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Original Article

Development of a Simple and Sensitive Terbium Sensitized Fluorescence Method for Determination of Epinephrine and Norepinephrine in Serum

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Abstract

A simple, rapid, selective and highly sensitive fluorimetric method for determination of two catecholamines, i.e. norepinephrine (NE) and epinephrine (EP), in serum samples was developed. The method is based on the fluorescence sensitization of terbium (Tb^{3+}) by complexation with both catecholamines in the presence of lanthanum (La^{3+}), as a co-cation, and in a Na_2SO_3 solution (chemical deoxygenating agent), which fluoresces intensely with an emission maximum at 545 nm when excited at 312 nm. We found that fluorescence enhancement effects were observed when La^{3+} was added to the system (co-luminescence effect). In the presence of this element the fluorescence of the Tb-catecholamines system was enhanced by a factor of about 15 compared with that of the system without La^{3+}. Optimum conditions for the formation of the complexes were investigated. Under optimum conditions, a linear relationship was obtained between the fluorescence intensity and catecholamines concentration in the range of 2.5×10^{-3} to 0.22 $\mu g.ml^{-1}$. The detection limits were 0.59 and 0.58 $\mu g.l^{-1}$ for EP and NE, respectively. Relative standard deviation (RSD) values for two compounds were in the range of 1.2-2.1% indicating excellent reproducibility. The proposed method was successfully applied to the determination of EP and NE in spiked serum samples after deproteinization of the samples with acetonitrile. Analytical recoveries from treated serum samples were in the range of 95-105%.

Keywords: Catecholamines; Co-luminescence effect; Sensitized fluorescence; Serum; Terbium.
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1. Introduction

Epinephrine (EP) or adrenaline is the main hormone of the adrenal medulla and norepinephrine (NE) or noradrenaline is the main neurotransmitter of the sympathetic nervous system. These biogenic compounds belong to catecholamines and regulate vital responses including blood pressure, cardiac

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output, sweating and many other important actions. EP is biosynthesized from tyrosine and several enzymatic reactions convert tyrosine to dihydroxyphenlyalanine (dopa), to dopamine, to NE and then to EP. These catecholamines are stored in complex secretory granules in the adrenal medulla consisting of 80% EP and 20 % NE. Both NE and EP are converted to methylated derivatives and also taken up by the nerve endings [1]. NE and EP exist as organic cations in the symphatetic nervous system and also body fluids. The concentration of catecholamines and their metabolites are elevated in urine and plasma in several diseases and are of clinical interest, especially in the diagnosis of pheochromocytoma, hypertension, Parkinson's disease, neuroblastoma and ganglioneuroma [2]. Quantitative determination of these compounds in biological fluids is important for developing nerve physiology, making diagnosis and controlling medicine [1]. Pharmaceutically, they are also widely used for treatment of many diseases such as allergic reaction emergencies, bronchial asthma, venticular bradycardia, cardiac arrest and glaucoma [3].

It is well known that some trivalent lanthanide ions in an aqueous medium at ambient temperature have fluorescence properties due to, principally, the transition within the 4f-shell but are weakly fluorescesing species due to their low molar absorptivities and poor quantum yields [4]. However, when these ions, in particular Eu^{3+} and Tb^{3+} are chelated with ligands that have a broad intense absorption band their fluorescence can be dramatically enhanced [5, 6]. Intramolecular transfer of energy from the excited organic molecule to a resonance level of the lanthanide followed by the lanthanide emission is responsible for excitation of lanthanide ion in the formed complex in the solution and also fluorescent enhancement. If there is efficient intramolecular energy transfer, the upper emitting levels of the lanthanides are more effectively excited by this technique than by the direct form, producing an enhanced fluorescence of the lanthanide by several orders of magnitude. This phenomenon has been named the lanthanide-sensitized fluorescence and is of great interest for the trace determination of lanthanide ions and a variety of medically important compounds [7]. The main features of the process are large Stokes shift, a narrow- emission bands and long fluorescence lifetimes, and hence avoid potential background fluorescent emission interferences from the biological matrix, so selectivity and detection limits are improved [7]. An efficient way for enhancing the fluorescence of lanthanide ions in aqueous solutions is the addition of co-cations, such as La^{3+} [8]. This phenomenon could be used for determination of catecholamines as described in the present work.

