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A sequential injection method for the determination of piroxicam in pharmaceutical formulations using europium sensitized fluorescence

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Abstract

A simple, selective and sensitive luminescence method for the assay of piroxicam (PX) in pharmaceutical formulation is developed. The method is based on the luminescence sensitization of europium (Eu^{3+}) by complexation with PX. The signal for PX–EU is monitored at $\lambda_{ex} = 358$ nm and $\lambda_{em} = 615$ nm. Optimum conditions for the formation of the complex in methanol were 0.01 M TRIS buffer and 0.2 mM of Eu^{3+} which allows the determination of 100–2000 ppb of pX in batch method and 100–1000 ppb with limit of detection (LOD) = 23.0 ppb using sequential injection analysis (SIA). The relative standard deviations of the method range between 2 and 3% indicating excellent reproducibility of the method. The proposed method was successfully applied for he assay of PX in pharmaceutical formulations (Feldene capsules and tablets). Average recoveries of 101.0 ± 0.3 and $98.8 \pm 2.7\%$ were obtained for capsules in methanol using batch and sequential injection (SI) methods, respectively.

Keywords: Sensitized fluorescence; Europium; Piroxicam; Sequential injection

1. Introduction

Piroxicam (PX) (4-hydroxy-2-methyl-*N*-2-pridyl-2H-1, 2-benzothiazine-3-carboxadiamide-1,1-dioxide) belongs to a class of non-steroidal pharmaceuticals with anti-inflammatory properties which are used to treat rheumatoid, arthritis and post operative inflammations. PX belongs to the oxicam group a class of enolic acids. Many diverse properties have been exhibited by oxicam group of drugs such as chemoprevention, chemosupression [1–3] and UV induced photosensitization of the skin [4]. The chemoprevention effect of PX is attributed to its property of reducing levels of prostaglandins which are associated with carcinogenesis in the colon [5]. However, the inhibition effect of PX on prostaglandin E_2 [PGE_2] synthesis is responsible for the ulcerogenic action of PX on the gastrointestinal tract [6].

Several methods have been described for the determination of PX in pharmaceutical formulations and biological fluids [7–10]. Recently, the use of a fluorescence technique for the quantification of PX in acidic medium and in the presence of cyclodextrin is reported [11]. A direct spectrofluorimetric method for the determination of piroxicam and pyridoxine in pharmaceutical formulation was reported [12]. The method required solid phase extraction of PX, and its determination on the solid surface. Good selectivity was obtained for the method. However, the method involved a pre-separation step which may be cumbersome. Escandar et al. [13] reported a direct method for the determination of PX in serums.

Spectrofluorimetic methods are highly sensitive and selective, and thus, well suited for the determination of trace amounts of drugs in biological materials and in some dosage forms. Fluorescence based assay is the method of choice in many unit dose analysis required by FDA. The simplicity, versatility, and economy of fluorescence techniques are desirable when higher levels of drugs are encountered.

The weakly fluorescent nature of PX limits its determination by direct spectrofluorometric measurements. PX has a β-diketone group and is known to have a chelating property with many metals such as copper, lead, cadmium, aluminium, and iron (III) [14,15]. On the other hand, tervalence lanthanide ions are known to have a very weak

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fluorescence because the absorption of these ions is very low. However, when these ions are chelated to organic ligands such as β-diketones their fluorescence can be dramatically enhanced [16–18]. The widely accepted explanation for this enhancement is that the excitation light is absorbed and collected by the organic ligand, which serves as an "*antenna chromophore*". This is followed by an internal transfer of the collected energy to the encapsulated lanthanide ion which is then emitted as the line spectrum of the lanthanide ion. The result of this process is a profound fluorescence enhancement of the lanthande emission due to the energy transfer. The efficiency of the energy transfer is governed by the nature of the ion, the ligand, the ligand ion bond, and the solvent. The energy gap between the excited and the ground state levels of the lanthanide ion and the rigidity of molecular structure also contributes to the enhancement of the luminescence intensity of the system [18].

