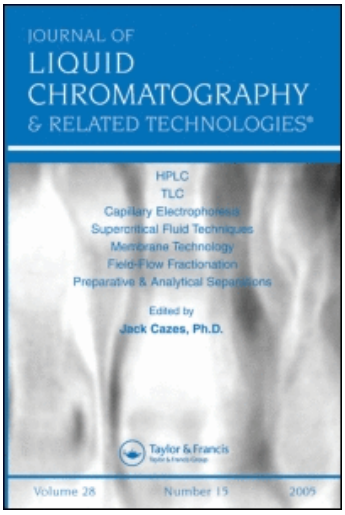


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Phosphorimetric Detection in HPLC VIA Trivalent Lanthanides: High Sensitivity Time-Resolved Luminescence Detection of Tetracyclines Using $Eu^3$ in a Micellar Post Column Reagent

Jeffrey X. Duggan[a]
[a] Perkin-Elmer Corporation, Oak Brook, Illinois

**To cite this Article** Duggan, Jeffrey X.(1991) 'Phosphorimetric Detection in HPLC VIA Trivalent Lanthanides: High Sensitivity Time-Resolved Luminescence Detection of Tetracyclines Using $Eu^3$ in a Micellar Post Column Reagent', Journal of Liquid Chromatography & Related Technologies, 14: 13, 2499 — 2525
**To link to this Article: DOI:** 10.1080/01483919108049335
**URL:** http://dx.doi.org/10.1080/01483919108049335

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(13), 2499-2525 (1991)

# PHOSPHORIMETRIC DETECTION IN HPLC VIA TRIVALENT LANTHANIDES: HIGH SENSITIVITY TIME-RESOLVED LUMINESCENCE DETECTION OF TETRACYCLINES USING $Eu^{3+}$ IN A MICELLAR POST COLUMN REAGENT

**JEFFREY X. DUGGAN**
*Perkin-Elmer Corporation*
*2000 York Road*
*Oak Brook, Illinois 60522*



## ABSTRACT

The detection of tetracyclines in HPLC by sensitized Europium phosphorescence has been reinvestigated using a dedicated LC detector with time-resolved luminescence capability. A micellar $Eu^{3+}$ post column reagent was developed which contained 1% Triton X-100 and 200uM tri-n-octyl phosphine oxide buffered at pH 10.5. This reagent provided optimized time-resolved detection and was found to be compatible with a several mobile phases used in reversed phase chromatography of tetracyclines. The sensitivity and quantitative linearity of the technique was determined using standard fluorometric and time-resolved modes of detection with both grating and filter emission monochromation. Peak areas were linear with concentration from 2 ug on column to the limit of detection. Subnanogram limits of detection were obtained for both tetracycline and oxytetracycline. The relative sensitivity of the technique for compounds tested was oxytetracycline = tetracycline > doxycycline > chlortetracycline >> minocycline. The high selectivity of this method promises to be useful in

simplifying chromatograms from complex matrices such as pysiological fluids and may also provide positive evidence for the presence of tetracyclines or structural analogs in a number of sample types.

## INTRODUCTION

### Characteristics of Organic Complexes of Trivalent Lanthanides

The trivalent lanthanides, $Eu^{3+}$ and $Tb^{3+}$, form complexes with a number of important molecules, including nucleic acids (1,2), aromatic aldehydes (3), and tetracyclines (4,5). In some cases, specific organolanthanide chelates are highly luminescent, exhibiting excitation spectra characteristic of the organic moiety and narrow line emission spectra corresponding to that of the lanthanide. The mechanism of sensitized phosphorescence of lanthanide chelates was described almost 30 years ago by Whan and Crosby (6). In these complexes, the organic chelator efficiently transfers excitation energy from its lowest excited triplet state to a higher energy level of the lanthanide by an intramolecular process. Lanthanide emissions are characterized by narrow luminescence bands with lifetimes in the submillisecond time frame. Sensitized lanthanide detection techniques are particularly well suited for HPLC by virtue of their sensitivity, selectivity, and simplicity. Unlike molecular phosphorescence, sensitized lanthanide luminescence occurs readily in liquid solution at room temperature; and because energy transfer from the organic triplet state to the lanthanide ion is an intramolecular process, luminescence is not quenched by oxygen (4,7).

### Sensitized Europium Luminescence for Detection of Tetracyclines

The use of $Eu^{3+}$ ions for the determination of tetracyclines (TCs) was first demonstrated by Hirshy et al. (4,7). These workers found that the TC

chelate of Europium had a luminescence peak ten times more intense than the fluorescence emission of uncomplexed TC and that the optimum tetracycline:$Eu^{3+}$ ratio for detection was 1:1. Recently, Wenzel et al. studied the use of $Eu^{3+}$ as a fluorogenic reagent for post column liquid chromatographic detection and flow injection analysis of TCs (5). They found that a number of published mobile phases were compatible with post column detection by sensitized lanthanide emission and that the use of this very selective technique with reversed phase HPLC helped to reduce background peaks from serum samples. Using a reagent buffered at pH 10.0 containing $10^{-4}$ M EDTA and $Eu^{3+}$, subnanogram detection limits were achieved using flow injection analysis with fluorometric detection. Attempts to use time-resolved detection, however, yielded higher detection limits, not lower as might be expected. Liquid chromatographic limits of detection for TC using the same reagent post colmn with fluorescence detection ranged from 1 to 4.8 ng (depending on mobile phase), and no l.o.d. for time-resolved HPLC detection was reported (5).



To date, published applications of sensitized $Eu^{3+}$ luminescence as applied to LC detection have utilized either filter fluorometric detection (3) or a benchtop grating luminescence spectrophotometer equipped with an LC flow cell (5). The purpose of this work was to reinvestigate the problem of sensitized $Eu^{3+}$ luminescence detection of TCs in reversed phase HPLC using a commercially available dedicated LC detector capable of time-resolved measurements and to optimize post-column conditions for maximum sensitivity and selectivity. The added selectivity of time-resolved detection has not yet been successfully utilized for this system. One reason for this is that the luminescence lifetime of this complex is too short (7) to permit efficient measurement by conventional pulsed source detectors which require delay times of at least 30 usec for full rejection of fluorescence and scatter (5). The approach taken here was to investigate the technique of micelle-stabilized luminescence as means of increasing both the luminescence yield and apparent lifetime of emissions from the TC/$Eu^{3+}$ chelate in order to take advantage of the added selectivity of time-resolved detection.

## MATERIALS AND METHODS

### Reagents

The hydrochlorides of tetracycline (TC), minocycline (MC), chlortetracycline (CTC), doxycycline (DC), and oxytetracycline (OTC) were purchased from Sigma Chemical Company, St Louis, MO. Trifluoroacetic acid (TFA), 2-amino-2-methyl-1-propanol (AMP), disodium ethylenediaminetetraacetic acid (EDTA), 3-(cylohexylamino)-1-propanesulfonic acid (CAPS), 3-(cyclohexylamino)-2-hydroxy-1-propane sulfonic acid (CAPSO), and tri-n-octylphosphine oxide (TOPO) were also from Sigma Chemical Company. Tris (hydroxymethyl)aminomenthane (TRIS) was from Boehringer Mannheim Biochemicals, Indianapolis, IN. Europium chloride was from GFS Chemicals, Columbus, OH. Triton X-100 (Scintillation grade) was obtained from Eastman Chemicals, Rochester, N.Y., and [polyoxyethylene (4) lauryl ether] (Brij30 (r)) was purchased from Aldrich Chemical Company, Milwakee, WI. Acetonitrille (ACN), methanol, and other chromatographic solvents were of HPLC reagent grade. All other chemicals were of Analytical Reagent grade.



