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ORIGINAL ARTICLE

Spectrofluorimetric Determination of Doxorubicin in Spiked Serum and Urine Samples

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KEYWORDS

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ABSTRACT: A simple spectrofluorimetric method is described for the determination of doxorubicin (DXR) 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 effects of pH, time, order of addition of reagents, concentrations of Tb^{3+} and DFX and the buffer volume on the quenched fluorescence intensity were investigated and optimized. In the optimum conditions, the decrease of the fluorescence intensity of the system showed a good linear relationship with the concentration of DXR in the range of 20-1000 μg L^{-1}, with a correlation coefficient 0.998. The detection limit (3s) was 6.1 μg L^{-1} and the relative standard deviation for four replicate determinations of different concentrations of DXR was in the range of 1.7–4.4%. The procedure was successfully applied to the determination of doxorubicin in urine and serum samples.

INTRODUCTION

Doxorubicin (DXR) (Figure 1) also known as hydroxydaunorubicin, is an anthracycline antibiotic and a drug used in cancer chemotherapy. It works by intercalating DNA and could cause some adverse effects such as life-threatening heart damage. Cardiomyopathies and myelo suppression are associated with the use of doxorubicin at high doses [1-3]. Therefore, the development or improvement of analytical methods for monitoring its level in urine and serum samples is of interest. Several methods have reported the determination of doxorubicin, based on high performance liquid chromatography [4–7], liquid chromatography [8-10], capillary electrophoresis [11, 12], room temperature phosphorescence spectra [13], fluorescence assay [14] and voltammetry detection [15].

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Lanthanide ions complexes are among the most important luminescence probes that have drawn great attention, due to their good luminescence characteristics like narrow spectral width, long luminescence lifetime and large stocks shift. In particular, attentions have been directed towards two rare earth cations, Tb^{3+} and Eu^{3+} [16]. The luminescence quantum yield increases on interaction with an organic ligand. On excitation of the ligand, a nonradiative energy transfer between the triplet level of the ligand and the resonance state of the ion occurs, followed by radiative emission. The emission is observed as sharp multiplet bands due to several electronic transitions [17].

In this study, a simple spectrofluorimetric method for determination of DXR in spiked human serum and urine samples is reported, based on the quenching effect of DXR on the florescence intensity of Tb^{3+}–deferasirox (DFX) complex as a fluorescent probe. The proposed fluorescence probe is inexpensive and the reagents are easily available and environmentally friendly. The Tb^{3+}- DFX complex has good stability and solubility in water and does not require the addition of luminescence enhancers.

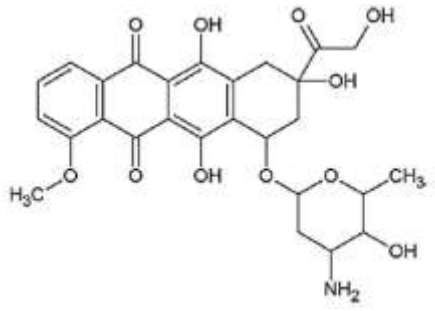

Figure 1. Chemical structure of doxorubicin

MATERIALS AND METHODS

Materials

Analytical grade hydrochloric acid (HCl), methanol, ethanol, 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), DXR powder (pure) and vials (nominal value of 2 mg mL^{-1} DRX) were purchased from Nano Eksire Sina Pharmaceutica Company (Tehran, Iran) and Darou Pars 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 DXR 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 7.8 with HCl.

Apparatus

All intensity measurements and fluorescence spectra scanning 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).

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 DXR and 1.0 mL of Tris (hydroxymethyl) aminomethane (Tris)-HCl (0.1 mol L^{-1} , pH= 7.8) 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 wavelength 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 DXR, respectively.

$$[\text{Te-DFX}] \xrightarrow{hv} [\text{Te-DFX}]^{*} \xrightarrow{DXR} [\text{Te-DFX}]$$

Sample preparation

Urine treatment

A 1 mL of urine sample was spiked with appropriate amounts of DXR stock solution. Spiked urine was diluted 500-fold with double distilled water. The final DXR concentrations were in the range of (0.02-0.9) mg L^{-1}.

Serum treatment

A 1 mL of serum sample was spiked with adequate amounts of DXR stock solution. Spiked serum was diluted 1000-fold with double distilled water to obtain the final concentrations of (0.02-0.9) mg L^{-1}.

RESULTS AND DISCUSSION

Fluorescence spectra

Fluorescence emission and excitation spectra of Tb^{3+}, Tb^{3+}- DXR, DXR, Tb^{3+}-DFX and Tb^{3+}-DFX- DXR are shown in Figure 2. It was found that free DFX and DXR had no intrinsic fluorescence in aqueous solution. Pure Tb^{3+} did 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 occurred at 328 nm, which corresponded 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 was much stronger. Therefore, the excitation and emission peaks were set at 328 and 545 nm, respectively. The fluorescence spectrum of the Tb^{3+}-DFX- DXR system was similar to that of Tb^{3+}-DFX; however, the fluorescence intensity of Tb^{3+}-DFX was decreased by DXR, and the complementary experiments showed that the decrease was proportional to the concentration of DXR.

