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LANTHANIDE IONS AS LUMINESCENT CHROMOPHORES FOR LIQUID CHROMATOGRAPHIC DETECTION

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SUMMARY

The chloride and nitrate salts of Tb(III) and Eu(III) can be employed as luminescent chromophores for reversed-phase liquid chromatographic detection. The method is applicable to specific compounds that are capable of either transferring energy to, or quenching the background luminescence of, a lanthanide ion. Addition of the lanthanide ion is achieved through a post-column reaction device. Mobile phases containing methanol and acetonitrile can be employed. Significant quenching of the lanthanide luminescence is observed in mobile phases containing water. This quenching can be reduced by the addition of potassium acetate. Higher temperatures increase the intermolecular energy transfer resulting in an increase in the sensitivity. Oxygen quenches the lanthanide luminescence and measures to remove oxygen from the mobile phase must be taken. The selectivity of the energy transfer can be used to both simplify chromatograms and aid in the identification of compounds.

INTRODUCTION

Detection methods employing luminescent compounds are of increasing importance in liquid chromatography. Most of these methods involve a process in which a fluorescent derivative of the analyte is prepared with a suitable chromophore either prior to chromatographic separation or in a post-column reaction system. Methods of liquid chromatographic (LC) detection based on chemiluminescence have been described in the literature[1–3]. A third procedure involves the use of sensitized phosphorescence[4]. In this technique the analyte transfers its triplet-state energy to a suitable acceptor and phosphorescence from the acceptor is detected.

In this report we describe the use of lanthanide ions as luminescent detection chromophores for LC. This method is based on the principle of sensitized luminescence. Organic compounds are excited by UV radiation and a transfer of energy from the triplet state of the organic to an excited state of the lanthanide ion occurs. Luminescence from the lanthanide ion is then detected[5]. The process is selective as only certain compounds have the necessary energy level match to complete the transfer. The sensitivity of this method is greatest for Eu(III) and Tb(III)[6]. The transfer effi-

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ciency from organic compounds to Eu(III) and Tb(III) differs because the ions have different excited state energy levels. We have also found in our work that some organic compounds can quench the background luminescence of the lanthanide ion. The ability of an organic compound to sensitize or quench the lanthanide luminescence can be useful in the identification of compounds separated by LC.

The results of preliminary studies that demonstrate the feasibility of employing lanthanide ions as detection chromophores for reversed-phase LC are reported. The lanthanide ions are most conveniently added to the LC system in a post-column mode. The compounds we describe in this report transfer energy by an intermolecular process. To our knowledge, this report represents the first use of lanthanide ions as detection chromophores in LC. Luminescent lanthanide ions have been utilized in the study and detection of mononucleotides[7–9], single-stranded DNA[10–12], RNA[12–17], proteins[18–23], and tetracyclines[24]. These classes of compounds are frequently separated by LC. Detection methods employing luminescent lanthanide ions are potentially applicable to a wide range of LC separations.

EXPERIMENTAL

Reagents

All reagents were used as received without further purification. The oxides of terbium and europium were purchased from Alfa Products (Danvers, MA, U.S.A.) in 99.9% purity. Salts of Tb(III) and Eu(III) were prepared with Ultrex hydrochloric or nitric acid purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade methanol and acetonitrile were purchased from Fisher (Fairlawn, NJ, U.S.A.). Water was doubly distilled prior to use. The organic compounds employed were reagent grade and were obtained from Aldrich (Milwaukee, WI, U.S.A.).

Preparation of the salts of the lanthanides

To prepare 1 l of a 0.01 *M* solution of $TbCl_3 \cdot xH_2O$, 1.829 g of Tb_2O_3 was added to approximately 75 ml of Ultrex hydrochloric acid. The use of Ultrex-grade acids in all lanthanide salt preparations is strongly recommended. The solution was heated with stirring until the Tb_2O_3 was completely dissolved. The volume of the solution was reduced to approximately 25 ml with heating. To avoid formation of an insoluble material, drying was completed on a rotary evaporator. A 1-l solution of the resulting salt in the appropriate solvent was then prepared. Solutions of the nitrate salt of Tb(III), and the nitrate and chloride salts of Eu(III) were prepared in a similar manner.

