Hydrochloride; (G) DTCDI; (H) Azure B; (I) Rhodamine B; (J) DPA; (K) NADH.

3400 detector (Soma Optics, Tokyo, Japan) equipped with an R26812 photomultiplier tube (PMT) and a 100 μL spiral flow cell. Peak areas were measured using a Shimadzu C-R6A Chromatopac recording integrator (Kyoto, Japan).

HPLC analysis was performed using a second reciprocating head pump (Altex model 110A, USA) attached to two pulse dampeners for post-column CL reagent addition (same as above) at a rate of 1 mL/min to the mixing device. The HPLC mobile phase of 35% ACN in H_2O containing 0.1% imidazole, pH = 7 was delivered at a rate of 0.5 mL/min from the Hewlett Packard pump. The samples were injected using a Rheodyne model 7125 injector (Rheodyne Inc., Cotata, CA, USA) into a 50 μL loop to a 300×3.9 mm i.d. C_{18} (5 μm particles) Phenomenex column (OOH-2117-CO, Torrance, CA, USA) prior to PO-CL detection.

Fluorescence measurements were obtained on an LS-50 spectrofluorometer (Perkin–Elmer Nederland, Gouda, The Netherlands) equipped with an R928 PMT using a 1 cm cuvette. Absorbance measurements were obtained on a Lambda 2S UV/vis spectrometer (Perkin–Elmer) using matched 1 cm cuvettes.

Reagents. HPLC-grade acetonitrile, ethyl acetate, methanol, and dimethyl formamide (DMF) were purchased from Baxter (Columbia, MD, USA). Toluene was purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was doubly distilled (Corning mega-pure, Corning, NY, USA). TCPO, hydrogen peroxide (30.8% in H_2O), imidazole (99+%), β-nicotinamide adenine dinucleotide, reduced from (NADH), 9,10 diphenylanthracene (DPA), fluorescein isothiocyanate (FITC), and amantadine hydrochloride (97%) were all purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Nile blue A (NB), Azure B, Toluidine Blue (TB), and Alkali Blue were purchased from Aldrich Chemical (Milwaukee, Wis, USA). Rhodamine B was obtained from Lambda Physik (Acton, MA, USA), and CY5, succinate ester was obtained from Amersham Life Sciences (Pittsburgh, PA, USA).

Flow-injection. 10^{-4} M solutions of Azure Blue, NB, DPA, TB, Rhodamine B, and CY5 were prepared in 95% ACN in H_2O. 10^{-4} M solutions of NADH, FITC, and Alkali Blue were prepared in 50% ACN in H_2O. Each of these nine solutions was then diluted to 10^{-5} M in 95% ACN in H_2O and injected (50 μL) onto the flow-injection system. The areas of the peaks were recorded for each compound.

Fluorescence measurements. The area of the fluorescence spectrum for each compound was obtained at either 10^{-7} M or 10^{-8} M in 95% ACN in H_2O depending on the intensity of the signal. The maximum absorbance and emission wavelengths were determined for each compound.

Absorbance measurements. The absorbance for each compound at the maximal excitation wavelength was measured at 5×10^{-6} M, 1×10^{-5} M M, or 1×10^{-4} M in 95% ACN in H_2O depending on the intensity of the signal. A solution of 95% ACN in H_2O was used as the blank.

Derivatization of amantadine hydrochloride. Stock solutions of amantadine hydrochloride (1-adamantanamine hydrochloride) were prepared at 250 and 2250 nmol/mL in methanol. Stock solutions of CY5.29.OSuc were made at 250 nmol/mL and 500 nmol/mL in 3% DMF by volume in ACN. An aliquot of one of the amantadine stock solutions was added to a Wheaton 2.5 mL glass reaction vial and evaporated to dryness using dry nitrogen (N-EVAP, model 112, Organomation, Northborough, MA, USA). 70 μL of 350 nmol/mL CY5 stock solution was added to the vial and then vortex mixed for 30 seconds. The vial was heated at 70°C for 90 min, conditions determined to be optimal in other work, and was vortexed every 10 min to facilitate mixing. After the heating procedure, the vial was evaporated to dryness and reconstituted in 2 mL of 50% ACN in H_2O. This solution was then injected onto the HPLC system.