### Apparatus

All chromatography was performed using the Perkin-Elmer Expert Analyst System consisting of a quaternary pump, autosampler, UV diode array detector and PC-based software for instrument control and data processing. The column used was a 25 x 0.46 cm, 5 micron C-18 column (Type 201H554) kindly provided by Vydac Corp., Hesperia, CA. The flow rate for both mobile phase and post column reagent (PCR) was 1.0 ml/min. Optimization of mobile phase parameters was assisted by automated searches through the PESOS program. The PCR was supplied by an isocratic pump equipped with a damping capacitor and a stainless steel backpressure coil (3m of 0.2mm tubing providing 400-600 psi at 1 ml/min). The column effluent and post column reagent were mixed in a straight stainless steel T

junction (SSI Corp.) which was connected to the detector inlet by 15 cm of 0.4mm i.d. PTFE tubing. Connections were made so that the angle between the column effluent and post column reagent tubes was 180°.

Absorption spectra were taken on a Perkin-Elmer Lambda 6 spectrophotometer with a slit setting of 1 nm using the UV Data Manager Software. The luminescence detector used was the Perkin-Elmer Model LC-240 double grating LC detector equipped with a red-sensitive sample PMT (Hammamatsu R928), an automated emission filter wheel accessory, and a total emission accessory. The slit widths were fixed at 10nm.

Post column reagent optimization experiments were performed in 10 mm quartz cuvettes using the PC-controlled Perkin-Elmer Model LS-50 luminescence spectrophotometer and the Fluorescence Data Manager Software. Fixed wavelength readings were performed using excitation and emission wavelengths of 395 and 617 nm, respectively, with EX/EM slit widths of 15/10 nm. For all fixed wavelength measurements, the luminescence signal was integrated for 1 second. Reported values represent the mean of 2 independent determinations. Time-resolved measurements were made using a delay time of 0.03 msec and a gate time of 1.0 msec. A 530 nm long pass cutoff filter was placed between the sample cell and emission monochromator to eliminate higher orders and reduce stray light. Luminescence excitation and emission spectra were scanned at 120 nm/min using a slit width for the scanned monochromator of 4.5 nm. Excitation spectra were corrected for the spectral output of the source, the spectral efficiency of the excitation monochromator and reference optics, and the spectral response of the reference PMT. Other settings are provided in the figure legends.

The benchtop fluorescence unit and the LC detector were equipped with a pulsed source and a digitally gated detector permitting both fluorescence and phosphorescence modes of operation. Using a combination of measurements in the fluorescence and time-delayed phosphorescence mode, emissions occurring during the lamp pulse were measured independently from delayed luminescence (See Fig. 1). Phosphorescence lifetimes reported here were determined using 10 gated measurements with durations of 20 usec

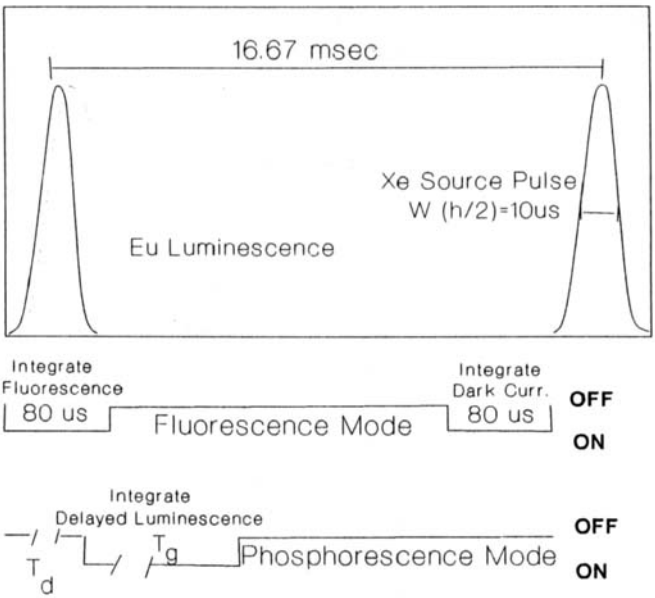


FIGURE 1.
Signal Gating Diagram for the LC-240 Pulsed Source Luminescence Detector. Source flashes at line frequency, producing a pulse with a duration at half height of 10 usec. In fluorescence mode, digitized signal is measured during each flash, and a measurement is taken immediately before the next flash for the purpose of dark current correction. In phosphorescence mode a single measurement is taken for each lamp cycle with an adjustable delay ($T_d$) time and gate ($T_g$) time.

each and delay times ranging from 0.03 to 0.35 msec. These readings were taken automatically, and the intensity data were fit to a single exponential decay model using an onboard application program which computed the lifetime values and correlation statistics.

In testing post column systems and mobile phases, equal volumes of the mobile phase and proposed post column reagent were added to a cuvette containing $5x10^{-5}$ M $EuCl_3$, $5x10^{-5}$ M EDTA, and other specified additives. Immediately after the addition of $3.33x10^{-5}$ M tetracycline and mixing by

inversion of the cell, luminescence intensities and lifetimes were determined. All PCR optimization experiments were performed using mobile phases containing acetonitrille and 0.1% aqueous TFA (27:73 or 24:76).

## Preparation of Standards and Post Column Reagent

TC, CTC, OTC, DC, and MC standards for chromatography were prepared immediately before use. One mg/ml stock solutions were prepared by dissolving the appropriate weight of antibiotic in mobile phase. These stock solutions were used to prepare mixed standards, and the desired levels for quantitation were obtained by serial dilution in mobile phase. Limits of detection were calculated by determining the weight of TC applied to the column which gave a peak height three times greater than the baseline noise (P/P).



After optimum conditions were determined, a post column reagent (PCR) containing 0.20 M AMP at pH 10.5, 1.0% Triton X-100, $5x10^{-5}M$ $EuCl_3$, $5x10^{-5}M$ disodium EDTA, 200 uM TOPO, and 1.5% acetonitrille was prepared as follows. The AMP was first dissolved in 80% of its final volume of $H_2O$ and brought to the proper pH with 2N NaOH. The EDTA and $EuCl_3$ were next dissolved together in a small amount (30 ml per L of PCR) of $H_2O$ then added to the buffer. Next the Triton X-100 was dissolved in the buffer/$EuCl_3$ mixture. The TOPO was weighed and dissolved in 15 ml (per L of PCR) of acetonitrille, then added as the last component of the PCR. The solution was turbid immediately after addition of the TOPO but cleared after 2 min of slow stirring and was then brought to its final volume.

