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Europium-Enoxacin Complex as Fluorescence Probe for the Determination of Folic Acid in Pharmaceutical and Biological Samples

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A simple, rapid and sensitive spectrofluorometric method was developed for the determination of folic acid (FA), based on its quenching effect on the fluorescence intensity of enoxacin (ENX)-europium (Eu^{3+}) complex as a fluorescent probe. Fluorometric interaction between ENX-Eu^{3+} complex and FA was studied using UV-visible and fluorescence spectroscopy. The quenched fluorescence intensity at an emission wavelength of 614 nm was proportional to the concentration of FA. Optimum conditions for the determination of FA were investigated. Under optimal conditions, the reduced fluorescence intensity at 614 nm was responded linearly with the concentration of FA. The linearity was maintained in the range of 1.25×10^{-9} to 1.50×10^{-7} M (R = 0.9986) with the limit of detection ($3S_b/m$) (where S_b is the standard deviation of blank and m is the slop of linear calibration curve) of 6.94×10^{-10} M. The relative standard deviation (RSD) for 9 repeated measurements of 1.0×10^{-9} M FA was 1.42%. This method was simple, cost effective, and relatively free of interference from coexisting substances. Successful determinations of FA in pharmaceutical formulation and biological samples with the developed method were demonstrated.

Key Words : Fluorescence, Europium, Enoxacin, Serum

Introduction

Folic acid (FA) is a yellow crystal and chemically made up of a bicyclic pterin linked by a methylene bridge (C9-N10) to *para*-aminobenzoic acid (pABA), which is joined by peptide linkage to a single molecule of L-glutamic acid, also called pteroylglutamic acid (PGA) (Fig. 1). It is also known as folate which is found in some enriched foods and vitamin pills. Folates are an important class of water-soluble B-vitamins that are essential for normal human cell division and cell growth. A deficiency of folate in the diet is closely linked to the presence of neural tube defects in newborns and to an increased risk of megaloblastic anemia, cancer, Alzheimer's disease and cardiovascular disease in adults.[1,2] It is produced by plants (green leaves, algae) and micro organisms (bacteria, yeast). It can promote the formation of red blood cells, and is believed to be a blood tonic, initially identified as an anti-anemia and growth factor. FA is also one of the important components of the haemopoietic system, being the coenzyme that controls the generation of ferrohaeme. A lack of FA gives rise to gigantocytic anemia, associated with leukopenia, devolution of mentality, psychosis, *etc.* Folate deficiency is believed to be the most common vitamin deficiency in the world due to food processing, food selection, and intestinal disorders.[3] Therefore, a lot of researches have been done for the development of novel analytical procedures for the determination of FA present in tablets and biological samples.

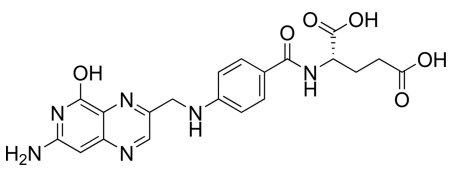

Figure 1. Molecular structure of folic acid.

In recent years, several methods for the determination of FA have been reported, including high-performance liquid chromatography (HPLC),[4-9] chemiluminescence (CL),[10-12] spectrophotometric,[13,14] electroanalytical,[15-17] and fluorometric methods.[18,19] However, HPLC technique has superiority in terms of separating and quantifying different forms of FA with the minimum interference from enzymes but it involves complicated system operation and maintenance, high consumption of samples and expensive reagents. Electroanalytical techniques also suffer from complex setup. spectrophotometric techniques undergo from disadvantages such as heating or extraction, long time for the reaction to be completed to form colour product and narrow range of determination. On this point of view spectrofluorometric technique involves inexpensive instrumentation, easy setup as well as higher sensitivity. Fluorescence quenching of emissive species by analyte is a great tool for the determination of target analyte. Some quenching methods involving ligand-sensitized fluorescence lanthanide complex were reported to form ternary complex with FA which caused the quenching of enhanced emission generated by the complex solution.[20,21] In recent years, the use of fluorescent lanthanide complexes is an important approach because of their long luminescence life times, large Stokes shifts, and narrow emission bands of lanthanide ions. Luminescence of lanth-

anide ions such as europium originates from forbidden 4f-electron transitions, which is too weak to be observed due to low absorption coefficients.[22-25]

