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Spectrofluorimetric Determination of Daunorubicin Using Terbium-Deferasirox as a Fluorescence Probe

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Abstract

A spectrofluorimetric method is proposed for the determination of Daunorubicin (DNR) based on its quenching effect on the fluorescence intensity of Tb^{3+}- deferasirox (DFX) complex as a fluorescent probe. The excitation and emission wavelengths were 328 and 545 nm, respectively. The optimum conditions for the determination of DNR were investigated considering the effects of various parameters on the quenched fluorescence intensity. In the optimum conditions the decrease of the fluorescence intensity of the system showed a good linear relationship with the concentration of DNR in the range of 15-1100 μg L^{-1}, with a correlation coefficient 0.998. The detection limit (3s) was 4.3 μg L^{-1} and the relative standard deviation for four replicate determinations of different concentrations of DNR was in the range of 1.6–4.7%. The procedure was successfully applied to the determination of daunorubicin in spiked urine and serum samples.

Keywords: Terbium-sensitized fluorescence, deferasirox, daunorubicin.

1. Introduction

Daunorubicin (DNR) is an anthracycline antibiotic with antiblastic and anticancer activity, which is linked by the formation of intercalative complexes with DNA and the inhibition of both DNA and Ribonucleic acid (RNA) synthesis [1]. DNR displays good properties against acute lymphocytic and acute granulocytic leukemias. When it surpasses a certain level, serious side-effects, such as cardiac toxicity, marrow suppression and oral ulcer are found. In the pharmacokinetic studies, the sensitive analytical techniques for the determination of these cytostatic drugs in biological fluids, including DNR, are highly demanded owing to their severe toxic side effects. A variety of methods, such as liquid chromatography with UV detection (LC-UV) [2], Rayleigh scattering [3], laser-induced fluorescent [4,5] or room temperature phosphorescence spectra [6], capillary electrophoresis (CE) [7-10] have been reported for DNR determination in biological fluids and in dosage forms. Electrochemical methods are also attempted [11,12]. The sensitization of the fluorescence of lanthanide ions by organic ligands, especially those of europium and terbium, has been widely employed in various applications, including the investigation of biological systems, immunoassays, DNA and RNA hybridization assays, quantification of organic compounds and chromatographic detections [13,14]. Rare-earth ions have

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luminescence characteristics of narrow spectral width, long luminescence lifetime, and large stocks shift and strong combination. Especially Tb^{3+} and Eu^{3+}, for their resonance energy levels overlap with ultraviolet light, are often used as the fluorescence probes for determining some substances because of the high fluorescence quantum efficiency of their chelates [15,16]. Terbium ions can form fluorescent complexes with several classes of organic compounds. Depending on the type of organic ligand, the formation of terbium complexes may occur either in slightly acidic (pH 5-6) or in strongly alkaline solutions (pH > 12) [13].

The aim of this study was to develop and validate a simple and rapid method for the determination of DNR in samples of urine and serum based on its quenching effect on the fluorescence intensity of Tb^{3+}-DFX complex. It is obvious that simple, cheap, rapid and selective analytical methods are highly in demand in pharmaceutical and biomedical analyses.

2. Experimental

2.1 Materials and Reagents

Analytical grade ethanol, hydrochloric acid (HCl), methanol, tetrahydrofuran, acetonitrile, propanone and tris-(hydroxymethyl) aminomethane (Tris) were obtained from Merck (Darmstadt, Germany), terbium (III) chloride hexahydrate ($TbCl_3.6H_2O$) from Acros Organics (Geel, Belgium), DNR powder were purchased from Behestan Pharmaceutica Company (Tehran, Iran) and DFX powder was donated by Osvah Pharmaceutical Company (Tehran, Iran). Double distilled water prepared by a Millipore-Q-plus water purification system (Bedford, USA) was used in this study. A (10^{-2} M) terbium (III) solution was prepared by dissolving the appropriate amount of terbium (III) chloride hexahydrate ($TbCl_3$ $6H_2O$) in double distilled water and stored in a polyethylene container to avoid memory effects of terbium adsorbed on glass vessels. A stock solution (400 μg mL^{-1}) of DNR was prepared by dissolving the compound in water. A stock solution (1.0×10^{-3} M) of DFX was prepared in ethanol and double distilled water and for experiments freshly diluted in water in order to have less than 2% of ethanol. A 0.1 M Tris-hydrochloric acid (Tris-HCl) buffer solution was prepared by dissolving a desired amount of Tris-base in 100 mL of water, adjusting the pH to 8.25 with HCl.