Several analytical methods have been proposed for the determination of catecholamines in biological fluids and pharmaceutical preparations during the last years. Among the numerous methods for this purpose, liquid chromatography (LC) with electrochemical [9, 10], fluorimetric [11, 12], or chemiluminescence [13, 14] detection were the most widely used. Flow injection [15], spectrophotometric [16], fluorimetric [17, 18], capillary electrophoresis [19], amperometric [20], electrochemiluminescence [21], noncompetitive enzyme immunoassay [22] and modified electrodes [23, 24] for the determination of catecholamines were also reported. Some of these methods are very expensive, complicated, time consuming, need extraction or derivatization and also suffer from insufficient sensitivity, poor selectivity and high consumption of samples [16, 25].

The purpose of this work was to develop a simple and very sensitive fluorimetric method with high selectivity for the

determination of catecholamines based on improving terbium sensitized fluorescence in serum using La^{3+} as a co-cation. This high performance procedure, is easily carried out, gives good precision and accuracy, provides an optimum detection limits and is successfully applied to the determination of NE and EP in human serum samples, without needing a separation technique in analysis, due to high both sensitivity and selectivity obtained with this system.

2. Materials and methods

2.1. Reagents

All reagents and solvents used were of analytical grade and were used without further purification. Doubly distilled water was used throughout.

A 10^{-2} M terbium (III) and a 5×10^{-3} M europium (III) solution were prepared by dissolving the appropriate amount of terbium (III) chloride hexahydrate ($TbCl_3.6H_2O$) and europium (III) chloride hexahydrate ($EuCl_3.6H_2O$) (Acros Organics, USA) in doubly distilled water and stored in polyethylene containers to avoid memory effects of terbium and europium adsorbed on glass vessels.

A stock standard solution of 10^{-2} M lanthanum (III) was prepared by dissolving a desired amount of La $(NO)_3$ aq (Fluka) in water. Stock solutions of gadolinium (III) and lutetium (III) (10^{-2} M) (Garden, London, E.C.1 England) were prepared.

The stock solution, 1000 $\mu g.ml^{-1}$ L-noradrenaline-L-tartarat monohydrate (Merck) and L-adrenaline (Wako, 050-04081) were prepared in water and stored at 4 °C in a refrigerator. The stock solution was stable for several months.

Also aqueous solutions of N-cetyl-N,N,N-trimethylamonium bromide (CTAB), polyoxyethylene lauryl ether (Brij-35), sodium dodecyl sulfate (SDS), EDTA, Triton X-114, hydrochloric acid, sodium hydroxide, ammonium chloride, boric acid, sodium carbonate (all were obtained from Merck) were prepared. All organic solvents were purchased from Merck. Bis (2-ethylhexyl) sulfosuccinate sodium (AOT) and 1, 10-phenanthroline (Phen) were obtained from Fluka.

A 0.05 M Tris-(hydroxymethyl) amino methan-hydrochloric acid (Tris-HCl) buffer solution was prepared by dissolving a desired amount of Tris-base (Merck) in 90 ml of water, adjusting the pH to 9.5 with HCl and making up the volume to 100 ml with water.

To prevent the oxidation of NE and EP the freshly prepared 1% aqueous solution of sodium sulphite (Na_2SO_3) (Merck) was used. Working standard solutions were prepared daily by dilution of the stock standard with water.

2.2. Apparatus

Fluorescence spectra and intensity measurements were performed using a Shimadzu RF-540 spectrofluorimeter (Kyoto, Japan) equipped with a 150 W xenon lamp, using 1.0 cm quarts cell. The excitation and emission monochromator bandwidths were 10 nm. The excitation wavelength was set at 312 nm and the fluorescence was measured using the peak height 545 nm. All measurements were performed at 25±0.1 °C by using a thermostated cell holder and a thermostatically controlled water bath (Rikakika, Japan). The pH of solutions was measured with Metrohm model 654 pH meter (Herisau, Switzerland).

2.3. Methods

All measurements were corrected for the background fluorescence of blank which was taken as the solution containing all reagents except the catecholamines. Optimization of terbium sensitized fluorescence was investigated by varying the pH or the concentration of one of the components while the rest remained constant.

2.4. Experimental procedure

Table 1. Comparison of results of the analytical characteristics of the proposed method with those of other methods for the determination of NE and EP.