Plasma extraction. A variation of the amantadine hydrochloride plasma extraction as reported by Rakestraw (Rakestraw, 1993) was performed. 90 μL of human plasma was spiked with 10 μL of amantadine solution in methanol in a 1.5 mL polypropylene micro centrifuge tube and vortex mixed for 10 s. 100 μL of 1.0 N NaOH was added, and the tube was vortex mixed for 10 s. To this, 200 μL of toluene was added and the tube was vortex mixed for five min. After centrifuging for 10 min, 100 μL of the toluene layer (upper) was transfered into a Wheaton 2.5 mL glass reaction vial using a 100 μL pipet, evaporated to dryness, and derivatized as previously described.

RESULTS AND DISCUSSION

Chemiluminescence yield of dye candidates

Honda *et al.* (1985) defined CL yield as the quantity Ydf' where:

$$Ydf' = e'I'I'_{CL}/I'_F \quad (1)$$

and where e',I',I'_{CL}, and I'_F are the relative molar absorption coefficient, relative intensity of the light source, relative chemiluminescence intensity, and the relative fluorescence intensity, respectively. All quantities are measured relative to DPA, thus these four quantities are each assigned a value of 1 for DPA. For the other compounds, each quantity is divided by the value obtained for DPA to obtain the value relative to DPA. YDf' is a quantitative measure of CL with respect to fluorescence corrected for differences in molar absorptivity and light intensity. Absorbance measurements

Table 1. Molar absorptivities (ε) of the dyes investigated measured at the excitation maximum

Name of Dye	Abs.	Conc. (M)	ε	ε rel
DPA	0.1497	1.0X-05	14970	1
CY-5	0.7897	5.0X-06	157940	10.55043
Rhodamine-B	0.5434	1.0X-05	54340	3.629927
FITC	0.4048	1.0X-05	40480	2.704075
Alkali Blue	0.1434	1.0X-04	1434	0.095792
Azure Blue	0.4909	1.0X-05	49090	3.279225
NADH	0.0394	1.0X-05	3940	0.263193
Toluidine Blue	0.2041	1.0X-05	20140	1.345257
Nile Blue	0.3869	1.0X-05	38690	2.584502

were used to calculate both absolute and relative molars absorptivities of the compounds and are shown in Table 1. Fluorescence measurements were corrected for differences in the sensitivity of the PMT to different wavelengths of light through a correction feature in the software used to obtain the fluorescence spectra. Both absolute and relative areas under the fluorescence curves are shown in Table 2. Flow-injection analysis ($n=3$) was used to determine the CL intensities shown in Table 3. A correction factor was included in the CL relative to DPA to account for the differences in sensitivity of the PMT to different wavelengths of light. The correction factor was obtained from a graph of the photocathode radiant sensitivity (mA/W) vs. wavelength (nm) for the PMT tube found in the Hamamatsu catalog (Hamamatsu Photonics, Hamamatsu, Japan). The differences in the intensities of the xenon arc light source at the wavelength range used in the LS-50 spectrofluorometer were considered to be negligible, thus I′ was assigned a value of 1 for all compounds. The *Ydf′* values are shown graphically in Fig. 2.

As expected, the far red dyes demonstrated higher *Ydf′* values than the compounds in the other spectral regions. Significant differences were observed in the *Ydf′* values for these far red dyes which spanned over two orders of

Table 2. Fluorescence characteristics of the dyes investigated

Name of Dye	AUC*	Conc (M)	AUC rel	λ ex	λ em
DPA	4341	1.0X-08	1	374	412
CY-5	961	1.0X-08	0.2214	647	670
Rhodamine	520.4	1.0X-08	0.11	548	572
FITC	699	1.0X-08	0.1610	489	530
Alkali Blue	6.82	1.0X-07	0.013	627	660.5
Azure Blue	73.5	1.0X-07	0.0169	637	662
NADH	28	1.0X-07	0.006	337	368
Toluidine Blue	88.3	1.0X-08	0.0203	631	654
Nile Blue	838.8	1.0X-07	0.1932	632	663

* Area under the fluorescence curve.

Table 3. Chemiluminescence characteristics of the dyes investigated

Name of Dye	Average CI intensity	Sd	Relative to DPA
DPA	96112	5037	1
CY-5	1130242	39189	11.76
Rhodamine	7471107	33858	7.773
FITC	34457	3766	0.358
Alkali Blue	1121258	3044	1.167
Azure Blue	1323	209	0.014
NADH	7830	1204	0.081
Toluidine Blue	218948	16678	2.27
Nile Blue	52468	364	0.54