2.2. Apparatus

Fluorescence spectra and intensity measurements were performed on a Shimadzu RF-5301 PC spectrofluorimeter (Kyoto, Japan) equipped with a 150 W xenon lamp. The pH of solutions was measured with Microprocessor Model 211 pH meter (Romania).

2.3. Experiment procedure

To a 10 mL test tube, the solutions were added according to the following order: 1.0 mL of 4.0×10^{-3} mol L^{-1} Tb^{3+}, 1.0 mL of 6.0×10^{-5} mol L^{-1} DFX solution, 1.0 mL of certain concentration DNR and 1.0 mL of Tris (hydroxymethyl) aminomethane (Tris)-HCl (0.1 mol L^{-1}, pH= 8.25) buffer solution. The mixture was diluted to 10.0 mL with water and allowed to stand for 3 min. Both the excitation and emission slit for all fluorescence measurements were maintained at 10 nm. The fluorescence intensity was measured with a 1-cm quartz cell by an excitation wavelength of 318 nm and an emission wave-length of 545 nm. The decreased fluorescence intensity was represented as $\Delta F = F_0 - F$. Where F and F_0 were the fluorescence intensities of the systems with and without DNR, respectively.

2.4. Sample Preparation

Urine Treatment

Urine (1 mL) was spiked with appropriate amounts of DNR stock solution. Spiked urine was diluted 500-fold with double distilled water. The final DNR concentrations were in the range of (0.02-1.1) mg L^{-1}.

Serum Treatment

Serum (1 mL) was spiked with adequate amounts of DNR stock solution. Spiked serum was diluted 1000-fold with double distilled water to obtain the final

concentrations of (0.02-1.1) mg L^{-1}.

3. Results and Discussion

3.1. Fluorescence Spectra

Fluorescence emission and excitation spectra of Tb^{3+}, Tb^{3+}- DNR, DNR, Tb^{3+}-DFX and Tb^{3+}-DFX-DNR are shown in

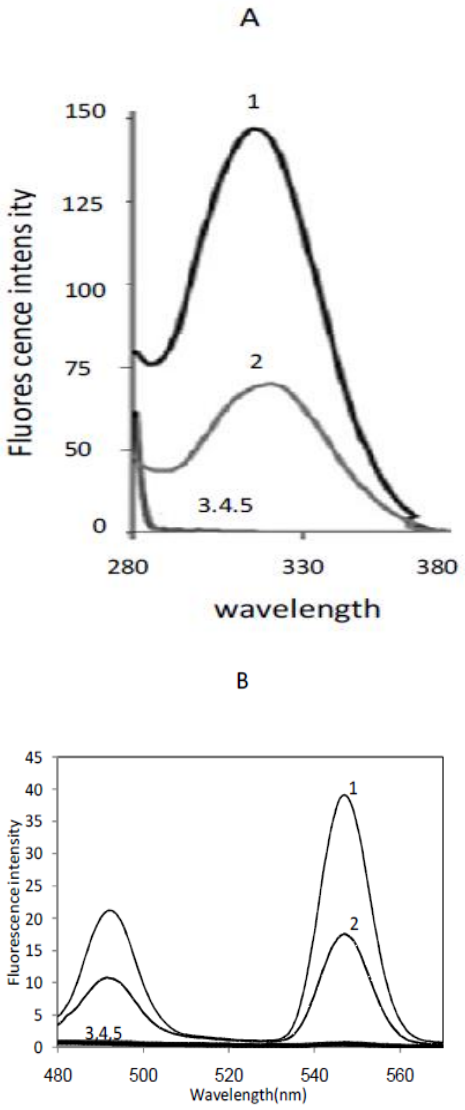

Figure 1. Fluorescence excitation (A) and Emission (B) spectra of Tb^{3+}–DFX (1), Tb^{3+}–DFX–DNR (2). Note that there are no emissions for Tb^{3+}, DNR, Tb^{3+}– DNR(3,4,5).