# RESULTS AND DISCUSSION

## Development of a Micellar Post Column Reagent

Figure 2a shows an overlay of the absorbance spectrum (Abs) and corrected luminescence excitation spectrum (LumEX) of $10^{-5}$ M tetracycline

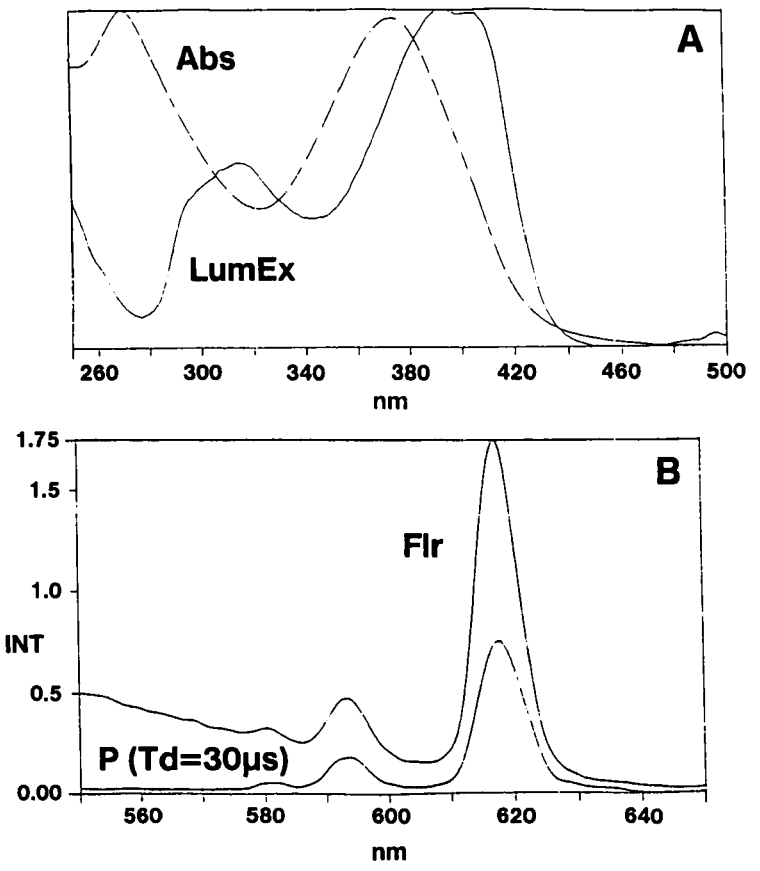




FIGURE 2.

Absorbance and Luminescence Spectra of the $Eu^{3+}$/Tetracycline Complex. Mixture contained: $5x10^{-5}M$ of both $EuCl_3$ and EDTA, 13.5% ACN, 0.05%TFA, 0.1M CAPS pH 10.0, and $1x10^{-5}M$ Tetracycline. **A:** Absorption (Abs) spectrum and time-delayed luminescence excitation (LumEx) spectrum of mixture. The luminescence spectrum was taken with the emission wavelength set at 617 nm; delay time was 0.03 msec, and gate time was 1 msec. Other conditions are described in Materials and Methods. **B:** Luminescence emission spectra of the mixture with the excitation wavelength set at 395 nm. Upper curve (Flr) scanned in fluorescence mode (Refer to Figure 1); Lower curve [P(Td=30us)] scanned in time-resolved mode with a delay time of 0.03 msec and a gate time of 1 msec. Other conditions are described in Materials and Methods.

in a mobile phase buffered at pH 10 containing $5x10^{-5}$M $EuCi_3$ and EDTA (5). The excitation spectrum is bathochromically shifted with respect to the absorbance spectrum, and the UV/VIS peak ratio has dropped from 1.03 to 0.54. Changes in the TC absorption spectrum due to $Eu^{3+}$ complexation were originally reported by Hirshy et al.(4). Their work and previous NMR studies suggested that at alkaline pH, the TCs bind Europium ions through Beta-diketone oxygens on rings C and D (4,5,7). The benzoyl moiety of the TC molecule is believed to be involved in energy transfer to $Eu^{3+}$ (5).

The fluorescence and phosphorescence (delay time = 30usec) emission spectra of this complex are shown in Fig. 2b. Both spectra exhibit the sharp emission bands characteristic of the lanthanide, except that background fluorescence and scatter is absent from the time-resolved spectrum. The combination of wavelength selectivity and time-discrimination offered by phosphorimetric detection could serve as an extremely selective analytical tool were it not for the lower sensitivity reported for time-resolved mode (5). In this study the luminescence lifetime of the complex at pH 10 was found to be 75 usec. Since conventional pulsed source luminescence spectrophotometers require a delay time of 30 usec in order to fully reject scatter and prompt luminescence, it would be desirable to seek conditions which might extend the luminescence lifetime of the lanthanide chelate and/or enhance the time-resolved component of its emission. The focus of this study was, therefore, to attempt to select a post column reagent which might optimize the total luminescence yield yet provide improved detection limits for time-resolved measurements.

Figure 3 shows the effects of pH on luminescence of the $Eu^{3+}$/ Tetracycline chelate as measured in the fluorescence mode (IF) and in the time-resolved mode using a delay time of 30 usec (IP). The optimum pH for both prompt and time-delayed emission was 10.5. CAPSO and CAPS buffers were tested at pH intervals overlapping the experimental optimum and were found to give comparable results; however, AMP buffer generated the largest relative time-resolved signal. Luminescence lifetimes varied from 76 to 93 usec between pH 9.0 and 10.5 and then dropped sharply at pH 11.0 to 37 usec (values not shown).

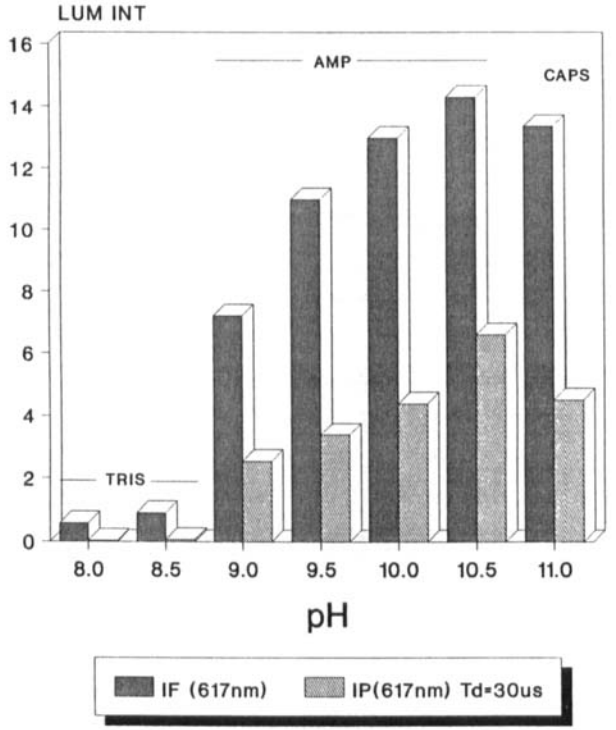


FIGURE 3.
Response of Sensitized $Eu^{3+}$/Tetracycline Luminescence to pH. Cuvettes contained: 0.1M buffer at indicated pH, 13% ACN, 0.036%TFA, $5x10^{-5}$M of both $EuCl_3$ and EDTA, and $3.33x10^{-5}$M tetracycline. Fixed wavelength measurements were made in the fluorescence (IF) and time-resolved (IP) modes. Details are given in Materials and Methods.