Method	Linear range (mol.l^{-1})	Detection limit (mol.l^{-1})	Reference
HPLC with fluorimetric detection	-	1.0×10^{-8} (NE)	12
		7.0×10^{-8} (EP)	12
Amperometric	5.0×10^{-5} - 3.5×10^{-4} (EP)	1.5×10^{-5}	20
Capillary electrophoresis	1.0×10^{-3} - 1.0×10^{-6} (EP and NE)	8.5×10^{-7} - 9.5×10^{-7}	19
Electrochemical	1.0×10^{-7} - 1.0×10^{-5}	1.0×10^{-8}	24
(Modified electrodes)	2.0×10^{-6} - 8.0×10^{-5}	2.0×10^{-7}	23
Spectrofluorimetric	2.0×10^{-8} - 6.0×10^{-6} (EP)	9.3×10^{-9}	17
Spectrophotometric	4.8×10^{-6} - 6.0×10^{-4} (NE)	2.5×10^{-6} (NE)	16
	4.8×10^{-6} - 8.0×10^{-4} (EP)	2.6×10^{-7}(EP)	16
This method	7.4×10^{-9} - 6.5×10^{-7} (NE)	1.7×10^{-9} mol.l^{-1} (NE)	-
	1.4×10^{-8} - 1.2×10^{-6} (EP)	3.2×10^{-9} mol.l^{-1} (EP)	-

For the terbium sensitized fluorescence determination, the analytical procedure used to construct the calibration graphs was as follows: to a 5 ml volumetric flasks, 0.1 ml of 1×10^{-2} M Tb^{3+} solution, aliquots of working catecholamines solutions, 0.2 ml of 1×10^{-2} M La^{3+} solution, 0.5 or 1 ml of 0.05 M Tris-HCl buffer (pH 9.5) solution and 1 ml of 1% w/v Na_2SO_3 solution were added. The mixture was then diluted to the mark with distilled water and allowed to stand for 30 min. The final catecholamines concentrations were in the range of 2.5×10^{-3}-0.22 μg.ml^{-1}. The solutions were thermostated at 25±0.1 °C and the fluorescence intensity was measured at 545 nm using an excitation wavelength of 312 nm against a blank solution. Both emission and excitation slits were set at 10 nm.

2.5. Analysis of serum samples

The proposed method was applied to the determination of catecholamines in spiked human serum provided by several healthy subjects and were used after deproteinization with acetonitrile.

Addition of untreated serum samples led to turbid solutions in the presence of Tb^{3+} and also high background signals were observed, owing to high protein concentrations. Therefore, a deproteinization process with acetonitrile was employed. Thus, an aliquot of 0.5 ml serum, previously spiked with convenient amounts of NE and EP stock solutions (100 and 1000 μg.ml^{-1}) for final concentrations in the range of 0.5-15 μg.ml^{-1} was placed in a test tube, each sample was deproteinized with 1 ml acetonitrile, by vortexing for 5 min. and centrifugating for 15 min. at 4000 r/min. to remove proteins. Then, 0.1 ml of the supernatant was pipetted in a 5 ml volumetric flask (dilution factor is 50-fold) (0.01-0.3 μg.ml^{-1}) and emission measured as explained for standard catecholamines against a blank solution (unspiked serum).

3. Results and discussion

3.1. Fluorescence characteristics of catecholamines and their complexes with terbium (III) in aqueous solution

Weakly acidic (pH 4.7) or neutral aqueous solutions of NE and EP show weak intrinsic fluorescence with $\lambda_{ex}/\lambda_{em}$ maxima at about 280/312 nm. The influence of pH in the range of 1-12 on the fluorescence of catecholamines was studied using NaOH and HCl (1 and 0.01 M). The fluorescence is pH dependent with maximum fluorescence observed over the pH range of 1-6. At pH 6-8, the intrinsic fluorescence is weak and at pH>9, it is negligible. A hypsochromic shift in the excitation and emission maxima is observed for both catecholamines due to ionization of dehydroxyphenyl group.

