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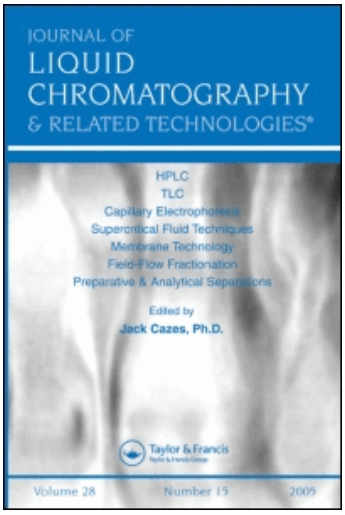

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LANTHANIDE LUMINESCENCE DETECTION OF BLEOMYCINS AND NALIDIXIC ACID

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ABSTRACT

Bleomycins and nalidixic acid transfer energy to Tb(III). Subsequent emission from Tb(III) provides for a selective and sensitive detection method. Temperature studies indicate that energy transfer for both compounds involves an intramolecular process. The efficiency of reversed-phase separations of bleomycins is improved by adding Tb(III) to the mobile phase. Tb(III) luminescence detection is compatible with a wide range of mobile phases suitable for the separation of nalidixic acid. Detection limits for bleomycin A_2 and nalidixic acid are 3×10^{-6} M and 4×10^{-7} M respectively.

INTRODUCTION

Luminescent methods of detection are important in liquid chromatography because of their sensitivity and selectivity. Since many compounds are not inherently luminescent it is common to use either a pre- or post-column derivatization scheme with a suitable luminescent chromophore. We have previously described the use of the luminescent lanthanide ions europium(III) and

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terbium(III) as detection chromophores in liquid chromatography[1-3].

These ions do not exhibit intense luminescence when directly excited. Instead, the lanthanide ions are excited indirectly by an energy transfer from the first excited triplet state of certain organic compounds. In particular, compounds with a benzoyl moiety often have triplet states close to that of the excited state of terbium and europium. Certain nitrogen heterocycles such as observed in nucleic acids also have triplet energies well matched with those of the lanthanides. The energy transfer can occur by an intermolecular or intramolecular process depending on whether the transferring compound has a strong binding group. Compounds such as dimethoxybenzophenone and naphthaldehyde transfer energy by an intermolecular process[1], whereas tetracycline[2,4], orotate[5], and single-stranded nucleic acids[2] transfer by an intramolecular process. Intramolecular transfer is generally preferred since it provides for more sensitive detection, and is less susceptible to variables such as the anion of the lanthanide salt and quenching by water or oxygen[1,2]. Non-transferring compounds such as aliphatic thiols have been detected by lanthanide luminescence by derivatizing with a transferring moiety such as 4-maleimidylsalicylic acid[6,7]. Compounds that quench lanthanide luminescence either by accepting energy from excited state lanthanide ions[1,8] or by ligand exchange with a transferring moiety[9] can also be detected.

This report investigates the liquid chromatographic detection of bleomycins(I) and nalidixic acid(II) by lanthanide luminescence. These compounds contain moieties that should have triplet state energies close to those of the lanthanides. They also have the potential to form chelate bonds with lanthanide ions.

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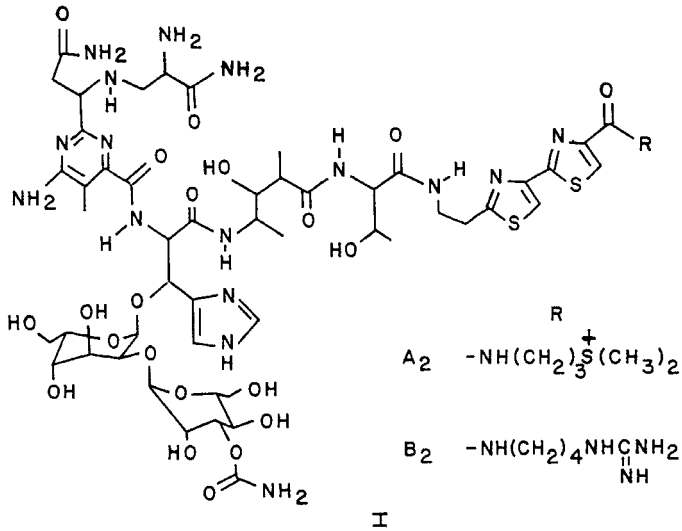