In an effort to both lengthen the phosphorescence lifetime and increase the overall luminescence yield for HPLC detection, the efficacy of a micellar post column $Eu^{3+}$ reagent was investigated. Micelle stabilization has been used to improve luminescence yields for the direct fluorometric determination of Europium and other lanthanides complexed with aromatic B-diketone chelators (8) and to improve detection limits in aqueous time-resolved $Eu^{3+}$

based fluoroimmunoassays (9). The basic principles of the micellar luminescence techniques were recently reviewed by Georges (10). In general, the $Eu^{3+}$ chelate is sequestered within a detergent micelle where it is protected from collisions with the external solvent molecules and from other interactions (notably with water) which might quench luminescence. A "synergistic agent", tri-n-octyl phosphine oxide (TOPO), is also added. The TOPO serves as a bulky nucleophylic ligand which forms a ternary complex with the $Eu^{3+}$ chelate, further shielding it from deactivating collisions and increasing its solubility in the hydrophobic portion of the micellar phase (See Ref. 10, Sec. 5.5).

Previous studies with Europium chelates have utilized detergent concentrations ranging from 0.1 to 1.5% with TOPO concentrations between 50 and 100 uM (8-10). The effects of varying the TOPO concentration in a PCR containing 0.5% Triton X-100 (TX) are shown in Fig. 4. While adding the detergent alone resulted in only a slight improvement in the luminescence yield, the addition of TOPO at 100 uM significantly increased the total luminescence intensity and resulted in a 21% improvement in the time-resolved signal. The addition of TOPO also caused a slight increase in luminescence lifetime over the untreated control (linear plot, Fig. 4). No further improvement could be achieved at higher TOPO concentrations in part because of solubility problems. When the TOPO concentration experiment was repeated at 1% Triton X-100 no significant increases in luminescence yield or lifetime were noted.

Other attempts were made to improve the performance of the micellar system. One approach was to select a different detergent in which the $Eu^{3+}$/TC complex might have a higher micellar solubility. The alternate detergent selected, Brij 30, is similar in structure to BL-9EX used in aqueous $Eu^{3+}$ time-resolved immunoassays (9). At 0.5% in the buffered PCR Brij 30 produced a turbid solution. A test PCR containing a mixture of 0.5% TX and 0.5%Brij 30 was optically clear; however, no additional luminescence enhancement was noted with this TX/Brij 30 heteromicellar reagent containing 100uM TOPO and other additives as noted in Fig. 4.

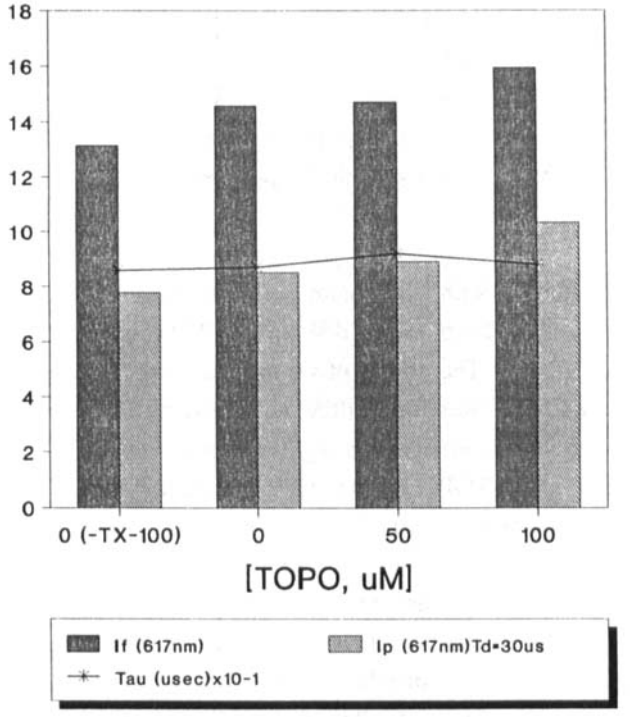


FIGURE 4.
Effects of Triton X-100 and TOPO on Luminescence Intensity and Lifetime of the $Eu^{3+}$/Tetracycline Chelate. Cuvettes contained: 0.1M AMP at pH10.5, 13% ACN, 0.036% TFA, $5x10^{-5}$M of both $EuCl_3$ and EDTA, $3.33x10^{-5}$M tetracycline, and the indicated concentration of TOPO with and without (-TX-100) 0.5% Triton X-100. Intensity measurements were made in fluorescence (If) and time-resolved (Ip) modes. Luminescence lifetimes for each experimental point are shown in the superimposed line graph (Tau). Instrumental details are provided in Materials and Methods.

The greater polarity and water solubility of the $Eu^{3+}$/ TC complex as compared to other aromatic beta diketone chelates assayed in micellar systems (8,9) might cause less of the TC chelate to partition into the detergent micelles, thus diminishing the micellar enhancement of luminescence. Addition of NaCl at 0.005 and 0.01M in an attempt to salt the complex out of the aqueous phase into the micellar phase caused no improvement in performance.

In summary, the optimized post column reagent (added to an equal volume of mobile phase) was found to contain 0.2M AMP pH 10.5, 1% Triton X-100, 200 uM TOPO, $5x10^{-5}$ M EuCl3, and $5x10^{-5}$M EDTA. A 21% increase in the time-delayed component of the signal along with a 14% improvement in the total luminescence yield realized in the presence of Triton X-100 and TOPO was deemed sufficient to warrant use of this micellar post column reagent for time-resolved HPLC detection of TCs.



## Optimization of Post-Column Conditions

To test the stability of the micellar luminescence, the fluorescence and phosphorescence (Td = 30usec, Tg = 1msec) signals were monitored immediately after mixing PCR, TC, and the ACN/TFA mobile phase. While the phosphorescence signal exhibited a very slow decline, both phosphorescence and fluorescence intensities were essentially constant during the first 20 seconds after mixing. There was actually a small rise in the phosphorescence signal during the first 5 seconds after mixing, presumably due to enhancement of luminescence after partitioning of the $Eu^{3+}$/TC complex into the micelles (data not shown). With the post-column system tested here, the time between mixing of the column effluent with PCR and entry of the mixture to the detector flow cell was less than 5 seconds.

In an attempt to optimize PCR addition and mixing prior to chromatographic sensitivity runs either a 1m x 0.4 mm teflon mixing coil or a 15 cm bead string mixer containing 250 uM silanized glass beads in narrow bore teflon tubing (Suppelco Corp., Philadelphia, PA.) was placed between

the mixing T and the detector inlet. Neither of these devices provided any improvement in peak height, so the straight T junction was used for all further work (Refer to Materials and Methods).

The storage stability of the reagent was evaluated during the course of chromatographic runs. The PCR exhibited good stability, showing no change in activity after 1 week of storage at room temperature.

## Mobile Phase Compatibility

A number of diverse mobile phases have been applied to TC chromatography over the last decade (11-16). Wenzel et al. examined the compatibility of a non-micellar $Eu^{3+}$ PCR for tetracyclines with several mobile phases (5). Figure 5 shows the response of the micellar PCR to 6 different solvent systems used for reversed phase chromatography of TCs as compared to that of distilled $H_2O$. In order to fully test the compatibility of each mobile phase with time-resolved detection, both fluorescence and time-delayed signals were measured and the luminescence lifetimes were determined. Of those tested, the aqueous methanol system containing TFA yielded the highest overall luminescence signal and the longest lifetime. It is interesting that both the TFA-containing systems gave better responses than the $H_2O$ control, indicating a positive synergistic effect. This was not completely unexpected, however, since these solvent systems might alter interaction of the chelate with water molecules which are known to quench $Eu^{3+}$ luminescence (3,10). The phosphate and EDTA systems gave lower responses. In the case of the latter a lower luminescence signal might be expected, since the large excess of EDTA ($5x10^{-4}$ M) might outcompete the TC for available $Eu^{3+}$ ligation sites. The lower performance of the phosphate-containing mobile phase might also be predicted due to the insolubility of Europium phosphate (4). In all cases, the calculated luminescence lifetime for a particular system closely mirrored the total luminescence signal.