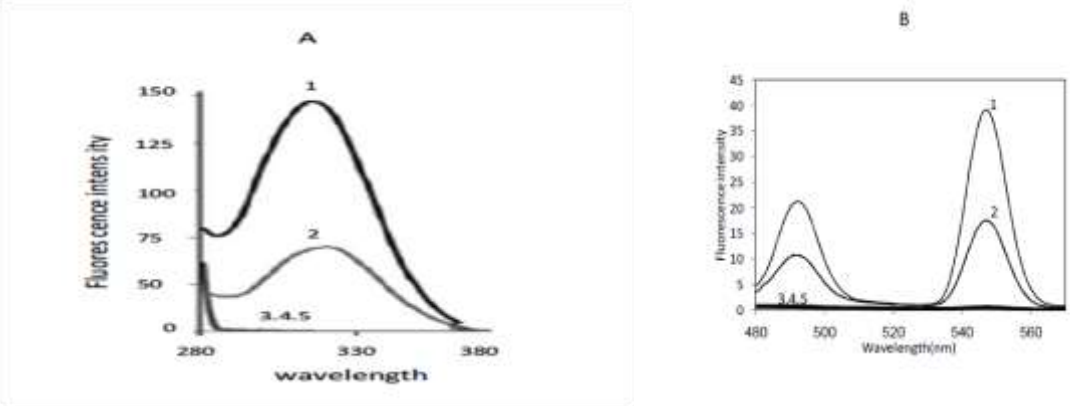

Figure 2. Fluorescence excitation (A) and emission (B) spectra of Tb^{3+}–DFX (1), Tb^{3+}–DFX– DXR (2). Note that there are no emissions for Tb^{3+}, DXR, Tb^{3+}– DXR (3, 4, and 5)

Optimization of experimental conditions

The changes of pH influence the compositions and stabilities of the fluorescent complexes and result in changes in the fluorescence characters. Therefore, a suitable pH is very important for the fluorescence characters of the metallic organic complexes. In order to study the effect of pH, a series of solutions with buffers of different pH values but the same concentrations of other reagents together with corresponding blank solutions were prepared and their fluorescence signals were measured at λex/λem = 328/545 nm. The results indicated that the decreased luminescence intensity (ΔF) of Tb^{3+}– DFX complex was strongly dependent on pH and reached a maximum at 7.8 (Figure 3). Thus, pH 7.8 (0.1 M Tris buffer) was selected for the following experiments.

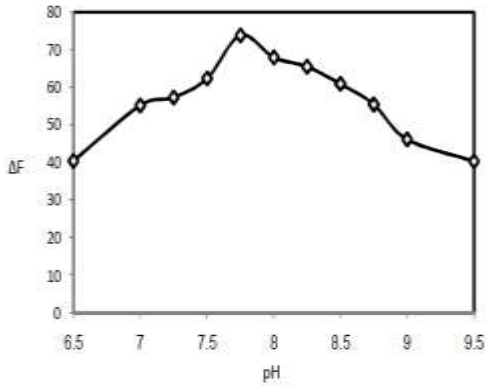

Figure 3. Effect of pH. $[Tb^{3+}] = 5\times10^{-4}$ M, [DFX] = 3×10^{-6} M, [DXR] = 1 mg L^{-1}, λex/λem = 328/545 nm

The concentration of Tb^{3+} is another important parameter, which influences the fluorescence intensity. The effect of the Tb^{3+} concentration on the decreased luminescence intensity (ΔF) of Tb^{3+}-DFX-DXR system was studied, at the constant concentration of 1.0 mg L^{-1}DXR. ΔF was the highest when the concentration of Tb^{3+} in the mixture was 2×10^{-4} M and the concentrations less than 2×10^{-4} M could not provide sufficient amount of Tb^{3+} for complex formation. Therefore, the concentration of Tb^{3+} in the mixture was chosen at2×10^{-4} M for further investigations.

The effect of DFX concentration on the decreased luminescence intensity (ΔF) of the Tb^{3+}-DFX-DXR system was studied. It was found that the quenched fluorescence intensity of the Tb^{3+}-DFX-DXR system reached a maximum when the concentration of DFX was 6.0×10^{-6} M. Therefore, this value was used for further study.

The effect of different buffer volumes on the decreased luminescence intensity (ΔF) of the system was also studied (Figure 4). At lower concentrations of Tris, the OH groups of water molecules surround the terbium ions and act as effective fluorescence quenchers due to OH oscillation, thus the fluorescence intensity is decreased. As the concentration of buffer is increased, Tris ligands can probably prevent Tb^{3+} ions from coordinating water and the fluorescence intensity is increased. It should be noted that, with the increase of the fluorescence intensity, ΔF is also increased. The results indicated that 1 mL of Tris-HCl buffer solution in 5 mL mixture was the optimum buffer volume.