The addition of terbium ions to weakly alkaline (pH 9.5) aqueous solutions of NE and EP results in the appearance of the narrower

and stronger new emission bands at the range of 450-600 nm (λ_{max}=545 nm), characteristic of terbium ion fluorescence, and a shift of about 32 nm of the excitation maximum of catecholamines (312 nm), compared with those of NE and EP, which is due to the complex formation of catecholamines with terbium (III) (Figure 1).

Additional evidences proving the formation of the Tb-catecholamines complex are: a) the free Tb^{3+} aqueous solutions under the same conditions fluoresces too weakly to be observed and b) in the presence of Tb^{3+}, intrinsic fluorescence of catecholamines decreases while the emission band of Tb^{3+} appears whose peak intensity is much greater than that of uncomplexed NE and EP. Therefore, it was decided to test whether NE and EP can be determined by the lanthanide-sensitized fluorescence method.

As an example, the fluorescence excitation and emission spectra of Tb^{3+} (1), La^{3+} (2), Tb-NE (3), Tb-La-NE-Na_2SO_3 (4), at pH 9.5 are shown in Figure 1. From spectra 1 and 2 in Figure 2, it can be seen that only the $Tb^{3+}$and La^{3+} solutions have little or nearly no peak. Under the same conditions, after the addition of the NE into Tb^{3+}solution, NE can from a binary complex with Tb^{3+} ion, which leads to energy transfer from NE to Tb^{3+}, so two little characteristic peaks of Tb^{3+} ion appear at 490 and 545 nm . The most intense band is the $^5D_4 \rightarrow {}^7F_5$ transition at 545 nm, thus 545 nm was selected for measurement. Comparing spectrum 3 with spectrum 4 in Figure 2, reveal that the characteristic peak of Tb^{3+}at 545 nm can remarkably be enhanced in the presence of appropriate amounts of La^{3+} ion (about 15 fold with respect to Tb-NE system), which indicated that the intramolecular energy transfer can occurs more easily than directly from NE to $Tb^{3+}$and a very stable complex was formed followed by a co-luminescence effect that occurred [8].

The experiments indicated that at the room temperature the fluorescence intensity of the system was very stable and reached maximum value within 30 min., and remained stable for more than 24 h.

3.2. Conditions of the complexation

As expected, complexation of catecholamines with Tb^{3+} ions depends strongly on the pH and occurs through dihy-

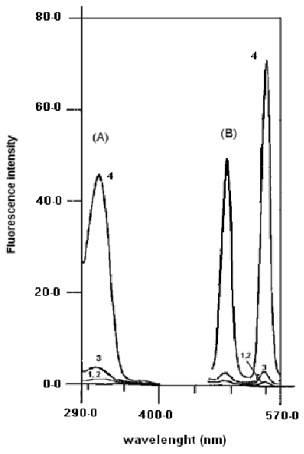

Figure 1. Terbium-sensitized fluorescence excitation (λem=545 nm) (A) and emission (λex=312 nm) (B) spectra of the Tb-La- NE complex. (1) Tb^{3+}, (2) La^{3+}, (3), Tb-NE, (4) Tb-La-NE. Conditions: $[Tb^{3+}] = 2\times10^{-4}$ M, $[La^{3+}] = 4\times10^{-4}$ M, [NE] = 0.2 μg.ml^{-1}, $[Na_2SO_3]$ = 0.2% w/v, [Tris]=0.05 M, pH=9.5.

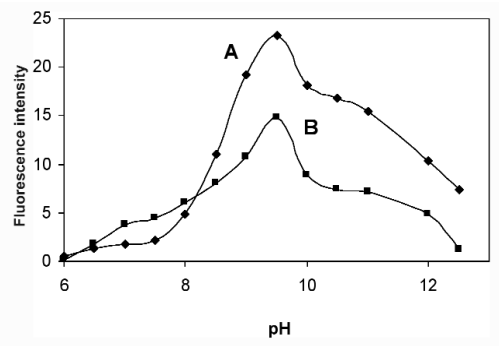

Figure 2. Effect of pH on fluorescence intensity of the (A) NE-Tb-La and (B) EP-Tb-La complexes. [NE], [EP]=0.2 μg.ml^{-1}, $[Tb^{3+}]=10^{-5}$ M, $[La^{3+}]=10^{-4}$ M, pH=9.5, [Tris]=0.05 M, $[Na_2SO_3]$=0.005% w/v.

droxyphenyl group [26]. This result indicates that complexation involves two negatively charged oxygens available in a single ligand to form a chelate. Therefore, in order to form fluorescent chelates between catecholamines and Tb^{3+} ions, it is necessary to work in alkaline media because the optimum pH for the complex formation seems related to the dissociation of the phenol group. However, the aqueous solutions of catecholamines, decompose rapidly in alkaline medium (pH=10.5, after 15 min.). Therefore, in order to prevent their oxidation in alkaline medium, that is necessary for the complex formation, sodium sulphite was used. The results indicated that in the presence of Na_2SO_3, the complexes were stable for 24 h and their fluorescence intensity were enhanced.