The problem due to low molar absorptivity was overcome by employing the technique of ligand-sensitized luminescence. Therefore, the weakly fluorescent europium (Eu^{3+}) ion is complexed with a ligand, enoxacin (ENX) that is capable of forming strong complexes with the metal ions because it contains an active group in the form of -COOH and C=O in the structure. Upon excitation, some of the excited energy is then transferred to the lanthanide ion by intramolecular energy transfer. It was reported that terbium and europium ion can form complexes with similar antibacterial agents such as norfloxacine, nalidixic acid, ciprofloxacine, gatifloxacine, and moxifloxacin.[26-29] In our previous study we have successfully determined adenosine disodium triphosphate (ATP) using Eu^{3+}-ENX complex as a fluorescence probe.[30]

In this study, it was observed that FA causes the fluorescence quenching of Eu^{3+}-ENX complex in alkaline media and the quenching was related with the concentration of FA. Therefore, fluorescence emission quenching of lanthanide complex by FA was used for its quantitative determination. The influences of involved reagents and other reaction conditions were to be systematically studied and optimized to utilize the quenching process for analysis of FA. Critical analytical performance parameters such as measurement linearity, detection limits, and repeatability should be assessed under the finally optimized conditions. If such parameters turned out to be satisfactory, the feasibility of applications to real samples such as biological and pharmaceutical formulation was to be tested.

Experimental

Reagents. Europium chloride hexahydrated, enoxacin (ENX), folic Acid (FA), and tris (hydroxymethyl) amino methane were obtained from Sigma-Aldrich (USA). Other reagents used for this study were of analytical grade in purity from a standard supplier. Double deionized (DI) water was used throughout the work. The primary standard stock solutions of europium chloride hexahydrated (1.0×10^{-2} M) were prepared by directly dissolving 0.183 g of the salt in 50 mL DI water. A stock ENX solution (1.0×10^{-2} M) was prepared by dissolving 0.16 g in 0.1 M NaOH and then was diluted up to mark using DI water in a 50 mL volumetric flask. An appropriate amount of FA was directly dissolved in DI water to prepare 1.0×10^{-3} M primary standard solutions. Tris-HCl buffer was prepared by dissolving an appropriate amount of tris (hydroxymethyl) amino methane in DI water, and its pH was adjusted using 0.1 M HCl to 8.0. The prepared stock solutions were stored in a refrigerator at 4 °C. Working solutions were prepared daily before use by making required dilutions of the stock solutions.

Apparatus. Fluorescence measurements were conducted with a spectrofluorometer (F-4500; Hitachi, Japan) equipped with a 150 W xenon lamp and a photomultiplier tube (R 928; Hamamatsu, Japan). A pH meter (520A; Orion, USA) was used for pH adjustment. UV-visible spectra were scanned by a UV-visible spectrophotometer (UV-1800; Shimadzu, Japan).

Procedure. The following procedure was adapted to measure fluorescence spectra. A certain volume of europium chloride, buffer solution, and ENX solution were added to a 10 mL volumetric flask. The mixture was diluted with 5 mL double DI water and allowed to stand for several minute and then a certain volume of FA-solution were mixed with it. The emission and excitation spectra of Eu^{3+}, Eu^{3+}-ENX complex and Eu^{3+}-ENX-FA were measured in a 1 cm quartz cell with the excitation and emission wavelengths of 395 nm and 614 nm respectively. Both excitation and emission slits for luminescence measurement were set to 10 nm. High voltage for the photomultiplier tube was set to 950 V. The quenching of fluorescence emission intensity of Eu^{3+}-ENX complex by conjugation of FA was represented as $\Delta F = F_0 - F$, where F and F_0 are the emission intensities of the system with and without FA respectively.