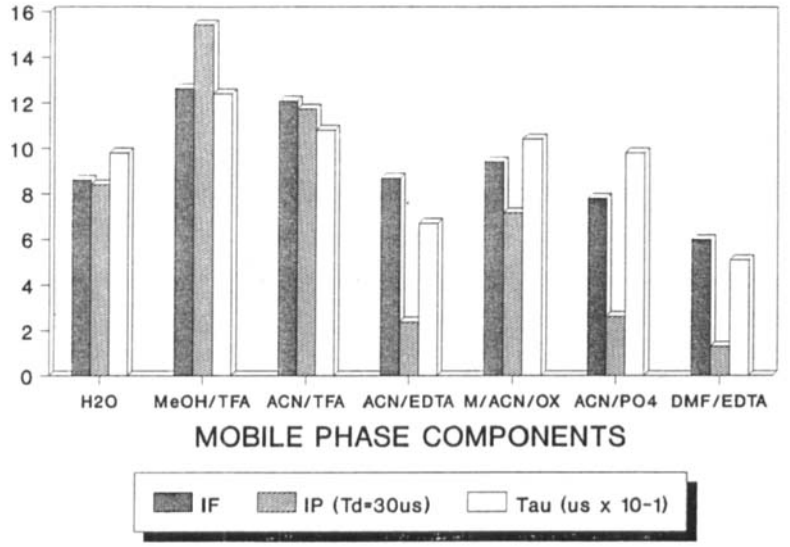


FIGURE 5.

Response of Sensitized $Eu^{3+}$/Tetracycline Luminescence from the Micellar Post Column Reagent to Various Mobile Phases. Cells contained equal volumes of the micellar post column reagent and the indicated mobile phase. Immediately after the addition of $1x10^{-5}M$ tetracycline, the fluorescence (IF) and time-resolved (IP) luminescence intensities and the luminescence lifetimes were measured as described in Materials and Methods. The post column reagent contained: 0.2M AMP at pH 10.5, 0.1% Triton X-100, 200 uM TOPO, $5x10^{-5}M$ of both $EuCl_3$ and EDTA, and 1.5% ACN. Mobile phases tested (versus $H_2O$ control) were: MeOH/TFA = methanol: 0.1% TFA (40:60); ACN/TFA = acetonitrille: 0.1% TFA (24:76); ACN/EDTA = acetonitrille: $1x10^{-3}M$ EDTA, pH6.6 (28:72); M/ACN/OX = methanol: acetonitrille: 0.01M oxalic acid, pH 2.0 (1:1.5:5); ACN/PO4 = 0.05M $Na\text{-}PO_4$, pH 2.5: acetonitrille (90:10); DMF/EDTA = $1x10^{-3}M$ EDTA with 0.12M $KNO_3$: dimethyl formamide (90:10).

## Chromatography

Despite the lower response of the ACN/EDTA mobile phase (Fig. 5) it has become very important in TC reversed phase chromatography, since it produces good peak shapes without degrading the stationary phase (11) and with a lower probability of forming TC epimers during chromatography (12). Figure 6 shows a reversed phase chromatogram from a mixture of TC, CTC and MC standards separated with the ACN/EDTA mobile phase where UV detection was used in-line before post column $Eu^{3+}$ luminescence detection in the time-resolved mode. Relative to UV detection, the system is most sensitive to TC and somewhat less sensitive to CTC. In agreement with previous work (5), the response to minocycline is relatively low. Impurities or degradation products from the TC and CTC standards at RT 3.27 and 6.16 min are detected by both the UV and $Eu^{3+}$/PCR techniques (Fig. 6).



## Chromatographic Sensitivity and Linearity

The sensitivity and linearity of the PCR system was studied with the detector in both the fluorescence and time-resolved modes. The efficacy of using grating/filter detection was also examined using the total emission accessory of the LC-240 detector. This microprocessor-controlled accessory drives a mirror in front of the emission grating and places a cutoff filter into the emission path to exclude scattered exciting light.

Figure 7 shows chromatographic separations of mixtures of OC, TC, and DC (5, 20, and 100 ng of each compound applied to the column) using time-resolved $Eu^{3+}$ luminescence detection. The high sensitivity of this technique for OTC and TC is contrasted with relatively low sensitivity for DC (Figure 7). This chromatographic system was used to determine linearity and limits of detection as described below (See Materials and Methods and figure legends for details).

Chromatographic linearity plots of the $Eu^{3+}$/PCR system with the detector in phosphorescence mode (Td=30 usec) and in fluorescence mode

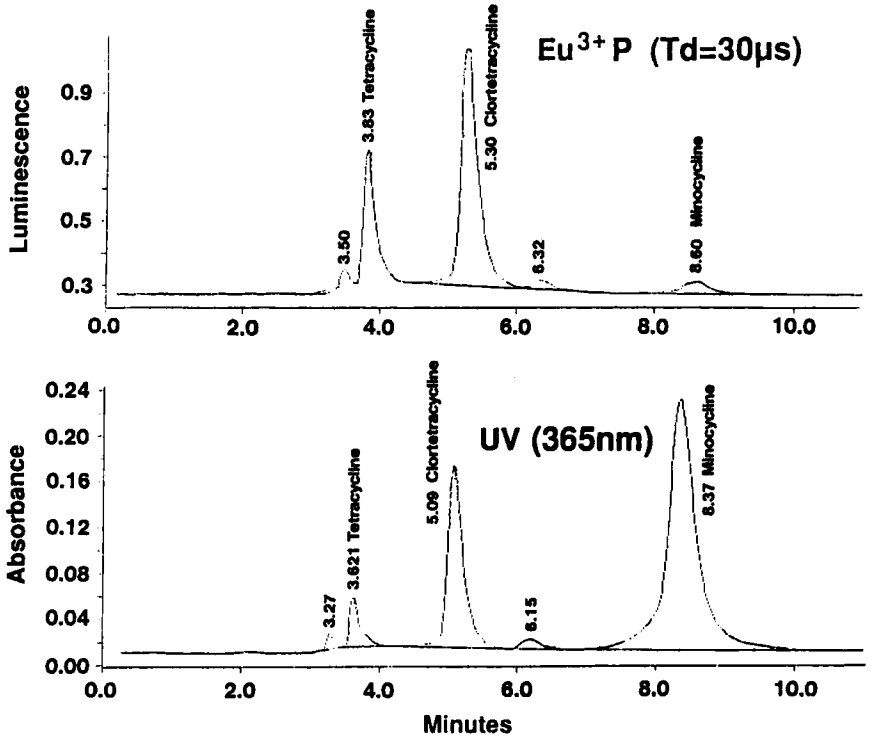




FIGURE 6.