In this work we report a simple, robust sequential injection (SI) method for the determination of PX in pharmaceutical formulation. The method uses the increase in sensitivity brought about by the fluorescence sensitization and enhancement of europium by PX and the merits of automation and optimal consumptions of reagents that characterize SI techniques. The luminescent properties of the europium–PX chelates were investigated in methanol. Factors affecting the enhancement of the fluorescence such as concentration of TRIS buffer and concentration of Eu^{3+} have been carefully studied. The method was then used to determine the concentration of PX in tablets and capsules.

2. Experimental

2.1. Apparatus

The SI system, FIAlab3500 (FIAlab Instruments, http://ww.flowinjection.com) used in this study consisted of the following components (Fig. 1): syringe pump (2.5 ml, Sunnyvale, CA), 200 cm holding coil (0.73 mm i.d. Teflon tubing, Upchurch Scientific, Oak Harber, USA), multiposition-value (eight ports, Valco, Houston, USA), 45 cm reaction coil (RC) (0.8 mm i.d. Teflon tubing, Upchurch Scientific, Oak Harber, USA), and a Hellma (Type 176.753-QS) cell. Aminco Bowman Series-2 Luminescence Spectrometer (SLM Instruments, NY, USA) was used for fluorescence measurements. A personal computer was used for fluid control using FIAlab for windows software (FIAlab Instruments, http://www.flowinjection.com). The SI-integrated fluidic system is connected to the computer via an RS-232C interface. The spectrofluorimeter and the data collection and evaluation were under the control of software using OS2-operating system (SLM Instruments, NY, USA).

Absorption spectra were recorded on a Varian 50 Con UV-Visible spectrometer with 1 cm matched quartz cell. The pH values were measured on a Hanna (Romania) HI 8314-membrane pH meter.

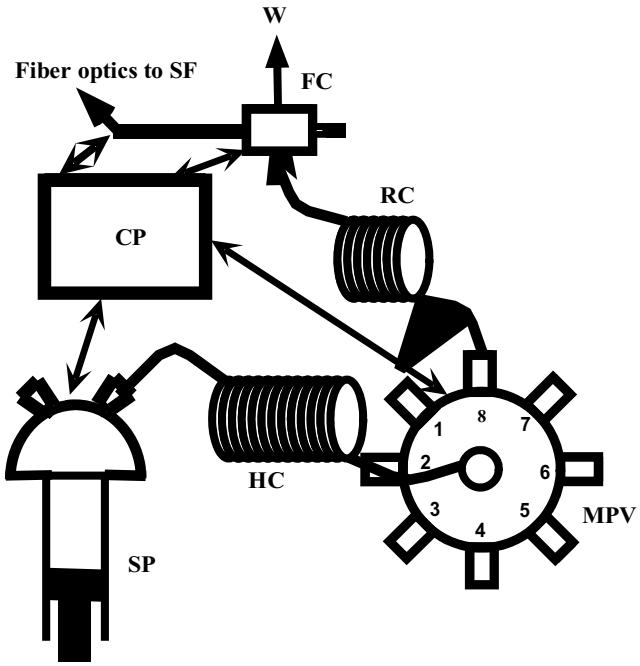

Fig. 1. Schematic diagram of the SI-manifold: SP, syringe pump; C, carrier; PC, computer; RC, 45 cm reaction coil 0.8 i.d.; HC, 200 cm holding coil 0.73 mm i.d.; MPV, eight ports selector valve; FC, flow through cell placed in spectrofluorometer (SF); W, waste.

2.2. Reagents

Tris (hydroxyl methyl) aminomethane (TRIS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Piroxicam (99.4% pure) was kindly donated by the Jordanian Pharmaceutical Manufacturing Company (Amman, Jordan). Pharmaceutical formulations of PX (Feldene tablets and Feldene capsules, Pfizer, NY, USA) were purchased from the local market. HPLC grade methanol was obtained from Panreac Quimica (Barcelona, Spain). Europium (III) chloride hexahydrate was obtained from Kanto (Tokyo, Japan). All reagents used were of analytical reagent grade and were used without purification.