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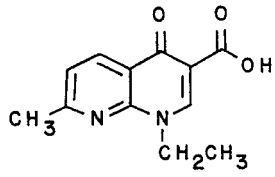

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EXPERIMENTAL

Reagents:

All reagents were used without further purification. Terbium was purchased as its oxide in 99.9% purity from Alfa Products (Danvers, MA, USA) and converted to its chloride salt using Ultrex hydrochloric acid (J,T. Baker, Phillipsburg, NJ, U.S.A.) according to published procedures[1]. Bleomycins were kindly provided by Mead Johnson Oncology Products (Evansville, IN, U.S.A.). Nalidixic acid was obtained from Aldrich Chemical Co.(Milwaukee, WI, U.S.A.). Solvents used in preparing mobile phases were HPLC grade except water, which was

purified by passage through a Milli Q water purification device. All mobile phases were prepared as specified in the literature.

Apparatus:

Chromatographic analysis was performed using a Beckman 110B pump and Beckman-Altex 210A injector with 20 uL sample loop. A Beckman 163 variable wavelength absorbance monitor was used for ultraviolet detection. A Beckman ODS column (15 cm x 4.6 mm, 5 um) was used for reversed-phase separations and an Alltech Anion R column (25 cm x 4.1 mm, 10 um) with Wescan Guard Cartridge for anion exchange separations. Fluorescence measurements were carried out on a Perkin-Elmer LS-5 spectrofluorometer fitted with either a cuvette holder or high pressure flow cell. Excitation wavelengths were 318 nm for bleomycins and 310 nm for nalidixic acid. The emission wavelength was 544 nm when Tb(III) was the emitting species. Excitation and emission slit widths were 5 nm. Response time was set at 1. For time-resolved measurements, the instrument was switched to the phosphorescence mode and appropriate delay and gate times were set.

The effect of temperature on the intensity of lanthanide luminescence was studied by flow injection analysis. A 25 ft. stainless-steel coil (0.01 in I.D.) was placed between the injector and spectrofluorometer and immersed in a constant temperature water bath.

Evaluation of Mobile Phases:

Preliminary evaluations of the compatibility of mobile phases with lanthanide luminescence detection were carried out by cuvette studies. Solutions were prepared by adding 30 uL of a 1 x 10^{-3} M solution of nalidixic acid (pH 6 by adding 1 M potassium hydroxide) to 3 mL of the appropriate mobile phase containing 0.01 M of Tb(III)

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either as its chloride salt or EDTA complex. The emission spectrum was then recorded.

RESULTS AND DISCUSSION

Bleomycins:

A comparison of emission from solutions of bleomycin with and without terbium(III) upon excitation of the bleomycin at 318 nm is shown in Figure 1. The two peaks in Figure 1b are characteristic of terbium emission. The greater intensity of the peak at 545 nm is typical. The pyrimidine ring of bleomycin is expected to be the moiety responsible for energy transfer to Tb(III). Although terbium is known to bond to bleomycin with an association constant of approximately 2.5×10^4, NMR experiments indicate that the site of binding cannot be determined with certainty[10]. Presumably the bleomycin associates with Tb(III) in a multidentate manner.

The efficiency of intramolecular transfer depends on the proximity of the lanthanide to the transferring moiety[2,4]. Given the relative concentration of terbium and bleomycin employed in liquid chromatographic detection, and the likely possibility that Tb(III) does not bind directly at the pyrimidine ring site, it is possible that energy transfer from bleomycin to Tb(III) can involve both intra- and intermolecular processes. Previous work on systems involving intermolecular transfer has shown that lanthanide luminescence increases as a function of temperature[1,11,12]. In these cases the increase in energy transfer more than offsets the increase in collisional deactivation, thermal quenching from low lying excited state levels in Eu(III)[13], or lanthanide to organic energy transfer[14]. The latter process can occur if the triplet energy of the organic is only slightly higher than the first excited state of the lanthanide.