Reversed-Phase Separation of Tetracycline, Chlortetracycline, and Minocycline Using In-line UV and Time-Resolved Sensitized $Eu^{3+}$ Luminescence Detection. Mixture of TC, CTC, and MC separated on a 25 x 0.46 cm, 5u, C-18 column using a mobile phase of acetonitrille: $1x10^{-3}M$ EDTA pH 6.6 (27:73) with a flow rate of 1 ml/min. Column effluent first passed through the UV detector which was set at 365 nm +/- 7.5 nm and was then mixed with the micellar $Eu^{3+}$ post column reagent (See Materials and Methods for PCR composition) flowing at 1 ml/min. The luminescence detector was set at EX WL=395 nm and EM WL > 530 nm (total emission) with a delay time of 0.03 msec and a gate time of 1 msec.

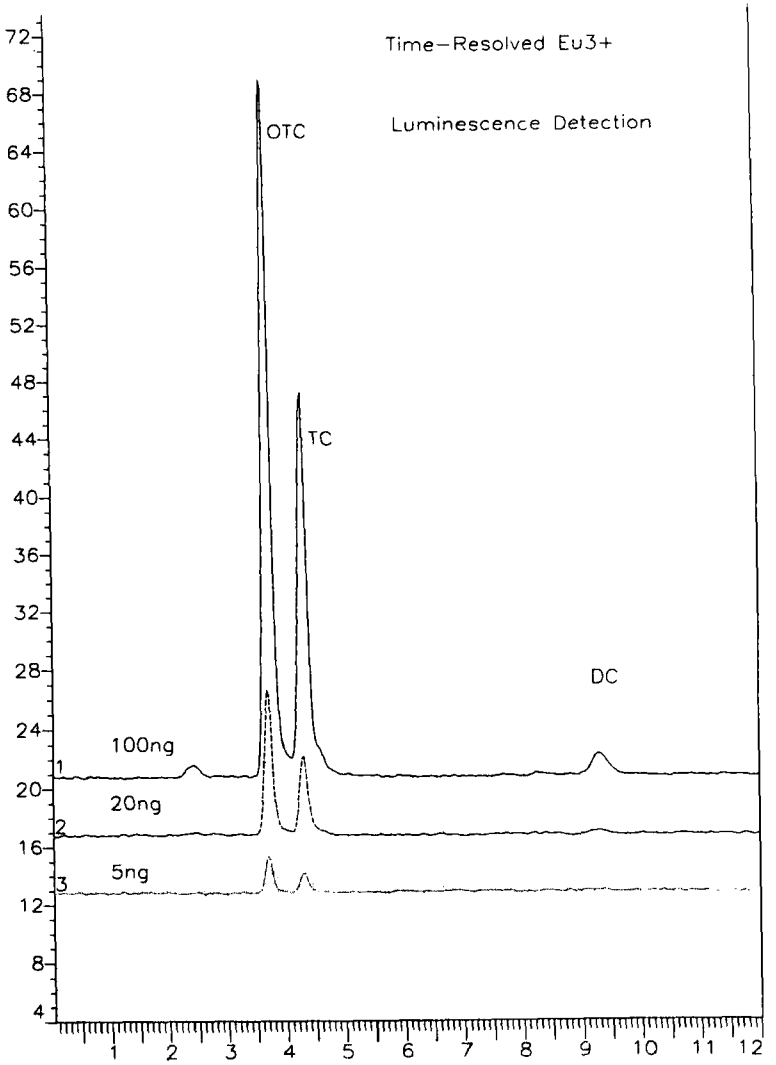


FIGURE 7.

Reversed Phase Chromatograms of Mixtures of Tetracycline, Oxytetracycline, and Doxycycline with Time-Resolved Sensitized $Eu^{3+}$ Detection. Chromatograms from mixtures of oxytetracycline (OTC), tetracycline (TC) and doxycycline (DC) at levels of 100, 20, and 5 ng on column (inj. vol. 10 ul), separated on a 25 x 0.46 cm, 5u, C18 column using a mobile phase of acetonitrille:0.1% TFA (24:76). Post column reagent composition is described in Materials and Methods. The detector wavelengths were EX WL=395nm and EM WL=617nm. The delay time was set at 0.03 msec and the gate time at 1 msec.

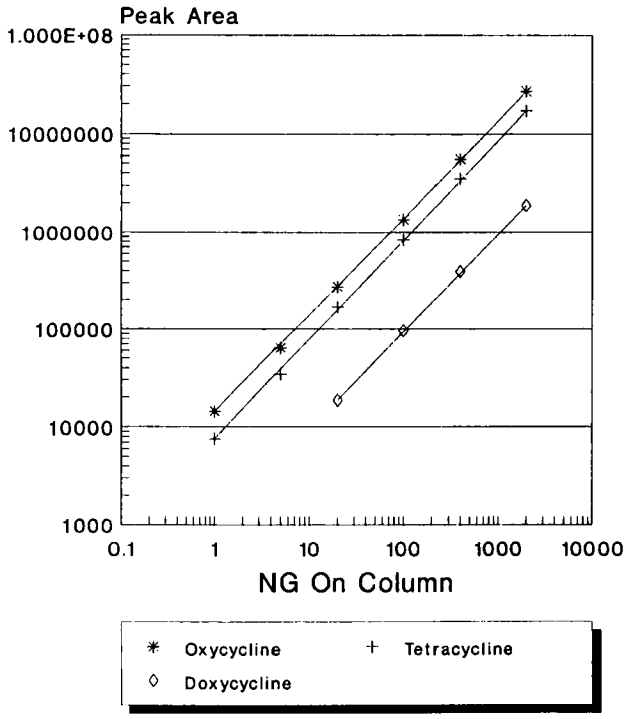


FIGURE 8.
Calibration Plots for Tetracycline, Oxycycline, and Doxycycline Separated by Reversed Phase Chromatography With Time-Resolved Sensitized $Eu^{3+}$ Luminescence Detection. Chromatographic and detection conditions were as described for Figure 7, except that the total emission accessory was used in combination with a 530nm longpass cutoff filter to remove scattered exciting light. The injection volume was 10 ul.

are shown in Figures 8 and 9, respectively. Chromatographic limits of detection for OC and TC in all 4 luminescence detection modes are provided in Table 1.

Based on peak area, the chromatography shows quantitative linearity ($r^2 > 0.998$) for all 3 TCs from 2 ug on column down to the limit of detection. The limits of detection (S/N = 3) for TC and OC were near or below 1 ng on column for all detection modes. Using UV detection at 280 nm (15 nm bandwidth) with the same column and mobile phase, the limit of detection for