Fig.1. It was found that free DFX and DNR have no intrinsic fluorescence in aqueous solution. Pure Tb^{3+} does not show the characteristic fluorescence spectrum, while by adding DFX to Tb^{3+} solution, intense fluorescence was observed. The maximal excitation wavelength of Tb^{3+}-DFX occurs at 328 nm, which corresponds to absorption peak of DFX. Under the same conditions, the characteristic peak of Tb^{3+}-DFX was observed, with two emission peaks at 545 and 490 nm, which are the characteristic fluorescence peaks of Tb^{3+} and correspond to the transitions $^5D_4 \rightarrow {}^7F_6$ and $^5D_4 \rightarrow {}^7F_5$, respectively, of which the emission at 545 nm is much stronger. Therefore, the excitation and emission peaks were set at 328 and 545 nm, respectively. The fluorescence spectrum of the Tb^{3+}-DFX- DNR system was similar to that of Tb^{3+}-DFX; however, the fluorescence intensity of Tb^{3+}-DFX was decreased by DNR, and the complementary experiments showed that the decrease was proportional to the concentration of DNR.

3.2. Optimization of Experimental Condition

Effect of pH

Effect of pH on the fluorescence intensity of the system was studied. Fluorescence intensities of series of 0.10 M Tris-HCl buffer solutions with the pH range of 6.5-9.5 were measured at λex/λem= 328 nm/545 nm. As shown in Fig.2, the decreased intensity (ΔF) of Tb^{3+}-DFX complex with DNR is strongly dependent on pH and reaches to a maximum value at pH 8.25. Below this pH, the hydroxyl groups of DFX are probably in protonated form, which disfavours the complex formation. Above this pH, Tb^{3+} ion is probably deposited in the strong alkaline medium, which blocks the coordination between DFX and the Tb^{3+} ion. In addition, the changes of pH influence the compositions and stabilities of the fluorescent complexes and result in changes in the fluorescence characters. So, a suitable pH was very important for the fluorescence characters of the metallic organic complexes. Thus, pH 8.25 (0.1 M Tris buffer) was selected for further research.

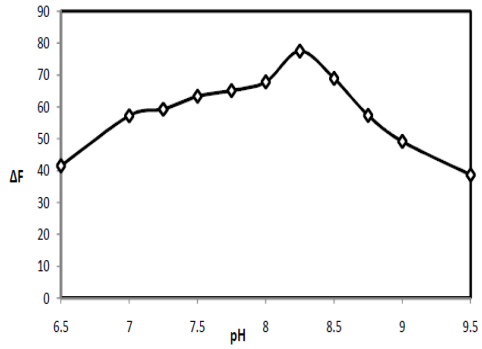

Figure 2. Effect of pH. $[Tb^{3+}] = 5\times10^{-4}$ M, $[DFX] = 3\times10^{-6}$ M, [DNR] = 1 mg L^{-1}, λex/λem = 328/545 nm .

3.2.1. Effect of Tterbium (III) Concentration

Another important parameter, influencing the fluorescence, is the Tb^{3+} concentration. The effect of the Tb^{3+} concentration on the decreased luminescence intensity (ΔF) of Tb^{3+}-DFX- DNR system was studied at a constant concentration of 1.0 mgL^{-1} DNR (Fig.3).The ΔF was the highest when the concentration of Tb^{3+} in the mixture was 4×10^{-4} M and the concentrations less than 4×10^{-4} M could not provide sufficient amount of Tb^{3+} for complex formation. Therefore, the concentration of Tb^{3+} in the mixture was chosen at 4×10^{-4} M for further investigations.

3.2.2. Effect of the Amount of DFX

The influence of the amounts of DFX on the fluorescence intensities was

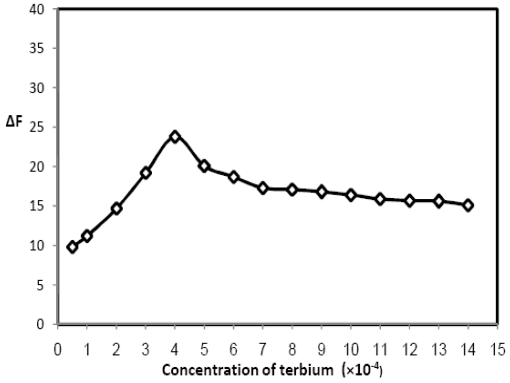

Figure 3. Effect of concentration of Tb^{3+}.
pH=8.25, $[DFX] = 3\times10^{-6}$ M, [DNR] = 1 mg L^{-1},buffer volume =0.5 ml, λex/λem = 328/545 nm .

studied (Fig. 4) and it was found that the quenched fluorescence intensity of Tb^{3+}-DFX- DNR system reached a maximum when the concentration of DFX was 6.0×10^{-6} M. So 6.0×10^{-6} M was used as optimum concentration of DFX for further study.