3.3. Optimization of analytical signal

It is well known that water molecules strongly quench terbium sensitized fluorescence [27]. The presence of synergistic agents, that complete the coordination sphere of lanthanide ion by displacing water molecules from ligand field, and the various surfactants, which protects these chelates from non-radiative processes, have been usually used to improve the fluorescence of these systems in solutions [27]. The use of organic solvents can also favor the efficiency of the energy transfer from the organic ligand to terbium ions [28]. Therefore, with the aim of minimizing radiationless deactivation processes, and in order to choose the adequate systems for the determination of catecholamines, based on lanthanide sensitized fluorescence, a series of preliminary studies were carried out. The influences of different concentrations of EDTA and Phen (10^{-5}-10^{-4} M), as co-ligands, and several concentrations of various surfactants such as Triton X-114 (0.01 to 0.05%), Brij-35 (0.01 to 0.4%), SDS, CTAB and AOT (5×10^{-4}-10^{-3} M) were studied for both complexes in solution. In all cases, the presence of a micellar medium and a co-ligand caused a slight increase in the fluorescence intensity of the systems. The results showed that the effect of concentrations of 5-10% of organic solvents, such as methanol, ethanol, propan-2-ol, dimethylformamide and 1, 4-dioxane the fluorescence intensity is insignificant, while in the presence of acetone and dimethyl-sulfoxide, the fluorescence was quenched. According to these different results, the use of a synergistic agent, a surfactant or an organic solvent was discarded. This is a major advantage compared to all lanthanide based methods because the addition of the enhancer always elongates the time for the determination of the sample and causes higher costs for the additional reagent.

This fluorescence behavior of the both systems could be ascribed to the fact that NE and EP are two dentate ligands where two strong donor from the phenolic group, giving a better stability to the system, so that it is less affected by non-radiative processes than other Tb^{3+} chelates.

Another efficient way for enhancing the fluorescence of lanthanide ions is called the co-luminescence effect. According to this phenomenon, the addition of certain lanthanide ions, such as La^{3+}, Ga^{3+}, Lu^{3+} and Y^{3+}, can considerably enhance the fluorescence intensity of the chelates of Tb^{3+}[8]. Therefore, the efficiency of the energy transfer in the presence of some of these cations (La^{3+}, Gd^{3+}, $Lu^{3+}$and Eu^{3+}) was also studied and the best result was obtained in the presence of La^{3+}.

The effect of pH on complexation and fluorescence intensity was studied in the range of 6 to 12 for both systems (Figure 2). Solutions containing reagents and their corresponding blank solutions with various pH values were prepared using HCl or NaOH. The results indicated that the fluorescence intensity reached maximum at pH 9.0-9.5. This is in accordance with the fact that the

Table 2. Tolerance limits of various interferents in the determination of 0.2 g.ml^{-1} of NE and EP.