2.3. Preparation of standard solutions

PX stock standard solution (200 ppm) in methanol was prepared by dissolving about 20 mg of PX in 100 ml of methanol. A working standard solution (50 ppm) was freshly prepared by appropriate dilution of the stock with methanol.

The stock solution was stored in a refrigerator at approximately 4 °C and remained stable for at least 1 month. Europium (III) chloride solution (3.0 mm) and TRIS buffer (0.1 M) were prepared in methanol.

2.4. Pharmaceutical preparation

Ten PX tablets were accurately weighed in order to find the average mass of each tablet. Then the tablets were finely

powdered in a mortar. An equivalent amount of the powder containing a known amount of active material was weighed and sonicated for 15 min, filtered into a volumetric flask and completed to the mark with methanol to make a stock solution of 1000 ppm. Working solutions of 50 ppm in methanol were prepared. Appropriate aliquots from the working solution were taken for the determination of PX in batch mode and in SIA. The components of 10 capsules were emptied mixed and treated in the same manner as described above for the tablets.

2.5. Method

2.5.1. Batch method

First a batch procedure was developed. In this way, a systematic variation of the parameters controlling the formation of the fluorescence complex of Eu–PX was carried out in the following way: into a 10 ml volumetric flask was added 1 ml of stock TRIS buffer (pH 9.0), 0.3 ml of stock Eu^{3+} solution and various concentrations of PX ranging from 0.1 to 2.0 ppm. The luminescence was measured within 10 min at 615 nm using an excitation wavelength of 358 nm.

2.5.2. Sequential injection method

The procedure starts by nesting the PX solutions (positions 2–7) and a mixture of Eu^{3+} and TRIS buffer (position #1) around the multi position valve (Fig. 1). Then the syringe and the holding coils are filled by the carrier solution, methanol. Further, aspiration of appropriate volumes of the solutions by selecting one port at a time fills the tubing around the valve. The excess of the solutions into the holding coils are then pumped to the waste through the detector point. Table 1 lists the steps of the procedure entered to the FIALAB for windows software (FIA labs Instruments)

Table 1
Experimental protocol as shown in the FIAlab for Windows software

```
variable Define New Portnum
Portnum = 1

Loop Start (#) 6
  Portnum = Portnum + 1
   Loop Start (#)2
    'Fill Syringe
      Syringe Pump Flowrate (µL/sec) 100
      Syringe Pump Valve In
      Syringe Pump Fill
      Syringe Pump Delay Until Done

    'clean detector
     Multiposition Valve port 8
    Syringe Pump Valve Out
    Syringe Pump Dispense (µL) 300
    Syringe Pump Delay Until Done

    'Standard to holding coil
    Multiposition Valve port 1
    Syringe Pump Flowrate (µL/sec) 20
'change volume
    Syringe Pump Aspirate (µL) 50
    Syringe Pump Delay Until Done

      Analyte New Sample
     if Portnum = 2 then
       Analyte Name StandardA
       Analyte Quantity 122.4
       Multiposition Valve Port 2
     End if
     if Portnum = 3 then
       Analyte Name StandardB
       Analyte Quantity 244.8
       Multiposition Valve Port 3
     End if
     if Portnum = 4 then
       Analyte Name StandardC
       Analyte Quantity 306
      Multiposition Valve Port 4
     End if
     if Portnum = 5 then
       Analyte Name StandardD
       Analyte Quantity 459
      Multiposition Valve Port 5
     End if
     if Portnum = 6 then
       Analyte Name StandardE
       Analyte Quantity 612
      Multiposition Valve Port 6
     End if
     if Portnum = 7 then
       Analyte Name StandardF
       Analyte Quantity 765
      Multiposition Valve Port 7
     End if

    Syringe Pump Aspirate (µL) 150
    Syringe Pump Delay Until Done

' send sample to detector
   Multiposition Valve port 8
   Syringe Pump Flowrate (µL/sec) 60
   Syringe Pump Dispense (µL) 2200
   'Delay (sec) 0.1
   'Spectrometer Reference Scan
   'Spectrometer Absorbance Scanning
   Syringe Pump Delay Until Done
   'Spectrometer Stop Scanning

  Loop End
```