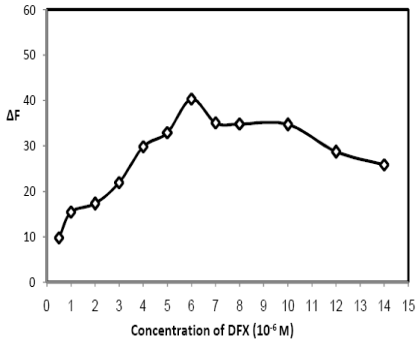

Figure 4. Effect of concentration of DFX. pH=8.25, $[Tb^{3+}] = 4\times10^{-4}$ M, , [DNR] = 1 mg L^{-1}, buffer volume =0.5 ml, λex/λem = 328/545 nm

3.2.3. Effect of Tris Buffer Volume

Tris buffer is known to have chelating properties with lanthanide ions. Hence, it is necessary to optimise its volume that will afford maximum sensitisation of the Tb^{3+}-DFX- DNR system. The influence of Tris buffer volume on luminescence intensity of Tb^{3+} was studied by varying the volume of the buffer in the range of 0.25-5 mL while keeping the concentration of Tb^{3+}, DFX and DNR as constants at 4×10^{-4} M, 6.0×10^{-6} M and 1.0 mg L^{-1}, respectively (Fig. 5). The coordination of Tb^{3+} ions by Tris prevents the OH groups of water molecules surround the terbium ions and reduces the complexation of DFX. In lower concentrations of Tris, the buffer could not coordinate terbium ions completely and the fluorescence intensity is decreased. The results indicated that 1 mL of Tris-HCl buffer solution in 10 mL mixture was the optimum buffer volume.

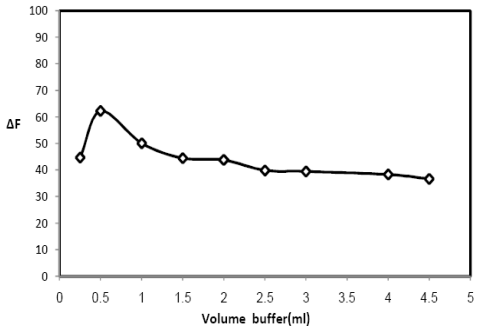

Figure 5. Effect of buffer volume. pH=8.25, $[Tb^{3+}] = 5\times10^{-4}$ M, $[DFX] = 3\times10^{-6}$ M,
[DNR] = 1 mg L^{-1}, λex/λem = 328/545 nm .

3.2.4. *Effect of Reaction Time*

Under the optimum conditions, variation of the fluorescence intensity as a function of time was studied at room temperature. It was found that the fluorescence intensity is stable for about 20 min after addition of all reagents. Hence, all measurements were made between 3 min after all reagents were added in further study.

3.2.5. Effect of the Addition Order of Reagents

Series of solutions were prepared with different addition orders of reagents but the same concentrations of reagents (F), and their corresponding blank solutions (F_0) were measured at λex/λem=328nm/545 nm. The experimental results showed that different orders of addition of

3.2.6. Effect of Organic Solvents

The effect of organic solvents, such as methanol, ethanol, 1-propanol and acetonitrile in the range of 0-60 v/v% was studied (Fig.6).The results indicated that, in the presence of large volumes of different organic solvents, the fluorescence intensity decreased.

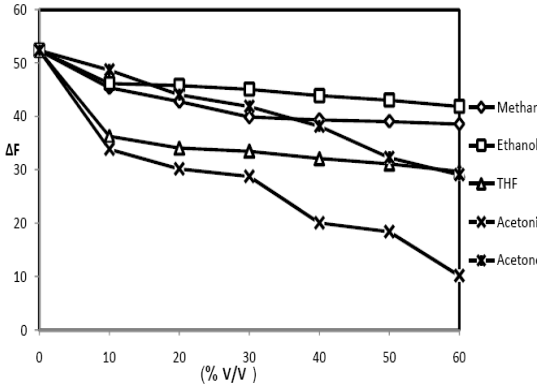

Figure 6. Effect of organic solvents. pH=8.25, $[Tb^{3+}] = 4\times10^{-4}$ M, $[DFX] = 6\times10^{-6}$ M, [DNR] = 1 mg L^{-1}, buffer volume =0.5 ml, λex/λem = 328/545 nm .components have little and insignificant impact on both F and ΔF.