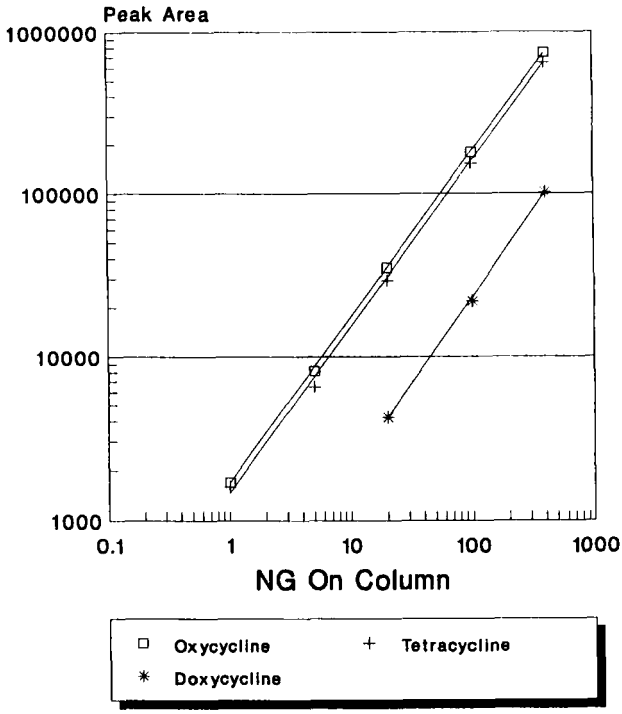


FIGURE 9.
Calibration Plots for Tetracycline, Oxycycline, and Doxycycline Separated by Reversed Phase Chromatography With Fluorescence Mode Sensitized $Eu^{3+}$ Luminescence Detection. Chromatographic conditions were as described for Figure 7. The detector was set in the grating/grating fluorescence mode with the EX/EM wavelengths at 395/617 nm, respectively. The injection volume was 10 ul.

TC was found to be 4 ng, in agreement with literature reports (17,18). It is interesting to note that the limits of detection for both TC and OTC as shown in Table 1 improve when the emission monochromation is changed from grating to total emission in the time-resolved mode. By contrast, the l.o.d.s actually deteriorate by 3 to 4 fold when the same change is made in fluorescence mode ( Table 1). This can be explained by higher baseline noise due to additional stray light and background fluorescence inherent in the filter

TABLE 1

HPLC Limits of Detection* for Tetracycline and Oxycycline Using Fluorescence and Time-Resolved Modes of Detection

| Mode of Detection | Oxytetracycline | Tetracycline |
|---|---|---|
| Time-resolved Grating/Grating | 0.69 | 1.36 |
| Time-resolved EM WL > 530nm | 0.36 | 0.60 |
| Fluorescence Grating/Grating | 0.21 | 0.26 |
| Fluorescence EM WL > 530nm | 0.58 | 1.04 |

* Ng on column for S/N=3; Mobile phase: ACN:0.1%TFA (24:76). See Materials and Methods for details.



fluorometric mode. All of these interferences are avoided through the use of gated detection, since only the longer-lived $Eu^{3+}$ emissions are measured.

The data for DC show a higher l.o.d. (4-25 ng, depending on mode of detection) as would be predicted by the lower response it exhibits with this postcolumn reaction. In general, the relative chromatographic sensitivity of this method for TCs tested appears to follow the order: TC = OTC > DC > CTC >> MC. These differences in sensitivity may be due to a) differences in efficiency of triplet state energy transfer from the TC to bound $Eu^{3+}$; b) Differences in the luminescence lifetimes of the various $Eu^{3+}$/TC chelates (this would be evidenced by large differences in sensitivity between the

fluorescence and time-resolved modes of detection for various TCs tested); c) different excitation maxima for each $Eu^{3+}$/TC chelate; d) increased limits of detection for more strongly retained TCs because of chromatographic band broadening; or e) a combination of the above factors. To test the relative sensitivity of the method on a more absolute basis, the corrected time-resolved excitation spectra and luminescence lifetimes of equimolar concentrations of each of the 5 tested TCs were compared under standard conditions. The results of this experiment are summarized in Table 2.

From Table 2 it can be seen that the large differences in relative sensitivity with the TCs tested cannot be due to differences in excitation maxima, since all excitation maxima are within 12 nm of the setting used for chromatographic detection, and the bands are very broad (See Fig. 2).



Further, variations in luminescence lifetime cannot be the reason, since OTC (Tau = 60 usec) yields more than 9 times the signal of CTC (Tau = 113 usec). It is more likely that the differences are due to differences in the efficiency of intramolecular excited triplet state energy transfer to the bound $Eu^{3+}$ ion. These data show clearly that, under standard conditions, all the TCs tested except MC yield analytically useful sensitized $Eu^{3+}$ signals. The very poor correlation coefficients for the single exponential decay model exhibited by MC and CTC are due in part to noise resulting from relatively low signal levels; however, correlation coefficients in the 0.91 to 0.94 range were commonly obtained throughout these studies (note OTC and TC, Table 2). This is consistent with previous work by Hirshy which reported the presence of 2 $Eu^{3+}$/TC species with differing lifetimes whose relative concentrations depend upon pH (7).

## Chromatograms from Physiological Fluids

One very important application noted in previous work by Wenzel et al. (5) and also by Hirshy (7) is the study of TCs and their metabolites in physiological fluids. Even when fluorescence methods are applied to such preparations, good chromatograms are difficult to obtain without extensive prior clean-up. Again, the combined specificity and sensitivity of time-

## TABLE 2

**Excitation Maxima, Relative Peak Heights, and Luminescence Lifetimes of Five $Eu^{3+}$/Tetracycline Chelates Recorded at Equimolar Concentrations Under Standard Conditions**

| Compound | Excitation Maximum (nm) (Normalized Intensity) | | | Apparent Lifetime (usec) [Corr. Coef.] |
|---|---|---|---|---|
| Oxytetracycline | 245 (0.18) | 305 (0.60) | 388 (1.00) | 60 [0.935] |
| Tetracycline | 246 (0.14) | 306 (0.48) | 390 (0.89) | 71 [0.906] |
| Doxycycline | 246 (0.08) | 303 (0.28) | 383 (0.79) | 102 [0.993] |
| Chlortetracycline | 245 (0.03) | 308 (0.07) | 395 (0.11) | 113 [0.835] |
| Minocycline | 245 (0.01) | 299 (0.02) | 395 (0.02) | 26 [0.892] |

Cuvettes contained: 1.47 ml of optimized PCR (See Materials and Methods) and 1.5 ml of acetonitrille: 0.1%TFA (24:76). Excitation spectra and luminescence lifetimes were measured immediately after addition of 0.03 ml of $1x10^{-3}$M of the indicated tetracycline and mixing. Excitation spectra were taken at an emission wavelength of 617nm in the time-resolved mode ($T_d$ = 0.03 msec, $T_g$ = 1.0 msec). See Materials and Methods for further experimental details.