which was used to program the complete procedure to run the method automatically. In this work, 50 μl of Eu^{3+} TRIS buffer mixture was aspirated via port # 1 followed by aspirating PX solutions (150 μl) one at a time into the holding coil (HC). A flow reversal is then used to pump the composite zone through port # 8 to the reaction coil and then to the detector. The fluorescence of the resultant Eu–PX complex is then monitored at λ_{em} 615 nm with the excitation at 358 nm. The time required to analyze one sample is approximately 1 min. The peak fluorescence intensity was used as the performance criterion for the optimization study and for quantitative analysis.

3. Results and discussion

3.1. Spectral characteristics

The fluorescence spectra of PX in methanol were determined by measuring a PX solution (10 ppm) in methanol. A weak fluorescence signal was obtained at λ_{em} 478 nm with excitation at 335 nm. This feeble signal at high instrumental setting has no useful analytical applications. Therefore, in order to obtain a better analytical characteristics for the determination of PX in pharmaceutical formulations, an attempt was made to sensitize Eu^{3+} fluorescence by complex formation with PX in the presence of TRIS buffer. Fig. 2 curve (1) shows the spectral characteristics of Eu(III) in the presence of TRIS buffer in methanol. A very weak signal is observed. However, the introduction of PX to this mixture resulted in an intense well known structured emission spectrum of Eu^{3+} (curve (2)). It is clear that the intensity of the emission line is greatly enhanced in the presence of PX as compared to Eu^{3+} alone.

The fluorescence enhancement of the Eu(III) ions upon complexation with PX is attributed to the efficient transfer of excitation energy from the PX moiety to the encapsulated europium (III) ion which can overcome the ions intrinsically low extinction coefficient. The successful sensitization process require the sensitizing moiety to be excited in the near ultra violet region since its triplet energy should be sufficient to provide efficient energy transfer to the rare earth ion. The criteria well satisfied by PX chelate since it absorbs at 356 nm. It has been argued that the longest wavelength at which sensitized luminescence from Eu^{3+} can be excited is at 385 nm [19].

The absorption spectrum of PX and the excitation spectrum of Eu–PX complex exhibited an excellent agreement between the absorption and excitation spectra ($\lambda_{max} = 360$ for absorption, $\lambda_{excitaion} = 358$), is an indication of the efficient sensitization process and that; the antenna chromophore is the only photo physical pathway leading to the observable luminescence in the system [20,21]. It is also noteworthy here to mention that there is a nearly complete