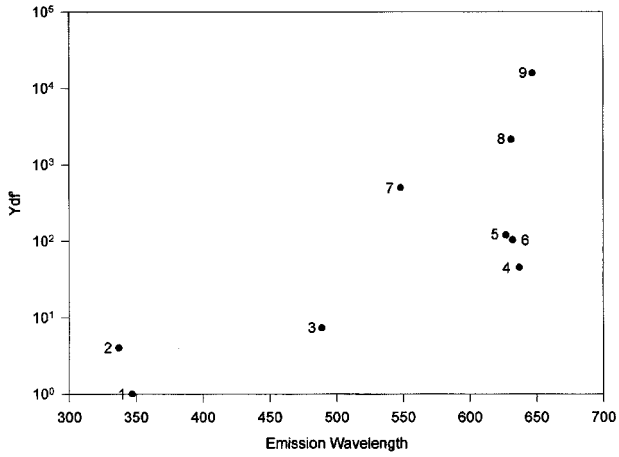

Figure 2. Plot of Ydf′ vs. Emission wavelength for the fluorescent dye compounds: (A) DPA; (2) NADH; (3) FITC; (4) Azure B; (5) Alkali Blue; (6) Nile Blue A; (7) Rhodamine B; (8) Toluidine Blue; (9) CY5.

magnitude. These differences may be attributed to the structural characteristics of the individual compounds. It has been reported that compounds which have low oxidation potentials are effectively excited by PO-CL and thus are good chemiluminophores (Honda *et al*., 1985). Because the structures of the far red dyes vary significantly, direct structural comparisons between all the dyes would be difficult. Two pairs of dyes however have similar structures and can be compared. Toluidine Blue and Azure B have identical ring structures and the only differences are the methyl and primary amine in TB and the hydrogen and secondary amine in Azure B. TB demonstrated a significantly higher *Ydf′* value than Azure B, yet their emission wavelengths are nearly identical. This difference may be a result of the electron withdrawing of the methyl substituent on TB. Electron withdrawing groups decrease the oxidation potential of the ring structure, and may thus have increased the excitation efficiency of the PO-CL which is consistent with the experimental observations. A second pair of dyes that have similar base ring structures are FITC and Rhodamine B. Rhodamine B has a significantly higher *Ydf′* value as compared to FITC which most likely can be attributed to the presence of the quaternary ammonium ion present in Rhodamine B. This is a highly electron withdrawing group when compared to the ketone present in FITC. FITC does have a tertiary amine present that is lacking in Rhodamine B, but this would not be expected to have the electron withdrawing capability of the quaternary ammonium ion in Rhodamine B, hence FITC has a higher oxidation potential and a less intense PO-CL response.

The *Ydf′* value of CY5 was found to be 16,000, and CY5 was found to be the most effective chemiluminophore. Kimoto *et al*. (1996), reported that 3,3′-Diethylthiadicarbocyanine iodide (DTDCI) had the lowest LOD (0.19 femtomoles, 2XS/N) of four near-infrared compounds which were investigated. The structure of DTDCI (Fig. 1) is similar to that of CY5 (LOD = 5 femtomoles 3XS/N, peak to peak). Qualitatively, the results of Kimoto's study agree with the results of this study that the dicarbocyanine structure results in the favorable PO-CL intensity, but a large difference in reported LOD exists between these two studies. This difference in LOD can be accounted for by two factors. First, Kimoto *et al*. (1996) used a red-sensitive R2228 PMT rather than the R26812 PMT used in this investigation. The R2228 is at least 10 times more sensitive to light in the red spectral region. Second, Kimoto *et al*.

(1996) used TDPO rather than TCPO. It has been reported (Honda *et al*., 1983) that up to ten fold increases can be realized in the PO-CL intensity when TDPO is used instead of TCPO. Assuming each of these factors contributed one order of magnitude of increased sensitivity, the theoretical LOD for CY5 would be 0.05 femtomoles, which is less than half the value obtained by Kimoto *et al*. (1996). Furthermore, DTDCI lacks a functional group capable of derivatization. CY5 has a succinimide group capable of being derivatized and the amantadine hydrochloride-CY5 derivative has been characterized.