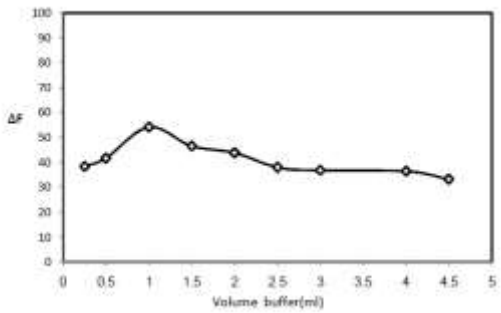

Figure 4. Effect of buffer volume. pH=7.8, $[Tb^{3+}] = 5\times10^{-4}$ M, [DFX] = 3×10^{-6} M, [DXR] = 1 mg L^{-1}, λex/λem = 328/545 nm

Concerning the variations of the fluorescence intensity as a function of time, it was found that the fluorescence intensity is stable for about 20 min after addition of all reagents, because the complex formation reaction between Tb^{3+} and deferasirox is rapid and the complex is stable enough. Hence, all measurements were made

between 3 min after all reagents were added in further study.

The effect of the order of addition was tested. For this purpose, a series of solutions with different addition orders of reagents and their corresponding blank solutions were measured at λex/λem = 328/545 nm. The experimental results showed that different orders of addition of components have little and insignificant impact on both F and ΔF.

The effect of organic solvents, such as methanol, ethanol, 1-propanol and acetonitrile on the fluorescence signal was studied. The results indicated that in the presence of these organic solvents the signal is decreased. However, the signal decreases little at larger volumes of organic solvents compared with lower volumes

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. Most of the coexisting substances were found to have no influence at their concentrations (normally found) in biological fluids [18, 19]. It should be noticed that after the 1000 and 500 fold dilution of all serum and urine samples respectively, possible interferences were eliminated. Therefore, the effects of these possible interferents could be ignored. Although the interference effects are not significant, the small increase or decrease of ΔF by some interfering species may be attributed to their quenching effect on the fluorescence of Te-DFX or inhibiting the quenching effect of DXR respectively.

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

Coexisting substance	Concentration of Coexisting substance (mg L^{-1})	(%)ΔF variation
K^+ (Cl)	100.00	3.7
Na^+ (Cl)	150.00	3.9
Cd^{2+} (Cl)	0.40	4.6
Al^{3+} (Cl)	0.50	3.8
Zn^{2+} (Cl)	0.40	3.6
Cu^{2+} (Cl)	0.40	-2.5
Phosphate	0.005	-3.6
Ca^{2+} (Cl)	50.00	4.7
Cr^{3+}(Cl)	0.20	-3.6
Fe^{3+}	0.20	-4.1
Uric acid	0.01	-3.6
L-Leucine	100.00	4.7
Sacarose	30.00	3.8
Glucose	100.00	3.5

Analytical figures of merit

In order to evaluate the analytical performance of the proposed method calibration graphs were obtained by plotting the decreased fluorescence intensities versus concentration of DXR in pure, urine and plasma samples. 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	0.070	2.459	0.999	20-1000	6.1	20.4
Urine	10	0.036	1.559	0.996	20-900	6.6	22.2
Serum	10	0.059	1.834	0.992	20-900	5.8	20.4

*Data point is the number of concentrations included in calibration graphs.
** r, regression coefficient of calibration graph. Each sample analysis was repeated four times.
*** The detection limit and quantification, calculated as 3Sb/m and 10Sb/m, where Sb and m are the standard deviation (SD) of the blank and the slope of the calibration graph, respectively.

Analysis of real samples

In order to validate the method, known quantities of DXR were added into samples of urine and serum and analyzed according to the procedure described in experimental section. The obtained results are shown in Table 3. Accordingly, the recoveries and precisions were 97.5-101.6 % and 1.7-4.4 % respectively. Biological matrices were very similar; therefore, most of the validation data for serum and urine were very close to each other.

The developed method was also applied to the determination DXR in injection samples. To perform an assay on DXR in parenteral solution samples, the samples were diluted appropriately within the linear range of the determination of DXR and analyzed by the method developed above. DXR vial contains 2 mg mL^{-1} DXR and the concentration of the samples determined by the proposed method was 1.8 ± 1.0 mg mL^{-1} with the RSD of 1.9% which is in agreement with the label claim.

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

	Added (μg L^{-1})	Found (μg L^{-1})	Intra-day RSD(%)	Inter-day RSD(%)	Recovery(%)
	0.00	0.00	-	-	
Urine	**300**	305	1.7	4.1	99.6
	600	594	2.5	4.4	99.0
	900	897	1.8	3.6	101.6
	0.00	0.00	-	-	-
serum	**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 five times

CONCLUSIONS

In this study, a spectrofluorometric method was developed, validated and applied for the determination of trace amounts of DXR in 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 DXR in biological samples, in therapeutic drug monitoring and in pharmacokinetic studies.

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