Apparatus

A Kratos FS 950 filter fluorometer was used for obtaining all fluorescence measurements. The excitation filter had an upper cutoff of 418 nm. The emission filter had a lower cutoff of 470 nm. The liquid chromatograms were obtained with a Beckman 341 Isocratic liquid chromatograph. A Varian post-column reaction (PCR) module was employed for the addition of a solution of the lanthanide salt to the effluent of the LC column. The PTFE mixing tee, which is standard equipment with the Varian PCR module, was replaced with a stainless-steel mixing tee. The connections from both the analytical column and PCR pump to the mixing tee were

0.01 in. I.D. stainless-steel tubing. A 25 ft. stainless-steel mixing coil (0.01 in. I.D.) was placed between the mixing tee and the fluorometer. If PTFE tubing is to be used for any of these connections, it is imperative that it be kept under an atmosphere of an inert gas such as helium or that some means of removing dissolved oxygen prior to the fluorometer be employed.

Procedures

The liquid chromatograph and PCR pumps were set at flow-rates of 1.0 ml/min. The PCR phase was typically a 0.01 *M* solution of the lanthanide salt. Therefore, the concentration of the lanthanide ion at the detector was 0.005 *M*. The time constant on the fluorometer was set at 5 sec. The organic sample was dissolved in a solvent identical to the LC mobile phase. In each case 20 μl of sample were injected from a sample loop. The column was maintained at room temperature. The temperature of the solution at the detector was adjusted by placing the 25 ft. mixing coil in a constant temperature water bath.

Several precautions were taken to avoid quenching by oxygen. All mobile phases and sample solutions were placed in an ultrasonic bath for 10 min prior to use. The LC and PCR phases were continually purged with helium during use. Since PTFE is permeable to oxygen, the PTFE lines leading from the solvent reservoirs to the two pumps were enclosed and maintained under an atmosphere of helium.

RESULTS AND DISCUSSION

The mechanism of energy transfer from an organic compound to a lanthanide ion has been determined by Crosby and co-workers[25–27] who were able to demonstrate that the energy transfer takes place from the triplet state of the organic to an excited state of the lanthanide ion. The efficiency of the energy transfer is dependent on the match between the energy levels of the organic and the lanthanide ion. In the case of energy transfer from an organic to the lanthanide, an increase in the luminescence of the lanthanide ion is observed. In LC detection, such a condition would result in the observation of a positive peak.

In our studies, which have employed a broadband excitation with a maximum intensity from 330 to 375 nm, the Tb(III) exhibits a fairly sizeable background luminescence. We have observed that certain organic compounds quench the background luminescence. In these cases the organic compound must have an excited state energy level below an excited state of the Tb(III). The transfer of energy from Tb(III) to the organic could from the first excited state of Tb(III) or higher excited states that are initially populated by the excitation source. In LC detection such an occurrence would result in the observation of a negative peak. In Table I our results have been summarized for the compounds 4,4'-dimethoxybenzophenone (DMB), *p*-diacetylbenzene, 1-acetonaphthone, and 1-naphthaldehyde with the nitrate and chloride salts of Tb(III) and Eu(III).

The larger sensitization or quenching observed with the chloride *versus* the nitrate salts is not surprising. The organic compounds listed in Table I are known to transfer energy by an intermolecular process[28–32]. Since the percentage of inner sphere complex with the lanthanide ions is greater for nitrate than chloride[33–38], the probability of collisions leading to energy transfer should be larger for the chloride salt.