Sample Preparations. Two commercial brands (Folin and Folda) of tablet containing FA were collected from local pharmacy. Ten tablets of each brand were pulverized into homogenized powder and 10 mg equivalent powder was transferred into a beaker. The powder was dissolved in methanol. The solution was filtered through a filter millex (0.22 μm pore size; Millipore, USA) to isolate the insoluble excipients and was washed with DI water five times. Finally, samples were diluted appropriately with DI water to meet the analyte concentration within the range of the calibration curve. Two samples, 1.0 mL of human serum and 1.0 mL of urine, were collected from a healthy person who offered the samples at the attaching hospital of our school. The serum sample was mixed with 5 mL of 10% trichloroacetic acid in a centrifuge tube for deproteinization. The mixture was left in a refrigerator overnight and centrifuged for 15 min at 8000 rpm, before 0.1 mL of the serum sample was spiked with a known amount of the standard FA solutions separately.

Results and Discussion

Absorption and Fluorescence Spectra. The absorption spectra of Eu^{3+}, ENX, FA, Eu^{3+}-ENX, Eu^{3+}-FA, ENX-FA and Eu^{3+}-ENX-FA are shown in Figure 2. The absorption maxima of Eu^{3+} and FA were recorded at 245 nm (Fig. 2(a)), and 280 nm (Fig. 2(c)) respectively. There were three absorption peaks exhibited by ENX at 220 nm, 263 nm and 340 nm (Fig. 2(b)). Addition of Eu^{3+} into an ENX solution caused the shifting of absorption maximum of ENX from 263 nm to 266 nm along with weakened absorbance intensity (Fig. 2(d)). The absorption peak of ENX at 220 nm disappeared. Thus the changes indicate the formation of Eu^{3+}-ENX complex. As observed in Figure 2(e), absorbance maxima of FA at 280 nm was not shifted upon addition of Eu^{3+} which indicated that there was no interaction only between Eu^{3+} and FA. After addition of FA into the Eu^{3+}-ENX complex, slight red shifting of the absorption maximum of ENX from 263 nm to 265 nm was occurred (Fig.

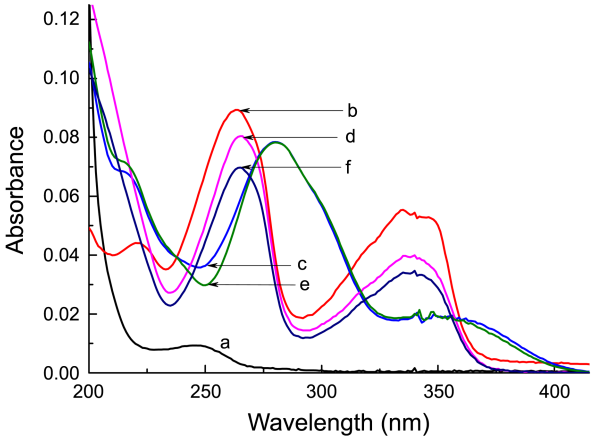

Figure 2. Absorbance spectra of (a) Eu^{3+}, (b) ENX, (c) FA, (d) Eu^{3+}-ENX, (e) Eu^{3+}-FA, and (f) ENX-Eu^{3+}-FA. Conditions: [Eu^{3+}], 2.5×10^{-6} M; [FA], 3.0×10^{-6} M; [ENX], 1.5×10^{-6} M.

2(f)). In this case, absorption intensity was lower than that of the Eu^{3+}-ENX complex. Therefore, the shifting and lowering of absorption maxima indicated the targeted effect of the formation of Eu^{3+}-ENX-FA ternary complexes.

Excitation and emission spectra of Eu^{3+}, Eu^{3+}-ENX, and Eu^{3+}-ENX -FA at pH 8.0 are shown in Figure 3. The excitation spectra of Eu^{3+} was very weak (Fig. 3A(I)) in aqueous medium but it was strongly enhanced after addition of ENX (3A(II)). A blue-shift occurred from 395 nm to 274 nm. But incorporation of FA into the Eu^{3+}-ENX complex caused a noticeable decrease of intensity (Fig. 3A(III)), with blue-shift (to 370 nm) which was obviously resulted from the interaction between FA and the Eu^{3+}-ENX complex. In the case of emission, weak luminescence peaks at 589 nm and 614 nm were observed when Eu^{3+} was excited at the wavelength of 395 nm,[29] which were corresponding to $^5D_0$-$^7F_1$ and $^5D_0$-$^7F_2$ transition of Eu^{3+} (Fig. 3B(i)), respectively. Incorporation of ENX into Eu^{3+} in presence of the buffer solution preferentially enhanced the emission peak 614 nm sharply (Fig. 3B(ii)). This is because of intra-molecular energy transfer from the triplet state of excited organic ligand, ENX to the resonance energy level of the Eu^{3+} at room temperature.[31,32] The rate of energy transfer depends upon the extent of overlap of the emission spectra of the donor with the excitation spectra of the accepter as well as the distance between them. Energy transfer easily occurs between Eu^{3+} and ENX because of the strong spectral overlap between the emission spectra of donor (ENX) and the excitation spectra of accepter (Eu^{3+}).[30]