3.3. Interference Studies

The interferences of coexisting substances on the reduced fluorescence intensity (ΔF) were tested under the optimal conditions. The examined concentrations of substances were in the range of their typical amounts in biological samples. The results are shown in Table 1.

Table 1. Effects of common interferents on the determination of 1 (mg L^{-1}) DNR

Coexisting substance (mg L^{-1})	Centration of coexisting substance	(%)ΔF variation
K^+ (Cl)	75.00	3.8
Na^+ (Cl)	120.00	2.3
Cd^{2+} (Cl)	0.40	4.3
Al^{3+} (Cl)	0.40	2.7
Zn^{2+} (Cl)	0.40	3.6
Cu^{2+} (Cl)	0.20	-4.5
Phosphate	0.003	-4.4
Ca^{2+} (Cl)	25.00	4.1
Cr^{3+}(Cl)	0.30	-4.8
Fe^{3+}	0.30	-3.2
Uric acid	0.02	-4.8
L-Leucine	150.00	3.3
Sacarose	12.00	2.1
Glucose	120.00	3.1

Most of the coexisting substances were found to have no influence at their concentrations (normally found) in biological fluids [17,18]. It should be noticed that after 1000 and 500 fold dilution of serum and urine samples respectively, possible interferences were eliminated. Therefore, the effects of these possible interferents could be ignored.

3.4. Calibration Line and Figures of Merit

In order to evaluate the analytical performance of the proposed method, calibration lines were obtained by plotting the decreased fluorescence intensities versus concentration of DNR in pure, urine and plasma samples. Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as $3S_b/m$ and $10S_b/m$ respectively (where S_b is standard deviation of the blank and m is slope of the calibration line). As mentioned before, the serum and urine samples were diluted 1000 and 500 times respectively. The analytical parameters obtained under optimal conditions, are shown in Table 2.

Table 2. Linearity parameters,limit of detection and quantification (μg L^{-1}) of the proposed method.

sample	Data point*	Slope	Y-intercept	r^{2} **	Range(μg L^{-1})	LOD***(μg L^{-1})	LOQ***(μg L^{-1})
standard	12	0.069	2.543	0.998	15-1100	4.3	14.3
Urine	10	0.035	1.443	0.994	20-1100	6.1	20.3
Serum	10	0.064	0.717	0.996	20-1100	5.8	19.3

*Data point is the number of concentrations included in calibration graphs.

** r, regression coefficient of calibration line. Each sample analysis was repeated four times.

*** The detection limit and quantification, calculated as $3S_b/m$ and $10S_b/m$,where S_b and m are the standard deviation (SD) of the blank and the slope of the calibration line, respectively.

Table 3. Precision and Recovery of daunorubicin in biological samples*.

Added (μg L^{-1})	Found (μg L^{-1})	Intra-day RSD(%)	Inter-day RSD(%)	Recovery(%)
		Urine		
0.00	0.00	-	-	-
600	605	2.6	4.7	100.8
800	791	1.6	3.4	98.8
1000	996	1.9	3.2	99.6
		Serum		
0.00	0.00	-	-	-
400	390	1.7	3.1	97.5
600	595	2.1	4.4	99.1
800	810	3.8	4.1	101.2

*Each sample analysis was repeated four times.

3.5. Analysis of Real Samples

In order to validate the method, known quantites of DNR were added into samples of urine and serum and analyzed according to the procedure described in Experimental. The obtained results are shown in Table 3. As shown in Table 3 the recoveries and precisions were 97.3-101.2 % and 1.6-4.7 % respectively. Biological matrices were very similar, therefore, most of the validation data for serum and urine were very close to each other.

Conclusions

In this study, a spectrofluorometric method was developed , validated and applied for the determination of trace amounts of DNR in spiked urine and serum samples. The results illustrated that this method was specific, linear, accurate and precise. These characteristics and the obtained LOD and LOQ values proved the reliability and applicability of the proposed method .In addition to the higher specificity and lower LOD and LOQ values of this method in comparison with the HPLC methods, its simplicity and the elimination of pretreatment could be considered as its main advantages, especially for routine analysis of DNR in biological samples, therapeutic drug monitoring and in pharmacokinetic studies.

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