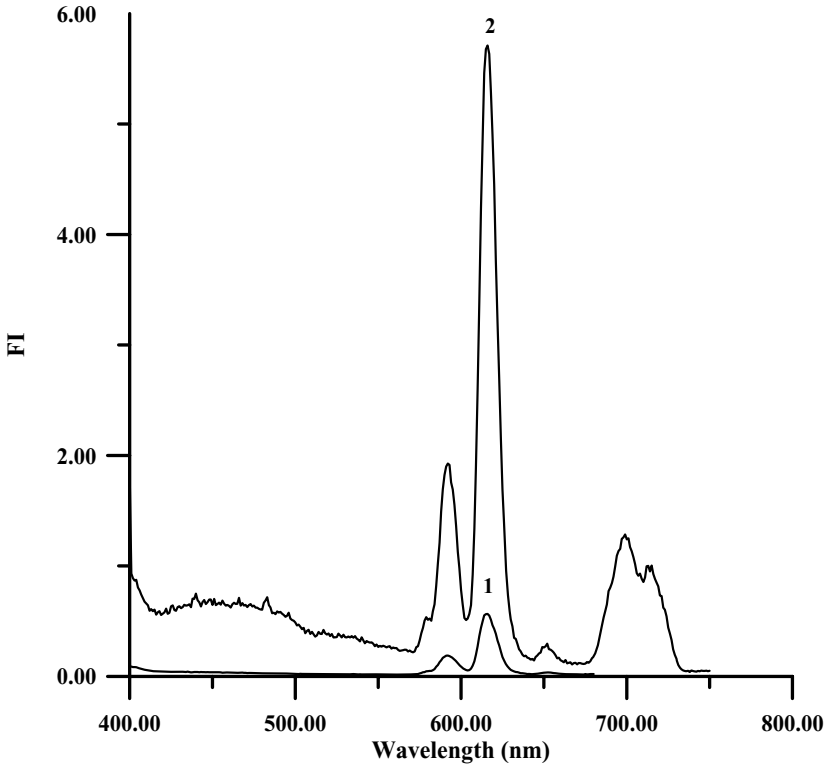

Fig. 2. (1) Luminescence of Eu^{3+} in TRIS buffer in methanol; (2) luminescence spectra of Eu^{3+}–PX system in TRIS buffer in methanol. $[Eu^{3+}] = 0.8$ mM; [PX] = 5 ppm; [TRIS] = 0.01 M. $\lambda_{ex} = 358$ nm, $\lambda_{em} = 615$ nm.

overlap between the absorption spectrum of PX and the excitation spectrum of Eu–PX complex.

The low reduction potential of Eu (−0.35 V versus SHE) may cause a ligand-to-metal charge transfer (LMCT) that decays non-radiatively [22,23]. If LMCT is efficient, it may lead to a dramatic reduction of the overall sensitized luminescence [22]. However, a large increase in fluorescence intensity of Eu^{3+}–PX complexation may suggest that LMCT may not have occurred in this system.

The emission spectrum of Eu–PX (Fig. 2) reveals the well known bands of Eu(III) luminescence based on the $^5D_0 \rightarrow {}^7F_0$ (580 nm) $^5D_0 \rightarrow {}^7F_1$ (590 nm), $^5D_0 \rightarrow {}^7F_2$ (615 nm), $^5D_0 \rightarrow {}^7F_3$ (650 nm) and $^5D_0 \rightarrow {}^7F_4$ (690 nm). Among these bands, the hypersensitive transition $^5D_0 \rightarrow {}^7F_2$ at 615 nm (orange red emission) [23] is the strongest and hence the peak height at 615 nm was used for monitoring of Eu–PX complex. Moreover, a large stoke shift of 255 nm was observed indicating that there is no overlap of the Eu^{3+} emission bands with the antenna chromophore absorption band. Furthermore, the reduction in fluorescence, intensity of PX upon complexation is a clear indication of an increase in singlet-to-triplet intersystem crossing due to the presence of Eu^{3+}, which induces a heavy atom effect [24].

In a previous study [25], on luminescence sensitization of Tm^{3+} using a β-diketone ligand 3-phenyl-2-4-pentanedionate (PPA), it was proposed that the energy absorbed by the phenyl group attached to Carbon number 3 in the PPA ligand was transferred to the metal ion through the enolate conjugated system [25]. Since PX contains a phenyl group in the oxicam nucleus, it is reasonable to assume that a similar transfer of energy from the phenyl group to Eu^{3+} will be realized as for (PPA-Tm^{3+}) system.

The use of the hypersensitive emission line of Eu^{3+} at 615 nm requires the investigation of different factors that affect the coordination environment. The effect of concentration of TRIS buffer, Eu^{3+} concentration and PX concentration were investigated in order to optimize the luminescence intensity.

3.2. Effect of concentration of TRIS buffer

The influence of TRIS buffer concentrations on luminescence intensity of Eu^{3+} was studied by varying the concentration of the buffer in the range 0.005–0.08 M while keeping the concentration of Eu^{3+} and PX a constant at 0.8 mM and 5 ppm, respectively. On increasing the concentration of buffer from 0.005 to 0.01 M an increase in the luminescence intensity was observed, after which the intensity started to decline.