The most intense peak of Eu^{3+}-ENX complex is located at 614 nm, thus this emission peak was chosen for spectral measurements throughout this work. When FA was added to the Eu^{3+}-ENX complex, luminescence intensity at 614 nm (Fig. 3B(iii)) was remarkably reduced, which apprises that there is a meaningful interaction between Eu^{3+}-ENX and FA. We propose that a stable ternary complex is formed by the interaction between Eu^{3+}-ENX ground state complex and FA. Subsequent experiments showed, the degree of decreasing of luminescence intensity was linearly related with the concentration of FA.

Optimization of the Reaction Conditions. In this work, the quenching of fluorescence emission intensity of Eu^{3+}-ENX complex by FA was investigated for the determination of FA. Thus, optimization of the reaction conditions is necessary to plot the calibration curve. Therefore, the required parameters were optimized for maximum decrease of emission intensity (ΔF) exhibited by Eu^{3+}-ENX complex at 614 nm.

The effect of pH on both the formation of Eu^{3+}-ENX complex and the binding capability of FA with Eu^{3+}-ENX complex could be substantial because it may affect the quantum yield of the fluorescent complex. The emission intensity of Eu^{3+}-ENX complex was significantly enhanced by increasing the pH from 6.0 to 8.0 and started to decline slowly above pH 8.0. The fluorescence intensity of Eu^{3+}-ENX system was examined at pH 8.0 using tris buffer. Experiments indicated that the chemical nature of the buffer has also considerable effects on the quenched fluorescence intensity. The results showed that 1 mL of Tris-HCl (0.05 mol/L, pH = 8.0) in a final 10 mL was the most suitable

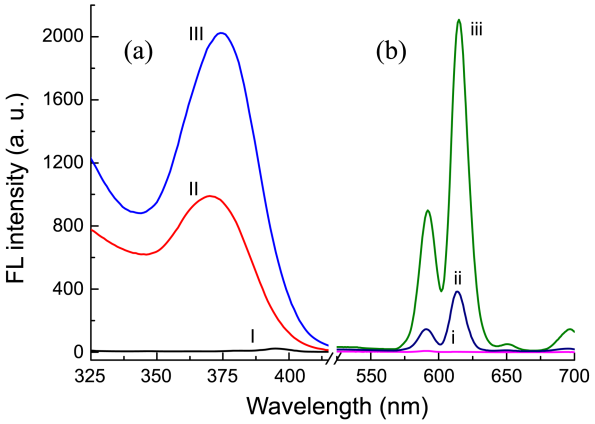

Figure 3. Excitation (A) and emission (B) spectra of Eu^{3+} (I and i), Eu^{3+}-ENX (II and ii) and Eu^{3+}-ENX-FA (III and iii). Conditions: [Eu^{3+}], 2.5×10^{-5} M; [FA], 2.5×10^{-6} M; [ENX], 4.0×10^{-4} M; Tris-HCL buffer; pH 7.5; $\lambda_{ex}/\lambda_{em}$ = 395/614 nm.

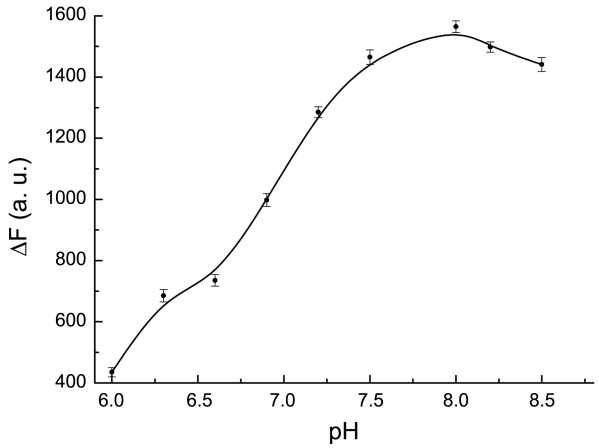

Figure 4. Influence of pH on ΔF. Conditions: [Eu^{3+}], 2.5×10^{-5} M; [ENX], 4.0×10^{-4} M; [FA], 2.5×10^{-6} M; Tris-HCL buffer; pH 6.0-8.5; $\lambda_{ex}/\lambda_{em}$ = 395/614 nm.