Analysis of amantadine hydrochloride using CY5 as the labeling reagent

Underivatized CY5 was injected onto the HPLC system and the LOD ($3\times$ S/N (p/p)) was found to be 5 femtomoles on-column. Amantadine hydrochloride was then derivatized with CY5 and separated from the unreacted CY5. Because CL is highly sensitive to imidazole concentration, solvent strength, and pH, all of these variables were optimized to produce the best separation without sacrificing CL signal intensity (Walters *et al*., 1994). Five different RP C18 columns were investigated with the Phenomenex being the only column to provide a CY5 retention time greater than four minutes, thus it was selected as the best column. Solvent strength was varied from 95% ACN in H_2O to 5% ACN in H_2O. CL intensity is directly related to the solvent strength of the mobile phase, so the largest percentage of ACN giving an adequate separation was chosen which was 35% ACN in H_2O. Imidazole, a known catalyst in peroxylate CL, was chosen as the buffer. The imidazole was investigated at three concentrations (0.05%, 0.1%, and 0.2%), with 0.1% giving the most intense signal. Finally, pH was investigated at 1.5, 3, 5, and 7, with pH = 7 giving the only suitable separation of the derivative from the unreacted CY5. The final mobile phase chosen was 35% ACN in H_2O, 0.1% imidazole, pH = 7. A typical chromatogram of the amantadine hydrochloride-CY5 derivative and the reaction blank are shown in Fig. 3. The derivative had a retention time of 6.5 min. A second, much smaller, broader amantadine concentration dependent peak was found at 9.5 min. This peak was consistently 14% of the larger peak indicating it is the result of an unidentified side-reaction product. This peak was not chosen to be used quantitatively. The relationship between the area of the peak eluting at 6.5 min and the concentration of amantadine hydrochloride is shown in Fig. 4.

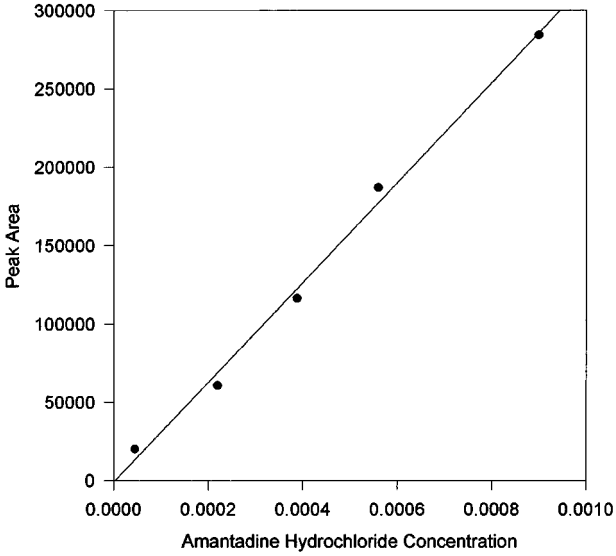

Figure 4. Plot of peak area vs. amantadine hydrochloride concentration (mol/L). Linearity was demonstrated in the range of 4.5×10^{-5} M to 9×10^{-4} M. Linear regression data: $m=3.2\times10^{8}$; $b=-770$; $r^2=0.99$.

The selectivity of the technique for analysis of amantadine hydrochloride in plasma was demonstrated by derivatization of extracts of blank plasma and plasma spiked

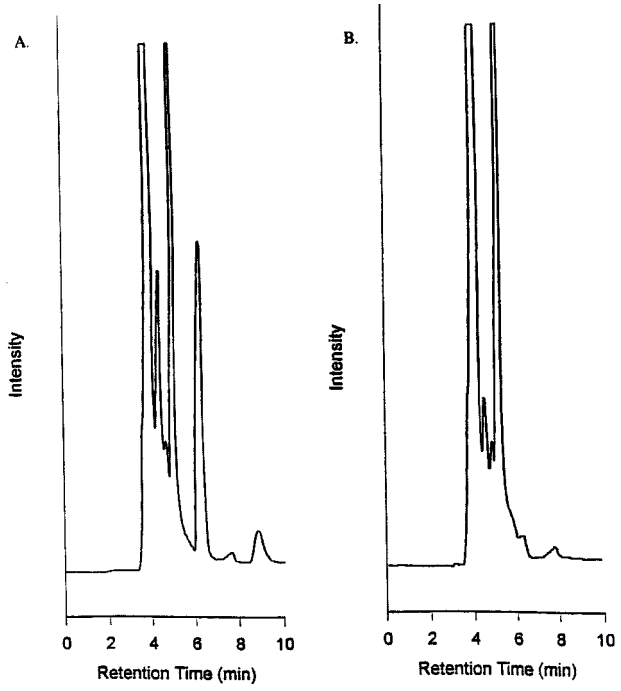

Figure 3. Typical LC chromatograms of CY5 labeled amantadine hydrochloride (75 pmol on column) (A), and CY5 reaction blank (B).