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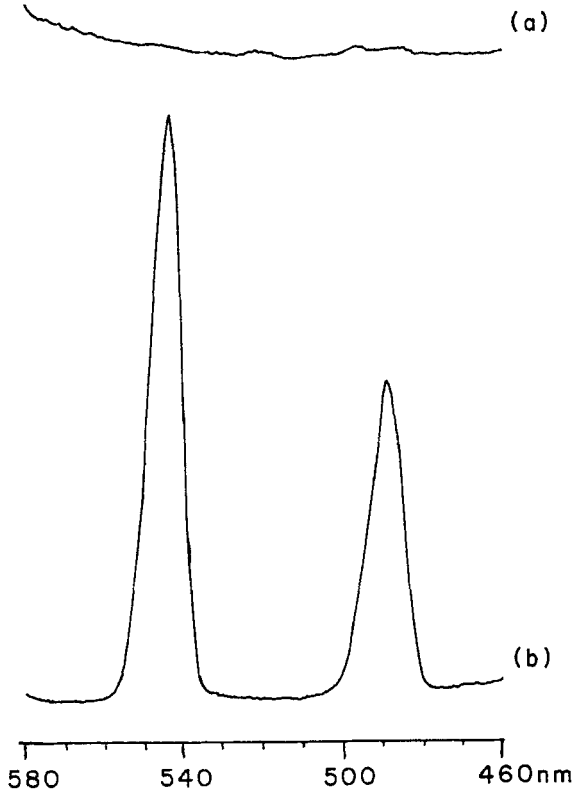

FIGURE 1. Emission spectra of a) Tb(III)chloride (10 mM) in MOPS (pH 6.8) and b) a 1:1 mixture of Tb(III)chloride (1 mM) in MOPS (pH 6.8) and bleomycin sulfate (0.1 mM) in PIPES (pH 6.8).

The intensity of terbium luminescence in a mixture of terbium and bleomycin decreases with increasing temperature (Figure 2a). This is similar to the temperature effect of europium with tetracycline (Figure 2b), a system known to involve intramolecular transfer[2,4]. Energy transfer between bleomycin and Tb(III) is therefore dominated by intramolecular exchange.

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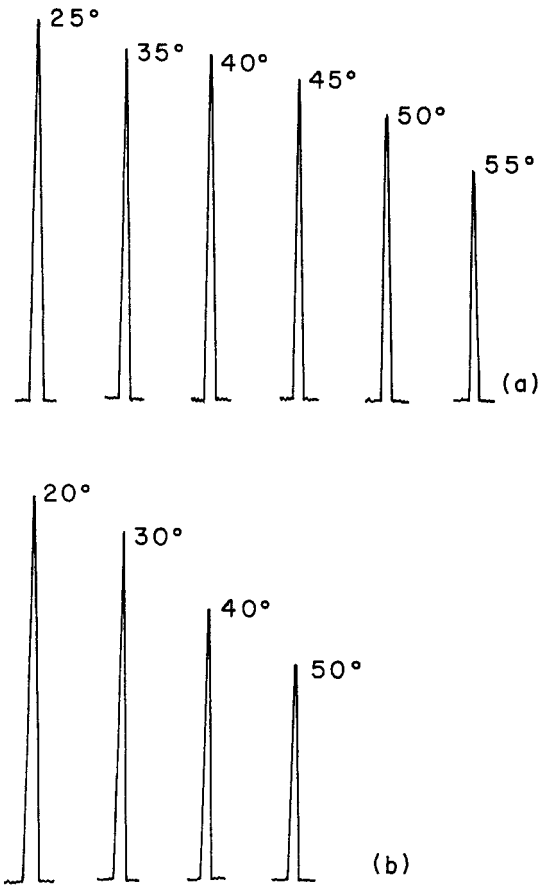

FIGURE 2. Effect of temperature on the intensity of lanthanide luminescence. Sample in a) was bleomycin (0.1 mM) and Tb(III)chloride (10 mM) in water (5 mM heptanesulfonic acid, 0.5 % glacial acetic acid, pH 6.8)-methanol, 55:45 v/v. Sample in b) was tetracycline (0.1 mM), Na[EuEDTA]·$5H_2O$ (0.1 mM), ammonium chloride (0.2 M) (pH 9) in water. Excitation wavelength, 392 nm; emission wavelength, 616 nm.