Substances	Interferent-to-analyte ratio	$\Delta F\%$	
		Norepinephrine	Epinephrine
K^+ (Cl^-)	500:1	-0.63	0.10
Na^+(Cl^-)	500:1	0.028	0.14
Ca^{2+}(Cl^-)	500:1	0.02	1.10
Mg^{2+}(Cl^-)	500:1	0.05	0.35
Al^{3+}(Cl^-)	40:1	0.80	2.00
Cr^{3+}(Cl^-)	20:1	-0.60	-0.30
Ag^+(Cl^-)	20:1	0.80	0.40
Fe^{2+}(Cl^-)	10:1	-0.51	-0.64
Fe^{3+}(Cl^-)	10:1	-0.57	-0.83
Zn^{2+}(Cl^-)	10:1	-0.30	-0.03
Mn^{2+}(Cl^-)	10:1	-0.97	-0.91
Ni^{2+}(Cl^-)	10:1	-0.98	-0.96
Cu^{2+}(Cl^-)	2:1	-0.30	-0.20
Cd^{2+}(Cl^-)	2:1	0.16	0.44
Hg^{2+}(Cl^-)	2:1	-0.16	-0.39
Co^{2+}(Cl^-)	2:1	-0.99	-0.94
L-Alanine	500:1	-0.01	-0.15
L-Cysteine	500:1	0.20	0.15
Tryptophane	500:1	-0.60	-0.64
Glycine	500:1	0.12	0.02
L-Leucine	500:1	0.45	0.13
Tyrosine	100:1	0.27	0.15
Uric acid	10:1	0.37	0.65
Sucrose	500:1	0.35	0.02
Glucose	500:1	0.52	0.21
BSA	40:1	1.20	1.90
EP	1:1	0.58	-
NE	1:1	-	0.43

$\Delta F\% = (F_1-F_2)/ F_2 \times 100$. Here, F_1 and F_2 are the fluorescence intensities of the systems with and without interferents, respectively.

ligand will coordinate with terbium ions more efficiently in its ionic forms. Dissociation of diohydroxyphenyl group in NE and EP increases with increasing pH, so that facilitates the formation of the Tb- catecholamines complexes, and enhances the fluorescence intensity. At higher values of pH (pH>10.0), the intensity decreases owing to the precipitation of terbium hydroxide and in the lower pH values the complex evidently dose not form or the degree of its formation is very low. Therefore, a pH value of 9.5 was chosen for the determination. Experiments indicated that the chemical nature of the buffer has also considerable effects on the intensity. The following buffers for the required pH were tested: Tris-HCl, $Na_2B_4O_7$-HCl, NH_4^+-NH_3 and HCO_3^--CO_3^{2-}. The results showed that 1 or 0.5 ml of Tris-HCl (0.05 M, pH=9.5) in a final 5 ml was the most suitable buffer.

Deoxygenating of the samples led to higher and stable fluorescence signals, as the triplet state of NE and EP in alkaline medium involved in terbium sensitized fluorescence is sensitive to oxygen quenching [29]. Oxygen in solution was removed using Na_2SO_3. The effect of Na_2SO_3 concentration was studied in the range of 0.005-0.5 % w/v (Figure 3). The concentration of 0.2% w/v concentration was required to eliminate oxygen completely from solutions. So a value of 0.2% w/v was selected for further studies.

Another important parameter influencing the fluorescence intensity is the terbium concentration which was studied in the range of (0-4)×10^{-4} M for both complexes. As

Table 3. Recoveries of NE and EP from human serum samples.

Sample	Norepinephrine			Epinephrine		
	Added[a] (μg.ml^{-1})	Found (μg.ml^{-1})[b]	Recovery (%)	Added (μg.ml^{-1})	Found (μg.ml^{-1})	Recovery (%)
Serum 1	0.22	0.217±0.0035	98.6	0.08	0.083±0.0017	103.7
	0.16	0.161±0.0046	100.6	0.06	0.058±0.0015	96.7
	0.076	0.077±0.0004	101.3	0.04	0.043±0.0011	105
Serum 2	0.24	0.232±0.0047	96.7	0.2	0.206±0.0023	103
	0.18	0.187±0.0058	103.8	0.1	0.096±0.0020	96
	0.014	0.014±0.0005	98.6	0.04	0.041±0.0011	102.5
Serum 3	0.28	0.276±0.0058	98.6	0.2	0.197±0.0052	98.5
	0.1	0.105±0.0021	105	0.1	0.095±0.0020	95
	0.046	0.048±0.0015	104.3	0.12	0.116±0.0020	96.7

[a]The given values are concentration in the final solutions.
[b]Average of three determinations ± SD.

shown in Figure 4, the intensity is enhanced with an increase in the Tb^{3+} concentration and reached a stable value for concentrations of above 10^{-4} M. Therefore a 2×10^{-4} M concentration was chosen for both systems. Above 2×10^{-4} M concentration it started to decline. This trend indicated that the excess of Tb^{3+} is required for complex formation. However, large excess above the optimum may result in quenching of the fluorescence due to non-radiative collisions of the singlet excited state of catecholamines [29].