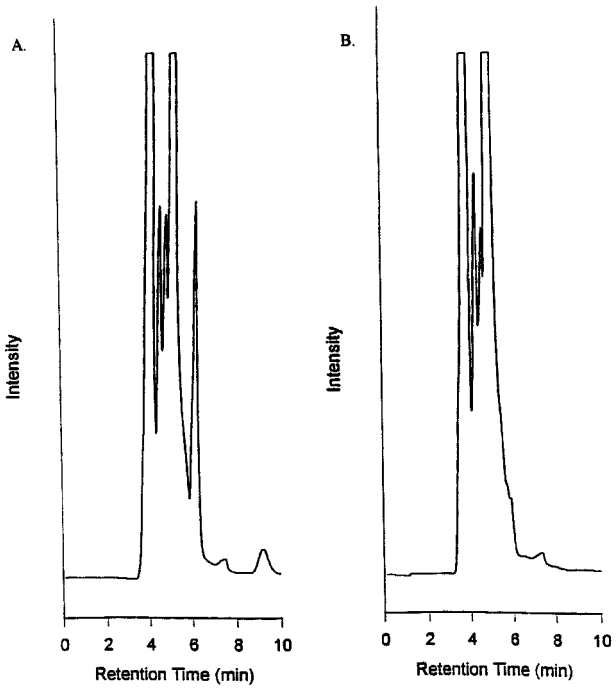

Figure 5. Typical LC chromatograms of human plasma spiked with 1200 ng/mL amantadine hydrochloride, extracted, and derivatized with CY5 (A), and blank human plasma extracted and derivatized with CY5 (B).

with amantadine hydrochloride. The absence of a detectable peak corresponding to the derivative in plasma blanks is evidence for selectivity of the method in the plasma matrix (Fig. 5).

The limit of detection for the current method determined by serial dilution was 4.0×10^{-9} M, or 200 femtomoles on column. The limit of derivatization was determined to be 1.0×10^{-5} M, or 500 picomoles on column for both plasma and neat solutions. Linearity for the derivative in neat solution established over the range of 4.5×10^{-5} M to 9.0×10^{-4} M. Rakestraw (1993), using electron capture detection, reported concentrations in plasma in the range of 2.3 to 402.9 ng/mL with the highest biologically relevant concentration found in their study to be 261.5 ng/mL using 1 mL of plasma. Mank *et al*. (1995) reported the limit of derivatization of *n*-octyl amine as 2×10^{-8} M and derivatized 5×10^{-7} M amantadine in urine. If the amantadine hydrochloride-CY5 derivative can be prepared at 5×10^{-7} M and an optimized separation of the derivative from the unreacted CY5 can be achieved, plasma concentrations as low as 0.05 ng/mL using 1.0 mL of plasma could be detected. This would result in an increase in detectability of two orders of magnitude as compared to Rakestraw's method (Rakestraw, 1993).

CONCLUSIONS

Far red dyes are more effectively excited by PO-CL, thus are better chemiluminophores than compounds which emit in other regions of the spectrum. CY5, a dicarbocyanine dye, was found to be the most efficiently excited far red dye. Chemiluminescence detection of the amantadine hydrochloride-CY5 derivative in human plasma has been demonstrated and potential has been shown for measurement at biologically relevant concentrations. With further optimization in the separation and derivatization procedure, detection limits below that which are currently available with electron-capture detection may be realized.

Acknowledgements

This work was supported by a summer fellowship from the APQ section of AAPS. Sriram Nagaraj is also gratefully acknowledged for his help with amantadine hydrochloride-CY5 derivatization.

REFERENCES

Givens, R. S., Jencen, D. A., Riley, C. M., Stobaugh, J. F., Chokshi, H. and Hanaoka, N. (1990). *J. Pharm. Biomed. Anal.* **8**, 477.

Honda, K., Sekino, J. and Imai, K. (1983). *Anal. Chem.* **53**, 940.

Honda, K., Miyaguchi, K. and Imai, K. (1985). *Anal. Chem. Acta* **177**, 111.

Kimoto, K., Gohda, R., Murayama, K., Santa, T., Fukeshima, T., Homma, H. and Imai, K. (1996). *Biomedical Chromatogr.* **10**, 189.

Kobayashi, S. and Imai, K. (1980). *Anal Chem.* **52**, 1548.

Mank, A. J. G., Laan, H. T. C., Lingerman, H., Gooijer, C., Brinkman, U. A. T. and Velthorst, N. H. (1995). *Anal. Chem.* **67**, 1742.

Rakestraw, D. (1993). *Biomedical Chromatogr.* **11**, 699.

Walters, D. L., James, J. E., Vest, F. B. and Karnes, H. T. (1994). *Biomedical Chromatogr.* **8**, 207.