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The intensity of terbium luminescence in terbium-bleomycin mixtures increases at higher pH. Deprotonation of the bleomycins, which have pKa values of 2.9, 4.7, and 7.3 for B_2[15] and 2.9, 5.0, and 7.7 for A_2[16] must either strengthen the terbium-bleomycin interaction or facilitate binding of the terbium closer to the pyrimidine ring. Terbium(III) chloride or nitrate form insoluble hydroxides at basic pH, and cannot be used above pH 7. Formation of EDTA complexes with lanthanide ions prevents such precipitates from forming. In previous work with tetracycline, efficient energy transfer was observed with Na[Eu(EDTA)] at basic pH[2]. In the case of bleomycin, however, the energy transfer to Na[Tb(EDTA)] was poor. Since the bleomycins are assumed to bind in a multidentate manner, the strong complexation of EDTA ($\log K_F \approx 17$)[17] must block bleomycin association with the terbium. Terbium(III)chloride was therefore used at a pH of 6.8 for detection.

The chromatographic efficiency in reversed-phase separations of bleomycins improves significantly when copper(II) is added to the mobile phase[18]. Complexation with copper would reduce the number of sites on the bleomycin capable of simultaneously adsorbing to the stationary phase. Such multi-site adsorption has been proposed as a reason for poor chromatographic efficiency in the reversed-phase separation of proteins[19]. Addition of a one hundred-fold excess of terbium(III) to a solution containing a one-to-one copper-bleomycin complex did not show significant enhancement of terbium luminescence. Terbium(III) forms a much weaker complex with bleomycin than copper(II) ($\log K_F = 18.1$)[20], and does not effectively compete for the binding site.

Terbium(III) can be added to the mobile phase in place of copper(II), and a similar improvement in chromatographic efficiency is observed. Because of the

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relatively weak complexation, a ten to one hundred-fold excess of terbium is needed to achieve good efficiency. Both bleomycin A_2 and B_2 are detected using Tb(III) luminescence, although the sensitivity for the A_2 congener is better than that of B_2 under the conditions employed(Figure 3). Both the A_2 and B_2 congeners transfer energy to Tb(III)[10]. Other congeners of bleomycin that do not involve modification at or near the pyrimidine moiety are expected to transfer energy as well.

By using a pulsed source and incorporating a delay between the excitation and detection, it is possible to discriminate the long-lived lanthanide luminescence from scatter and short-lived fluorescence from impurities[2,21]. For bleomycins, an approximately five-fold increase in sensitivity was realized using time-resolved methods (Figure 4). At the optimum conditions of delay time of 0.01 msec and gate time of 3.5 msec, the limit of detection, defined as three times the signal-to-noise was measured to be 3×10^{-6} M for bleomycin A_2, or approximately 80 ng of injected sample. Linear response was observed over two orders of magnitude. This detection limit is not as low as what was measured with ultraviolet detection (2×10^{-7} M), but the excellent selectivity of lanthanide luminescence detection should facilitate the analysis of bleomycins in complex mixtures.

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Nalidixic Acid:

Nalidixic acid transfers energy to Tb(III). Binding is expected to occur at the carboxylate group. A pH above six ensures deprotonation of the acid moiety and maximizes transfer to Tb(III). The effect of temperature on the intensity of Tb(III) luminescence (Figure 5) indicates that intramolecular transfer dominates the process. The signal decreased from 30°C to 50°C at