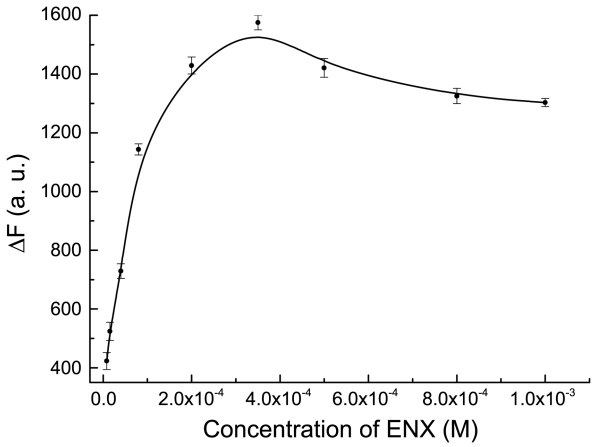

Figure 5. Influence of concentration of ENX on ΔF. Conditions: [Eu^{3+}], 2.5×10^{-5} M; [FA], 2.5×10^{-6} M; [ENX], 8.0×10^{-6} to 1.0×10^{-3} M; Tris-HCL buffer; pH 7.5; $\lambda_{ex}/\lambda_{em}$ = 395/614 nm.

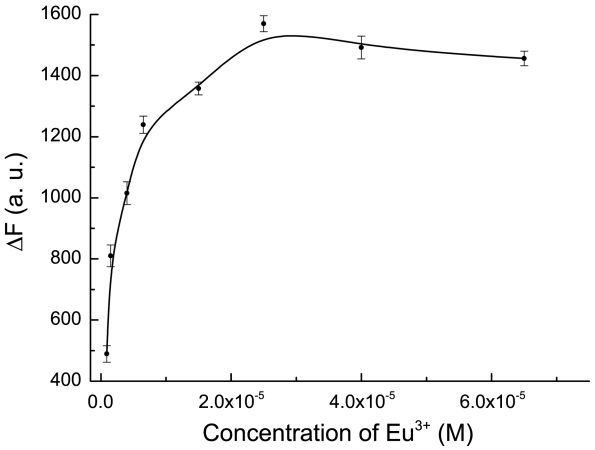

Figure 6. Influence of concentration of Eu^{3+} on ΔF. Conditions: [Eu^{3+}], 9.0×10^{-7} to 6.5×10^{-5} M; [ENX], 4.0×10^{-4} M; [FA], 2.5×10^{-6} M; Tris-HCL buffer; pH 7.5; $\lambda_{ex}/\lambda_{em}$ = 395/614 nm.

concentration. In presence of FA, maximum ΔF intensity was also achieved at pH 8.0 as shown in Figure 4. Therefore, pH 8.0 was selected as the optimum pH.

ENX concentration has an important effect on fluorescence emission intensity of Eu^{3+}-ENX complex. In the tested range of 7.5×10^{-6} - 1.0×10^{-3} M of ENX, the maximum intensity was observed at 4.0×10^{-4} M ENX with good reproducibility and above which the luminescence intensity began to decline. The maximum formation of the complexes might be reached at this concentration, and the excess of ENX would merely absorb or quench the luminescence. The maximum ΔF intensity after introducing FA in the complex was achieved at the same concentration of ENX (Fig. 5). Thus 4.0×10^{-4} M of ENX was determined as the optimum concentration for Eu^{3+}-ENX-FA system.

The influence of Eu^{3+} concentration on the maximum ΔF intensity was also investigated in the range of 4.0×10^{-5} – 1.0×10^{-3} M. Results showed, in Figure 6 that ΔF reached maximum at 2.5×10^{-4} M. Therefore, Eu^{3+} concentration was maintained at 2.5×10^{-4} M for the following experiments.