The concentration of La^{3+}in the range of (0-4) × 10^{-4} M was also investigated (Figure 5). It was found that when the lanthanum concentration was increased, the fluorescence intensity of the system also increased and the maximum intensities were obtained at 4×10^{-4} M.

The experimental results showed that the order of the addition of the various components has large effects on the fluorescence intensity of both systems. Therefore, a series of solutions with different addition orders of the reagents and their corresponding blank solutions were investigated. Considering the stability of the system along with the fluorescence intensity enhancement, the following order was chosen for further study: Tb^{3+} solution, NE (or EP) standard solution, La^{3+} solution, Tris-HCl buffer solution and Na_2SO_3 solution.

The effect of reaction time on signal intensity was also studied. The intensity

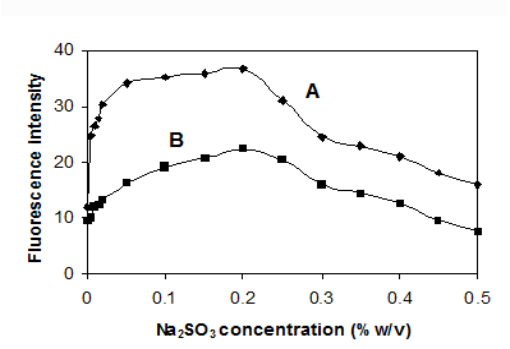

Figure 3. Effect of the Na_2SO_3 concentration on fluorescence intensity of the (A) NE-Tb-La and (B) EP-Tb-La complexes. [NE], [EP]=0.2 μg mL^{-1}, [Tb^{3+}]=10^{-5} M, [La^{3+}]=10^{-4} M, pH=9.5.

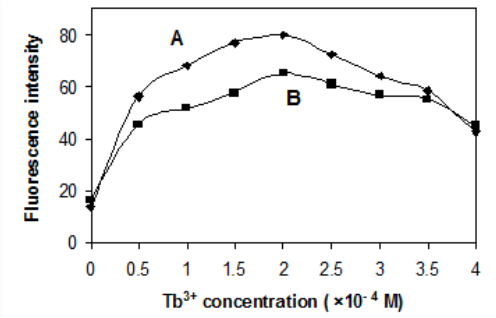

Figure 4. Effect of the concentration of Tb^{3+} (A) NE-Tb-La and (B) EP-Tb-La systems on fluorescence intensity. [NE], [EP]=0.2 μg.ml^{-1}, [La^{3+}]=10^{-4} M, [Na_2SO_3]=0.2% w/v, [Tris]=0.05 M, pH=9.5.

reached the highest values after 30 min. and remained constant for at least 24 h for both systems. Hence, all chelation reactions were carried out for 30 min. and measurements were made within 3-4 h.

3.4. Mechanism for the co-luminescence effect in the Tb-La-catecholamine system

Figure 1 shows that the excitation spectrum of Tb-La-NE (EP)-Na_2SO_3 system is similar to that of the system without La^{3+} indicating that La^{3+}dose not form a new complex with the Tb-catecholamine system. We believe that the Tb-NE (EP) and La-NE (EP) complexes are both formed in the Tb-La-catecholamine-Na_2SO_3-buffer system. The two complexes are in close proximity in the system and the light energy absorbed by NE or EP in the La-NE (EP) complex can be transferred to the fluorescence level of the Tb^{3+} in the Tb-NE (EP) complex through an intermolecular transfer of energy. As the concentration of the La complex is greater than that of Tb complex, the fluorescence intensity of Tb^{3+} is considerably enhanced.

3.5. Analytical figures of merit

By using the optimized conditions described above, a spectrofluorimetric method was developed for the determination of NE and EP. The calibration graphs (n=14) were found to be linear in the range of 2.5×10^{-3} and 0.22 $\mu g.ml^{-1}$ (7.4×10^{-9}-6.5×10^{-7} and 1.4×10^{-8}-1.2×10^{-6} $mol.l^{-1}$ for NE and EP, respectively) and their equation were F=450.75 (±3.69)C+3.15 (± 0.31) (for NE) and 448(±3.67)C+3.67(±0.30) (for EP), where F is the fluorescence intensity and C is the concentration of catecholamines expressed in $\mu g.ml^{-1}$. The limits of detection were calculated as $3S_b/m$ (where S_b is standard deviation of the blank and m is slope of the calibration graph) were found to be 0.58 and 0.59 $\mu g.l^{-1}$(1.7×10^{-9} and 3.2×10^{-9} $mol.l^{-1}$) for NE and EP, respectively.