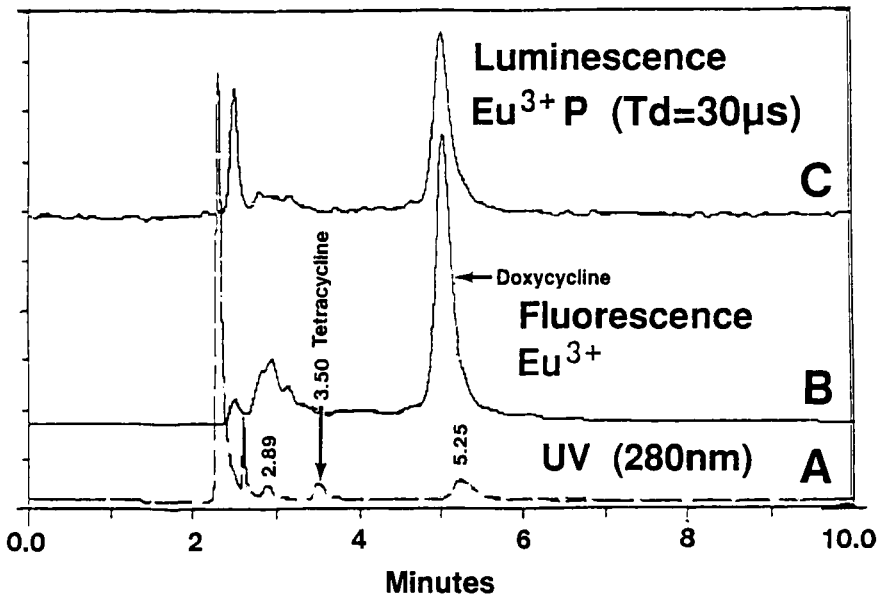


FIGURE 10.
Chromatograms of Three Identical Injections of Deproteinized Human Serum Spiked With Doxycycline using Three Different Modes of Detection. Mobile Phase: acetonitrille: 0.1%TFA (28:72) at 1 ml/min.; Column and post column reagent are described in Materials and Methods; **A:** UV detection (280 nm +/- 7.5 nm); **B:** Sensitized $Eu^{3+}$ luminescence, fluorescence mode, EX/EM wavelengths = 395/617 nm; **C:** Sensitized $Eu^{3+}$ luminescence, time-resolved mode, Td=0.03 msec, Tg=1 msec, EX/EM wavelenghts = 395/617 nm.

resolved sensitized $Eu^{3+}$ detection could be very useful for such applications. Figure 10 shows the results of chromatography of a deproteinized human serum sample which had been spiked with a pharmaceutical doxycycline preparation. Three identical injections were made using three different modes of detection: UV (lower), $Eu^{3+}$ luminescence in fluorescence mode (middle), and $Eu^{3+}$ luminescence in time-resolved mode (top). While doxycycline is detected in the UV chromatogram, many early eluting components from the serum clutter the chromatogram. One of these

components at RT=3.5 min, as indicated by its label, is located in the peak detection window of tetracycline (Figure 10a). With post column micellar $Eu^{3+}$ luminescence detection in fluorescence mode, relative sensitivity toward doxycycline is greatly increased, and the background is reduced (Figure 10b). Using time-resolved luminescence detection (Figure 10c) background peaks at the beginning of the chromatogram are nearly eliminated, and a large peak appears near the void volume. This peak is not seen in control serum and is probably due to sensitized $Eu^{3+}$ luminescence. The observation that its relative peak height is greater than in fluorescence mode might be explained if the component had a longer luminescence lifetime than the doxycycline/$Eu^{3+}$ chelate. Because of the high inherent selectivity of sensitized $Eu^{3+}$ luminescence detection, doxycycline provided excellent results in this complex matrix despite its relatively low response in this postcolumn system (See above).



The combination of high sensitivity and selectivity this detection technique offers might well be useful in other areas such as pharmaceutical purity testing or the analysis of synthetic products. Because so few compounds can bind and transfer excitation energy to $Eu^{3+}$, this chromatographic method could provide immediate evidence that a given peak is due to a TC or a structurally analogous compound.

## ACKNOWLEDGEMENTS

The author would like to thank Mr. Frank Padera for expert assistance with UV and fluorescence spectroscopy and Mr. Charles Woll for providing computing equipment and software expertise. Support for this project was provided by the Perkin-Elmer field sales organization.

## REFERENCES

1. Topal, M. and Fresco, J. , Fluorescence of Terbium Ion-Nucleic Acid Complexes: A Sensitive Probe for Unpaired Residues in Nucleic Acids, Biochemistry, 19, 5531, 1980.

2. Haertle, T. , Augustiniak, J., and Gusschlbauer, W., Is $Tb^{3+}$ Fluorescence Enhancement Only Due to Binding to Single Stranded Polynucleotides?, Nucl. Acids Res., 9, 6194, 1981.

3. DiBella, E., Weissman, J., Joseph, M., Schultz, J., and Wenzel, T., Lanthanide Ions as Chromophores for Liquid Chromatographic Detection, J. Chromatogr., 328, 101, 1985.

4. Hirshy, L., Dose, E., and Winefordner, J. , Lanthanide-Sensitized Luminescence for the Detection of Tetracyclines, Analyt. Chim. Acta., 147, 311, 1983.

5. Wenzel, T., Collette, L., Dahlen, D., Hendrikson, S., and Yarmaloff, L., Liquid Chromatographic and Flow Injection Analysis of Tetracycline Using Sensitized Europium (III) Luminescence Detection, J. Chromatogr., 433, 149, 1988.

6. Whan, R., and Crosby, G., Luminescence Studies of Rare Earth Complexes: Benzoyl-acetonate and Dibenzoylmethide Chelates, J. Mol. Spect., 8, 315, 1962.

7. Hirshy,L., Lanthanide Ions as Luminescence Probes, Ph.D. Thesis, University of Florida, Gainesville, Dept. of Chemistry, 1983.

8. Takehatsu, T., Spectrofluorometric Determination of Terbium, Europium, and Samarium with Pivaloyltrifluoroacetone and Tri-n-Octyl Phosphine Oxide in Micellar Solution of Nona-oxyethylene Dodecyl Ether, Talanta, 29, 397, 1982..

9. Hemmila, I., Dakubu, S., Mukkala, V.-M., Siitari, H., and Lovgren, T., Europium as a Label in Time-Resolved Immunofluorometric Assays, Anal. Biochem., 137, 335, 1984.

10 . Georges, J., Molecular Fluorescence in Micelles and Microemulsions: Micellar Effects and Analytical Applications, Spectrochim. Acta. Rev., 13, 41, 1990.

11. Aszalos, A., Haneke, C., Hayden, M., and Crawford, J. , Analysis of Tetracycline Antibiotics by Reversed-Phase High-Performance Liquid Chromatography, Chromatographia, 15, 367, 1982.

12. Knox, J. and Jurand, J., Mechanism of Reversed-Phase Separation of Tetracyclines by High Performance Liquid Chromatography, J. Chromatogr., 186, 763, 1979.

13. Hasan, T. and Cooperman, B., Reversed-Phase High-Performance Liquid Chromatographic Separations of Tetracycline Derivatives Using Volatile Mobile Phases, J. Chomatogr., 321, 462, 1985.

14. Naidong, W., Roets, E., and Hoogmartens, J., High Performance Liquid Chromatography of Chlortetracycline and Related Substances on Poly(styrene-divinylbenzene) copolymer, Chromatographia, 30, 105, 1990.

15. Sokolic, M., Filipovic, B., and Porkorny, M., High Performance Liquid Chromatographic Procedures in Monitoring the Production and Quality Control of Chlortetracycline, J. Chromatogr., 509, 189, 1990.

16. Torel, T., Cillard, J., and Cillard, P., Determination of Oxytetracycline, Sulphamethazine and Sulphamethoxypyridazine in Feed Premixes, J. Chromatogr., 330, 425, 1985.

17. Mack, G.D. and Ashworth, R.B., A High Performance Liquid Chromatographic System for the Analysis of Tetracycline Drug Standards, J. Chromatogr. Sci., 16, 93, 1978.

18. Oka, H., Uno, K., Harada, K.-I., Yasaka, K., and Suzuki, M., A Simple Method or the Analysis of Tetracyclines Using Reversed-Phase High-Performance Liquid Chromatography, J. Chromatogr., 298, 435, 1984.