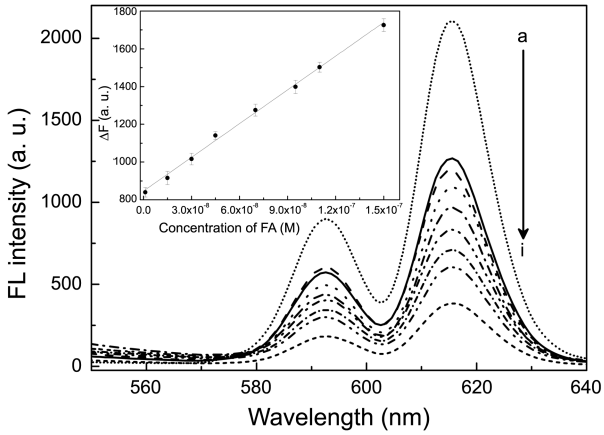

Figure 7. Fluorescence quenching of Eu^{3+}-ENX probe upon addition of increasing amount of FA; (a) 0 M, (b) 1.2×10^{-9} M. (c) 1.5×10^{-8} M, (d) 3.0×10^{-8} M, (e) 4.5×10^{-8} M, (f) 7.0×10^{-8} M, (g) 9.5×10^{-8} M, (h) 1.1×10^{-7} M, (i) 1.5×10^{-7} M. Inset: Calibration plot of concentration of FA against F intensity. Conditions: [Eu^{3+}], 2.5×10^{-5} M; [ENX], 4.0×10^{-4} M; Tris-HCL buffer; pH 7.5; $\lambda_{ex}/\lambda_{em}$ = 395/614 nm.

Possible influence of reagent-mixing order on the ΔF intensity of Eu^{3+}-ENX-FA was also investigated, and the maximum ΔF was observed at the following mixing order: Solution of Eu^{3+}, ENX, tris buffer, and FA. The effect of time on the fluorescence intensity was studied under the optimum conditions. The ΔF of the system was observed maximum 10 min after the mixing of all reagents, and the intensity was stable for approximately 2 hours. Thus, measurements of fluorescence emission intensity were made 10 min after mixing of the reagents at $\lambda_{ex}/\lambda_{em}$ = 395/614 nm.

Analytical Figures of Merit. The linear range for the determination of FA was investigated under aforementioned optimal conditions, when the standard deviation for a series of six reagent blanks was 1.38%. The emission quenching of Eu^{3+}-ENX complex by the conjugation of FA was found regular at 614 nm with the concentration of FA (Fig. 7). Therefore, the measured ΔF of Eu^{3+}-ENX-FA system was found to be responded linearly with the concentration of FA (Inset, Fig. 7) in the range of 1.25×10^{-9} to 1.50×10^{-7} M with a correlation coefficient of 0.9986. The regression equation for standard solutions was $\Delta F = 841.63 + 5.96 \times 10^9$ C in which C is in M. The limit of detection as defined by IUPAC, $C_{LOD} = 3S_b/m$ (where S_b is the standard deviation of the blank signals and m is the slope of the calibration curve) was calculated to be 6.94×10^{-10} M. The precision of the method was verified by calculating the relative standard deviation (RSD) for 9 repeated measurements of 1.0×10^{-9} M of FA which was found to be 1.42%. The analytical characteristics of the developed method such as limit of detection and linear range was compared with most of the previously reported spectrofluorimetric and other method in Table S1 (Supplementary Information). The developed method shows relatively better sensitivity and lower limit of detection comparing to other spectrofluorometric method.

Interferences. The effects of some potentially interfering

ions that can coexist in tablet, urine and vegetables samples were studied. They can affect the analytical performance as interferents by suppressing or enhancing the emission intensity of the investigated system. Interfering effect was therefore investigated by preparing a set of solutions in which 1.0×10^{-7} M each FA and a potentially interfering chemical coexisted. The tested chemical was considered as non-interfering component when the deviation of ΔF of the proposed system is less than 5%. The degrees of interferences of various chemicals are summarized in Table 1. The presence of amino acids in urine samples can affect the luminescence spectra from the emission of Eu^{3+}-ENX-FA system. Certain amino acids such as glycine, tryptophan, phenylalanine, tyrosine, and leucine are reported to be coordinated with lanthanide ions through the carboxylic acid group and capable of enhancing europium emission.[34] Therefore, the interfering effect of amino acids on the luminescence intensity of the systems was explored. The concentration of investigated species present in real samples affects the system luminescence intensity by less than 5%. The obtained tolerance level has been considered the maximum acceptable concentration of the foreign species. Therefore, the existence of the interferents could be ignored up to the tolerable concentration in the present study.