TABLE I

RELATIVE PEAK HEIGHT OF ORGANIC COMPOUNDS WITH SALTS OF Tb(III) AND Eu(III) IN 100% METHANOL

*Organic**	$TbCl_3$**	$Tb(NO_3)_3$	$EuCl_3$	$Eu(NO_3)_3$
4,4'-Dimethoxybenzophenone	100	12	1	0
p-Diacetylbenzene	45	11	5	5
1-Naphthaldehyde	−66	−9	37	9
1-Acetonaphthone	−22	−10	4	3

* Concentration of injected sample: 0.001 *M*.
** Concentration of lanthanide ion at the detector: 0.005 *M*.

Other reports differ on whether dissolved oxygen quenches the lanthanide luminescence in mixtures involving an energy transfer[24,30–32,39]. It appears, however, that those processes that involve in intermolecular transfer are susceptible to oxygen quenching. As a result measures to remove dissolved oxygen from both the LC mobile phase and PCR phase were taken. The first was to replace the PTFE mixing tee and 25 ft. reaction coil with stainless-steel counterparts.

Treating both the mobile and PCR phase in an ultrasonic bath for 10 min, and then continuously purging them with helium during use resulted in greater than 100% enhancement for both positively and negatively detected compounds. Covering the heavy walled PTFE lines from the two solvent reservoirs to the pumps so that the helium purge gas swept over these lines caused further enhancement of the peaks. This enhancement was more pronounced for positively detected compounds. When using the lanthanide ions as luminescent detection chromophores in systems that involve an intermolecular transfer of energy it is essential that efforts be taken to remove dissolved oxygen.

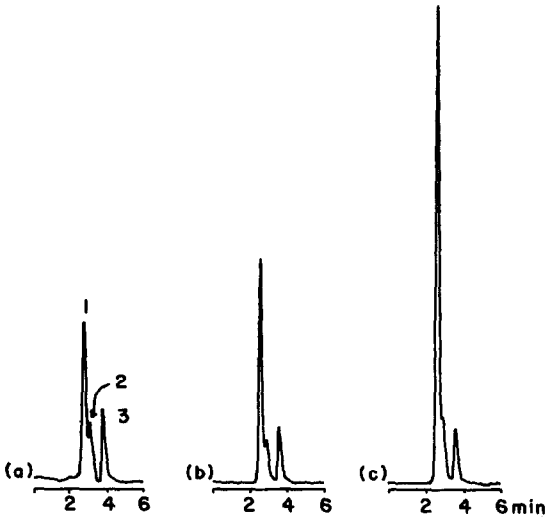

Fig. 1. Detection of 1-naphthaldehyde (1) at (a) 4.5°C, (b) 25°C, and (c) 36°C. Mobile phase: methanol, 1 ml/min. PCR phase: 0.01 *M* $EuCl_3$ in methanol, 1 ml/min. Concentration of sample injected: 0.001 *M* in methanol. Peaks 2 and 3 are impurities.

TABLE II

RELATIVE PEAK HEIGHTS WITH METHANOL AND METHANOL–ACETONITRILE (90:10)

Mobile phase: 100% methanol; PCR phase: 0.01 *M* $TbCl_3$ in methanol–acetonitrile (80:20); concentration of injected sample: 0.001 *M*.

Organic	*Methanol*	*Methanol–acetonitrile (90:10)*
4,4'-Dimethoxybenzophenone	100	117
p-Diacetylbenzene	45	57
1-Naphthaldehyde	−66	−76
1-Acetonaphthone	−22	−24

It has been reported that raising the temperature of lanthanide–organic mixtures increases the intensity of the lanthanide luminescence[30,40]. Higher temperatures will increase the collisional deactivation of the lanthanide ion which should cause a decrease in the intensity of luminescence. Since the process we are monitoring requires a collision between the organic and lanthanide ion, however, increased collisions will also increase the energy transfer process. In mixtures of lanthanide ions and organic compounds at elevated temperatures, the increase in energy transfer is greater than the decrease of lanthanide luminescence due to collisional deactivation. As a result the intensity of lanthanide luminescence increases.