This behavior indicated that an optimum concentration of TRIS buffer is required for maximum complex formation between Eu and PX. On exceeding the optimum concentration TRIS buffer compete with PX ligand for Eu^{3+} binding sites. This may result in less Eu–PX chelate being formed and hence a decrease in luminescence intensity. Similar behavior was observed previously by Rieutord et al. [26] where it was reported that TRIS buffer may have chelating properties towards lanthanide ions. Hence, the optimum concentration of TRIS buffer for this study was taken to be 0.01 M.

3.3. Effect of europium (III) concentration

Since luminescence sensitization is believed to occur via complex formation between Eu and PX, it is necessary to optimize the ratio of the concentration of Eu^{3+} to ligand that will afford maximum complexation and hence maximum intensity of the emission line.

The optimum concentration of Eu^{3+} was determined by measuring the luminescence intensity with an increase in concentration of Eu^{3+} ranging from 0.03 to 0.3 mM, while keeping the concentration of PX and TRIS buffer constant at 2 ppm (6.0 μM) and 0.01 M, respectively.

A gradual increase in the luminescence intensity was observed with an increase in concentration of Eu^{3+} reaching a maximum at 0.2 mM above which it started to decline (Fig. 3). This is a typical result, as an excess of Eu^{3+} is required for complex formation. However, large excess above the optimum may result in quenching of the luminescence due to non-radiative collisions where the free ions may act as a quencher of the singlet excited state of PX. Similar results were previously reported by Arnaud and George [27].

3.4. Effect of varying the concentration of PX

In order to probe whether sensitization is brought about by the presence of PX ligand, emission intensities in the presence of various concentrations of PX in the range of

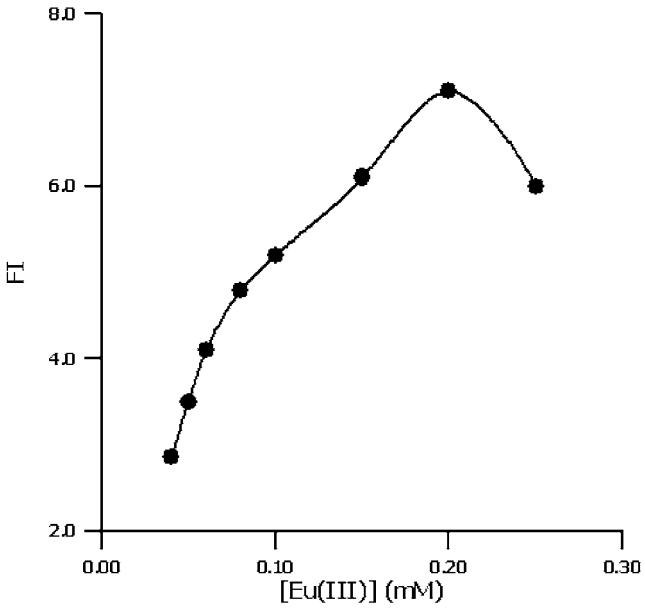

Fig. 3. The effect of changing Eu^{3+} concentration on the emission of PX–Eu^{3+}-system. [PX] = 5 ppm; [TRIS] = 0.01 M. λ_{ex} = 358 nm, λ_{em} = 615 nm.