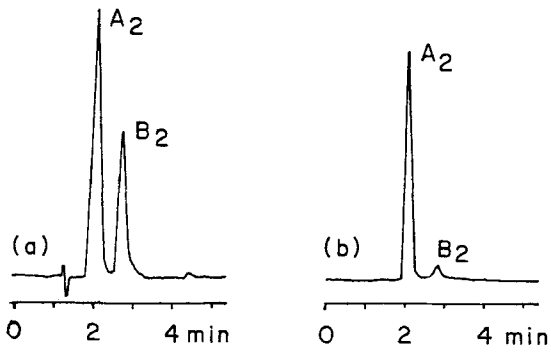

FIGURE 3. Chromatograms of bleomycins (50 uM) using a) ultraviolet detection (318 nm) and b) fluorescence detection. Mobile phase: Tb(III)chloride (10 mM) in water (5 mM heptanesulfonic acid, 0.5 % glacial acetic acid, pH 6.8)-methanol, 55:45 v/v. Flow rate, 1 mL/min.

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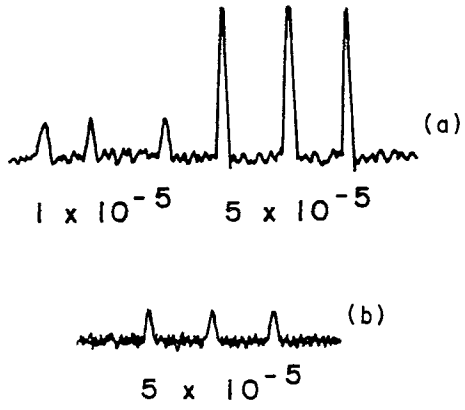

FIGURE 4. Comparison of the sensitivity of a) time-resolved and b) normal measurements on the detection of bleomycin. Sample was bleomycin (concentration shown in figure) and Tb(III)chloride (10 mM) in water (5 mM heptanesulfonic acid, 0.5 % glacial acetic acid, pH 6.8)-methanol, 55:45 v/v. Excitation wavelength, 318 nm; emission wavelength, 544 nm.

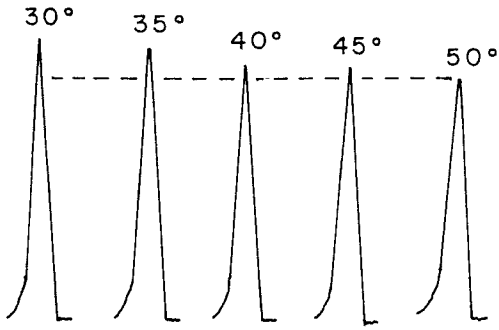

FIGURE 5. Effect of temperature on the intensity of Tb(III) luminescence. Sample was nalidixic acid (10 uM) and Tb(III)chloride (1 mM) in water (10 mM sodium acetate).

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Tb(III)-nalidixic acid ratios of 20:1, 100:1 and 200:1. The use of time-resolved methods did not enhance the signal-to-noise ratio or detection limits for this system.

A number of separations of nalidixic acid and similar compounds have been reported[22-34]. The compatibility of each of these phases with lanthanide luminescence detection was assessed. Insoluble complexes formed when terbium was mixed directly with phases containing phosphate, citrate, or oxalate ions. In most cases the insoluble complexes could be eliminated by adding EDTA to the mixture. Transfer from nalidixic acid to Tb(III) was observed after addition of the EDTA. Lanthanide luminescence detection is therefore compatible with a range of available mobile phases.

Two reversed-phase separations using sodium dihydrogen phosphate (5 mM), Na[Tb(EDTA)] (10 mM)-acetonitrile (60:40)[29] and oxalic acid (5 mM), Na[Tb(EDTA)] (10 mM)-acetonitrile (55:45)[34] as mobile phases and one anion exchange separation using sodium

tetraborate (10 mM), sodium sulfate (3 mM), and Na[Tb(EDTA)] (10 mM) at pH 9.2[23] as the mobile phase were attempted. In all three cases we were unable to reproduce the literature results. Either no elution or extremely inefficient separations with severely tailed peaks were observed. The detection limits and linearity was therefore assessed in a flow-injection mode. The measured detection limits were 7×10^{-7} M for the two reversed-phase conditions and 4×10^{-7} M in the anion exchange phase. Linearity was observed over more than two orders of magnitude. Work is continuing in an attempt to find efficient chromatographic conditions for the separation of nalidixic acid.

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