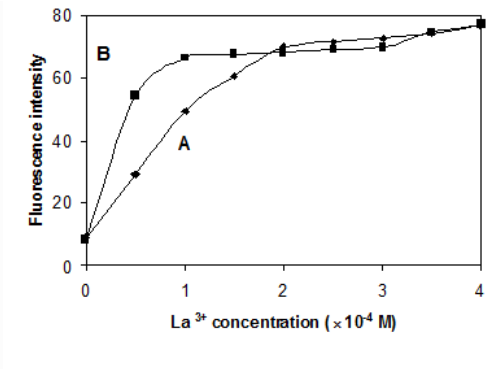

Figure 5. Effect of the concentration of La^{3+} (A) NE- Tb-La and (B) EP-Tb-La on fluorescence intensity [NE], [EP]=0.2 $\mu g.ml^{-1}$, $[Tb^{3+}]=2\times10^{-4}$ M, $[Na_2SO_3]$=0.2 % w/v, [Tris]=0.05M, pH=9.5.

In order to study the precision of the proposed method, series of six solutions of 0.2 and 0.1 $\mu g.ml^{-1}$ of catecholamines were measured on the same day. By applying the IUPAC definition, the relative standard deviation (RSD) for six analyses was 1.2-2.1%.

The analytical characteristics of the proposed method are compared with some published methods for the determination of NE and EP in Table 1. As can be seen, our proposed method has comparable or better detection limit (about 10-10.000 fold lower) than the methods studied. Also this proposed method is very simple, rapid, precise, and more sensitive and selective. Under experimental conditions, the analytical parameters, specially the sensitivity significantly improve (100 fold) in the presence of Tb^{3+} and La^{3+} with respect to native fluorescence of the two compounds in aqueous solution at pH 9.5.

3.6. Interference study

Under the optimum conditions, a systematic study of various substances in the determination of NE and EP was carried out. In order to study potential interference, samples containing 0.2 $g.ml^{-1}$ of NE and EP were mixed with potential interferents.

Fluorescence intensities were compared with a sample containing 0.2 $g.ml^{-1}$ of NE and EP in the absence of interferents. A substance was considered to interfere when its presence produced a variation of more than 5% in the fluorescence intensity of the analyte. The tolerance levels of various interferents (e.g. ions, amino acids, proteins and saccharides) are summarized in Table 2. From Table 2, it can be seen that most substances were found to show no influence. These results allow the interference-free determination of NE and EP in serum samples.

3.7. Application to serum samples

To examine the applicability of the method, the proposed method was applied to the determination of the catecholamines in spiked serum samples. It was found that acetonitrile is the most efficient agent for deproteinization of serum. For the determination of catecholamines in serum with a content 0.5-15 $\mu g.ml^{-1}$, 0.1 ml of deproteinized serum was analyzed by the proposed method using the standard addition method. Under these conditions, we compared the calibration graphs obtained for catecholamines with aqueous and serum standards, the slopes of the standard addition graphs in these media were approximately 1.5% lower than those of the aqueous standards and no effect on the fluorescence of the blank (unspiked serum). Recoveries obtained were in the range of 95-105%.

Accuracy and precision of the method were checked by the added-found procedure and statistical treatment of data of catecholamines determination in the matrix. The recoveries found for three different serum samples and for three replicates are summarized in Table 3. Statistical analysis of the assay results showed satisfactory precision of the proposed method with no significant difference between certified and experimental results. These finding confirmed that the developed method was easy to perform, and afforded good precision, accuracy and recovery when applied to serum samples.

4. Conclusions

A spectrofluorimetric method was developed for the determination of catecholamines in serum samples based on terbium sensitized fluorescence. The proposed method is relatively simple, precise, rapid and more selective and sensitive than the many methods that have been used for the determination of catecholamines until now [16, 17, 19, 20]. The developed method was easily applied to the determination of catecholamines in serum samples with excellent reproducibility. This method doses not require previous separation techniques due to the fact that there is no interference between catecholamines and the serum matrix.

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