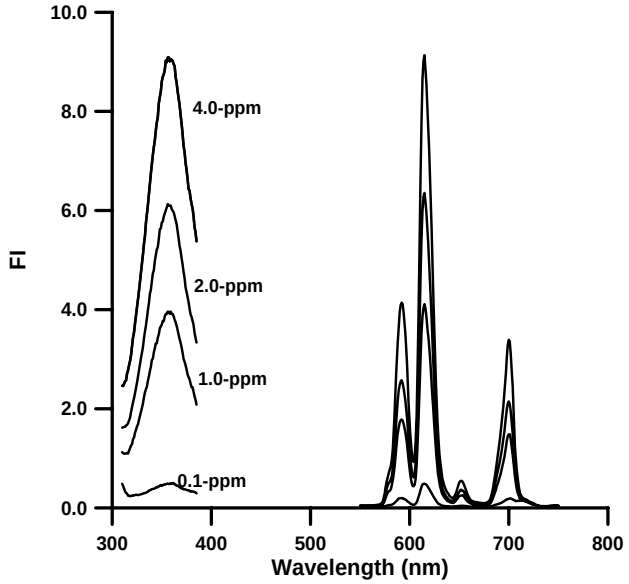

Fig. 4. The influence of PX concentration on the luminescence of PX–Eu^{3+}-system. [Eu^{3+}] = 0.2 mm; [TRIS] = 0.01 M. λ_{ex} = 358 nm, λ_{em} = 615 nm.

0.1–4.00 ppm were monitored while keeping the concentration of Eu^{3+} a constant at 0.2 mm, and TRIS buffer at 0.01 M (Fig. 4). An increase in the luminescence intensity was observed with an increase in the concentration of PX in the range studied. The excitation spectra exhibited a broad band at 358 nm which may be assigned to the efficient $\pi \rightarrow \pi^*$ transition based on the conjugated double bonds of the PX ligand while the emission spectra showed the characteristics, line spectrum of Eu^{3+} ion with the strongest emission band at 615 nm which comprise 53% of the total luminescent emission. This is a typical luminescence characteristic of Eu^{3+} which is well documented in the literature [18,28].

3.5. Optimization of SI parameters

The flow injection variables such as aspirated sample and reagent volumes were optimized in a univariate approach as follows: into a fixed reagent volume (50 μl of Eu^{3+}/TRIS buffer 0.01 M) were aspirated various sample volumes (30–200 μl) of 2 ppm PX. The luminescence intensity was monitored and was observed to increase with an increase in sample volume till 150 μl where the intensity leveled off. On the other hand the optimum reagent volume was determined by fixing the aspirated volume of PX at 150 μl and aspirating reagent volumes ranging from 30 to 200 μl of 0.2 mm Eu^{3+} in 0.01 M TRIS buffer. The luminescence intensity increased with an increase in the reagent volume and reached a maximum at a reagent volume of 50 μl. Therefore, 50 μl was considered the optimum reagent volume for this study.