Table 1. Effect of Interfering substances

Interfering substances	Change in ΔF (%)	Tolerable concentration (M)
Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}	3.5	3.5×10^{-5}
K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}	3.0	3.0×10^{-5}
phosphate	−3.5	2.5×10^{-6}
thymine	−2.5	5.5×10^{-5}
adenine	−3.5	6.0×10^{-5}
tryptophane	−3.7	4.5×10^{-5}
ascorbic acid	−5.2	4.0×10^{-5}
L-alanine	−3.5	3.5×10^{-5}
L-glutamic	−4.8	4.0×10^{-4}
L-glutamic acid	−2.9	5.0×10^{-4}
glucose	+4.2	6.0×10^{-5}
sorbitol	+3.8	5.5×10^{-5}
manitol	+4.2	4.5×10^{-5}
uric acid	−4.5	5.5×10^{-5}
ascorbic acid	2.5	4.5×10^{-4}
epinephrine, norepinephrine, dopamine	+3.5	3.5×10^{-5}

Application to Real Samples. The method was applied to determine FA in pharmaceutical, serum, and urine samples to examine its validity for real sample analysis. The results obtained for determining FA in the tablet samples using the linear calibration curve are summarized in Table 2. There was no significant difference between the labeled content and that obtained by the presented method. Recovery studies were also performed on the analyzed tablet samples by standard addition method. Recoveries were observed in the range of 95-98.2% and 96.3-101% for Folin and Folda, respectively.

Known amounts of FA in urine and serum samples were also recovered by standard addition method. The added concentration of FA was determined by the method. The recoveries were in the range of 98.5-99.7% for serum samples. The urine samples did not require any further pretreatment except proper dilution in order to bring the FA concentrations within the working range. The recovery

Table 2. Determination of FA in pharmaceutical samples and results for recovery test

Sample	Amount Found mg ± RSD[a] (%)		Standard Addition Method		
	Labeled (mg)	Proposed method	Added ($\times 10^{-7}$ M)	Found ($\times 10^{-7}$ M) ± RSD[a] (%)	Recovery (%)
Folin	5	4.98 ± 2.25	1.0	0.95 ± 2.75	95
			3.0	2.94 ± 1.56	98
			5.0	4.91 ± 2.05	98.2
Folda	1	0.96 ± 1.98	1.0	1.01 ± 1.36	101
			3.0	2.89 ± 2.19	96.3
			5.0	5.02 ± 1.23	100.4

[a]Relative standard deviation for six replicate measurements (n = 6)

Table 3. Recovery of FA in serum and urine samples

	Standard addition method					
	Serum			Urine		
Samples	Added ($\times 10^{-8}$ M)	Observed ($\times 10^{-8}$ M) ± RSD[c] (%)	Recovery (%)	Added ($\times 10^{-8}$ M)	Observed ($\times 10^{-8}$ M) ± RSD[c] (%)	Recovery (%)
FA	2.00	1.97 ± 2.23	98.5	2.00	2.02 ± 2.54	101
	4.00	3.95 ± 1.89	98.7	4.00	3.94 ± 1.59	98.5
	6.00	5.98 ± 2.14	99.7	6.00	6.05 ± 1.98	100.8
	8.00	7.97 ± 1.75	99.6	8.00	7.95 ± 2.36	99.3

[c]Relative standard deviation for three replicate measurements.

ranges were from 98.5-100.8% in the urine samples. The results are summarized in Table 3.

In conclusions, a new spectrofluorometric method is developed for the determination of trace amount of FA. The reduction of emission intensity at the wavelength of 614 nm of Eu^{3+}-ENX fluorescence probe after conjugation of FA was the basis of this technique. The method has good stability, sensitivity, selectivity, precision, wider linear range, and very low limit of detection which allowed application in determination of FA in tablet, serum, and urine samples analysis.

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