To vary the temperature of our detection system the 25 ft. stainless-steel mixing coil was placed in a water bath. The chromatograms shown in Fig. 1 illustrate the results of temperature on the sensitivity. The sample compound is 1-naphthaldehyde and Eu(III) is the detection chromophore. The two smaller peaks are fluorescent impurities that are detected when no Eu(III) is added to the LC effluent. The chromatogram in Fig. 1a was obtained at 4.5°C. With the reaction coil at 25°C, Fig. 1b, the peak for naphthaldehyde was larger. It can be seen in Fig. 1b that the size of the two impurity peaks decreases as the temperature is raised. Since these peaks are the result of fluorescent impurities, more collisional deactivation, and therefore less fluorescent intensity, occurs. Heating the coil to 36°C, Fig. 1c, brings about a further enhancement of the naphthaldehyde peak. Temperatures up to 50°C were evaluated with methanol as the mobile phase and continued improvements in sensitivity were observed. Matovich and Suzuki[40] have studied samples of Eu(III) in acetophenone

TABLE III

EFFECTS OF WATER ON RELATIVE PEAK HEIGHT

Mobile phase: 100% methanol; PCR phase: 0.01 *M* $TbCl_3$ in methanol–water; concentration of injected sample: 0.001 *M*.

Organic	*Methanol–water at detector*				
	100:0	*95:5*	*90:10*	*85:15*	*80:20*
4,4'-Dimethoxybenzophenone	100	15	12	8	7
p-Diacetylbenzene	45	14	15	13	14
1-Naphthaldehyde	−66	−13	−9	−6	−5
1-Acetonaphthone	−22	−2	0	0	0

and reported increases in sensitivity up to 90°C. The upper temperature limit in LC detection is probably dependent only on the volatility and thermal stability of the particular solvent and sample employed.

If the lanthanide ions are to be useful detection chromophores in LC, it is essential that they can be employed in all of the common reversed-phase solvents. The two most important solvents, in addition to methanol, are water and acetonitrile. Table II lists a comparison of the results obtained in methanol and methanol–acetonitrile (90:10). A 10–30% gain in sensitivity was observed with the 90:10 mixture. Acetonitrile appears to neither interupt the energy transfer nor quench the Tb(III) luminescence and can be employed in mobile phases with these chromophores.

It has been reported that water quenches the lanthanide luminescence in organic–lanthanide mixtures[30,39,40]. These reports found that the relationship between quenching and the concentration of water was not linear. Small amounts of water produced significant quenching after which the luminescence intensity remained essentially constant[40]. We have observed a similar result as shown by the data presented in Table III. A large loss in sensitivity was observed on changing the phase at the detector from pure methanol to methanol–water (95:5). The sensitivity leveled off as greater amounts of water were added to the mobile phase. It should also be noted that the background luminescence of the lanthanide species decreases on addition of water.

In solutions of the lanthanide chlorides in methanol–water solvents, the water molecules occupy the first coordination sphere of the lanthanide ion[36,38]. These water molecules could reduce the effectiveness of collisions and thereby reduce the energy transfer. An alternative mechanism is that the energy transfer occurs and the excited Tb(III) ions are more effectively quenched by the water molecules in the first coordination sphere. Since water is an important solvent in reversed-phase LC, this quenching poses a significant problem. It has been reported in the literature that addition of acetate to aqueous solutions of $TbCl_3$ increases the background luminescence of Tb(III)[39]. The acetate ion displaces water from the first coordination sphere of Tb(III)[33,35,41]. The larger background luminescence could be due to a reduction of quenching by water or to a transfer of energy from the acetate to Tb(III).

The effects of adding various concentrations of potassium acetate to a PCR

TABLE IV

EFFECTS OF POTASSIUM ACETATE ON RELATIVE PEAK HEIGHTS IN MOBILE PHASES CONTAINING WATER

Mobile phase: 100% methanol; PCR phase: 0.01 *M* $TbCl_3$ in methanol–water (80:20); concentration of injected sample: 0.001 *M*.