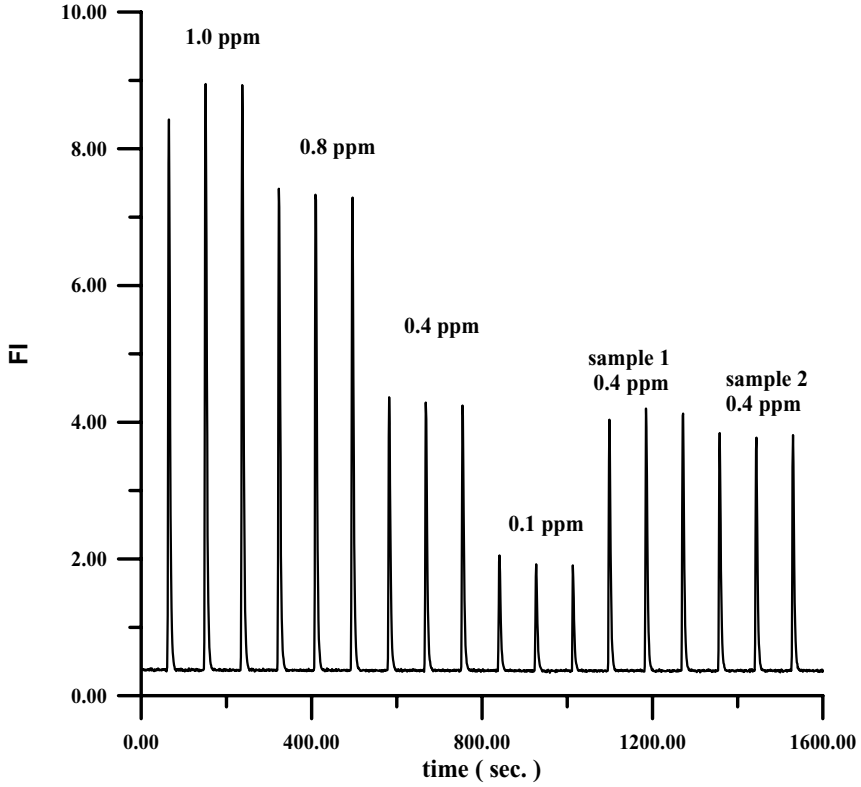

Fig. 5. A typical siagram obtained by the proposed method for a series of standards ranging from 0.1 to 1.0 ppm PX and one sample of tablet (sample 1) and a sample of capsule (sample 2). [Eu^{3+}] = 0.2 mM; [TRIS] = 0.01 M. λ_{ex} = 358 nm, λ_{em} = 615 nm.

3.6. Analytical features

3.6.1. Batch method

Using steady state experiments under the optimized conditions discussed above, the fluorescence intensity I versus the PX concentration C (ppm) was determined for the region of 0.1–10.0 ppm. A linear calibration curve was obtained for the range 0.1 and 2.0 ppm above which the graph begins to curve. The calibration equation ($n = 6$) is given by $I = 3.763C + 0.491$, with a correlation coefficient (R^2) of 0.995.

3.6.2. SI method

The analytical performance of the proposed SI-system was studied using the optimum conditions discussed above. A series of PX standards were aspirated in triplicate into the sequential injection system. A typical siagram is shown in Fig. 5. The fluorescence intensity, I versus PX concentration C was found to be linear over the concentration range of 0.1–1.0 ppm with the following calibration equation: $I = 7.774C + 1.1528$, with a correlation coefficient (R^2) of 0.999. The detection limit of the signal to noise ratio (S/N = $3s$, s is the standard deviation of the blank ($n = 12$)) is 23.0 ppb. The quantification limit was calculated (S/N = $10s$) is 76 ppb. The repeatability of the method was checked for all standard solutions where the maximum value of the relative standard deviation (R.S.D) obtained was 3.8% (Table 2) indicating that the results are within the acceptable range for good repeatability.

3.6.3. Determination of PX in pharmaceutical samples

To examine the applicability of the method, the proposed method was used to determine the concentration of PX in feldene capsules. Six samples prepared as described above were analyzed using initially the batch procedure. The mean percent recovery was found to be 101.0 ± 0.3%. It is clear from this result that excellent recovery with no interference from the excipients was obtained. The relative standard deviation was 3% ($n = 6$) showing excellent precision.

Using the SI method, six samples of both tablets and capsules were analyzed. The results of this analysis are shown in Fig. 5 and Table 2. It is clear from these results that excellent recoveries and precision are obtained for the samples. Also these results suggest that the proposed method can be employed for the determination of PX in pharmaceutical formulation in both capsules and tablets without any interference from the excipients present in these samples.

Table 2
Repeatability, accuracy, and precision of the method in SI

Concentration of PX claimed (ppm)	PX concentration found (ppm)	Recovery (%) ± R.S.D.
0.1	0.099	99.0 ± 3.8
0.4	0.40	100.0 ± 1.8
0.8	0.82	100.0 ± 1.6
1.0	0.99	99.1 ± 3.8
2.5 mg (tablet)	2.53 mg	101.2 ± 1.5
2.5 mg (capsule)	2.47 mg	98.8 ± 2.7

4. Conclusion

The development of a sensitive and robust microanalysis procedure has been described for the determination of PX in pharmaceutical preparations based on sensitized europium fluorescence. The enhancement of the europium fluorescence upon complexation with PX has enabled the assay of this drug with high sensitivity and selectivity. The procedure was successfully applied for the determination of PX in capsules and tablets with excellent reproducibility and no interference was observed from excepients commonly found in pharmaceutical preparations.

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