Acetate concentration	*4,4'-Dimethoxybenzophenone*	*1-Naphthaldehyde*
0	12	−9
0.03	19	−60
0.05	64	−65
0.10	38	−66
0.25	26	−65
0.50	29	−64
1.00	24	−60

phase of 0.01 *M* $TbCl_3$ in methanol–water (80:20) were studied. The data are listed in Table IV. The background luminescence of Tb(III) rose substantially on addition of acetate. In addition larger sensitivity was observed for both positively and negatively detected compounds. The sensitivity for naphthaldehyde is similar to that observed in 100% methanol. For DMB sensitivity on the order of that observed in 100% methanol was not achieved, but addition of acetate did improve the sensitivity by an order of magnitude. The largest enhancement was observed at 0.05 *M* potassium acetate. It could be that increasing the concentration of acetate beyond 0.05 *M* interferes with the energy transfer between the organic compound and Tb(III). Lanthanide ions can be used as detection chromophores in aqueous mobile phases provided potassium acetate is added in appropriate concentration.

The chromatograms shown in Fig. 2 illustrate the practical applications that result from the selectivity of the transfer process between an organic compound and Tb(III). Compounds that do not influence the intensity of the Tb(III) luminescence are not detected resulting in a simplification of the chromatogram. The nature of a peak, positive or negative, provides a useful piece of information in the identification of a compound. The mixture in Fig. 2 consists of DMB, 1-naphthaldehyde, and naphthalene. The chromatogram in Fig. 2a is that observed with fixed-wavelength UV detection at 254 nm. In Fig. 2b the chromatogram obtained using fluorescence

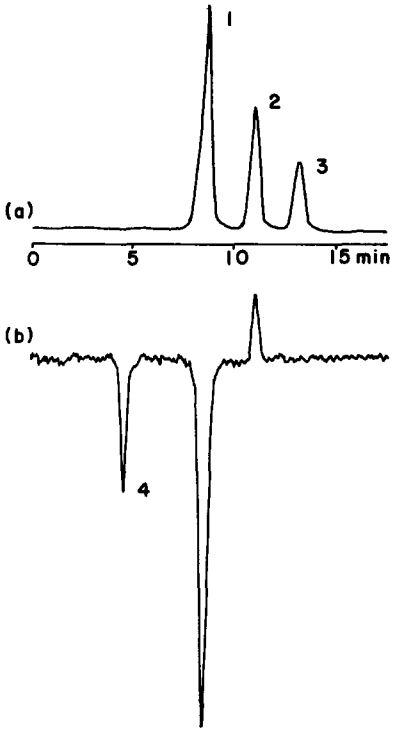

Fig. 2. Chromatogram of 1-naphthaldehyde (1), DMB (2), and naphthalene (3) with (a) UV detection at 254 nm, and (b) fluorescence detection with Tb(III). Mobile phase: methanol–water (70:30), 1 ml/min. PCR phase: 0.01 *M* $TbCl_3$ and 0.05 *M* potassium acetate in methanol, 1 ml/min. Concentration of sample injected: 0.001 *M* in methanol. Peak 4 is an impurity.

detection and post-column addition of 0.01 *M* Tb(III) is shown. The 1-naphthaldehyde is observed as a negative peak, DMB is observed as a positive peak, and naphthalene is not detected. In addition, a peak with a retention time of 4.5 min, which has been found to be an impurity in 1-naphthaldehyde, is detected in the presence of Tb(III). This compound was not observed in the chromatogram obtained with UV detection.

Lanthanide ions can be employed as luminescent detection chromophores in LC. The selectivity of the energy transfer between an organic compound and a lanthanide ion can be used to both simplify chromatograms and aid in the identification of compounds separated by LC. The method is potentially applicable to aromatic aldehydes and ketones[28–32] as well as a variety of biologically important compounds[7–24]. Applications of the lanthanide ions as detection chromophores for biologically important compounds are presently under investigation.

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