ISSN: 2357-0547 (Print)
ISSN: 2357-0539 (Online)

Research Article / JAPR
Rizk et al., 2020, 4 (4), 161-168

Journal of Advanced Pharmacy Research

Spectrofluorimetric Determination of Nepafenac Through Quenching Lanthanide Luminescence in Drug Substance and in Ophthalmic Suspension

Mohamed Rizk[1] , Ali K. Attia[2], Emad. M. Hussein [2], Fatma M. Aboul-Alamain[3], Mona S. Elshahed[1]*

[1]*Department of Analytical Chemistry, Faculty of Pharmacy, University of Helwan, Cairo, Egypt.*
[2]*National Organization for Drug Control and Research, Cairo, Egypt.*
[3]*VACSERA Holding Company for Biological Products and Vaccines, Giza, Egypt.*

****Corresponding author:** Mona S. Elshahed, Department of Analytical Chemistry, Faculty of Pharmacy, University of Helwan, Cairo, Egypt. Tel+2001006506715*

Email address: monaelshahed@gmail.com

Submitted on: 07-06-2020; Revised on: 25-06-2020; Accepted on: 08-07-2020

To cite this article: Rizk, M.; Attia, A. K.; Hussein, E. M.; Aboul-Alamain, F. M.; Elshahed, M. S. Spectrofluorimetric Determination of Nepafenac Through Quenching Lanthanide Luminescence in Drug Substance and in Ophthalmic Suspension. *J. Adv. Pharm. Res.* **2020**, *4* (4), 161-168. DOI: 10.21608/aprh.2020.32097.1111

ABSTRACT

Objective: A simple, selective and sensitive spectrofluorometric method is established for the quantitative assay of Nepafenac (NEPA) in pure form and in ophthalmic suspension dosage form. **Methods:** The fluorescence quenching of Terbium-Tris complex is measured at λ em580 nm after using λ ex 237 nm. The effect of pH, volume of terbium solution and reaction time on fluorescence quenching were studied. The complex formation was found to be highly dependent on the pH. The optimum fluorescence quenching was achieved by using 1.0 ml of (2×10^{-3}M) Tb^{3+} solution and 1.0 ml of Tris buffer solution (pH 10.0). **Results:** The formed complex was stable for 15 minutes from the starting time of the reaction at room temperature. The fluorescence quenching of Terbium-Tris complex was linear within the concentration range of 0.25 -10 μg ml^{-1} with mean percentage recovery of 99.85 ± 0.77 and Correlation Coefficient of 0.9996 (n=5). The sensitivity of the method could be evaluated by limit of detection (LOD) (0.008 μg ml^{-1}) and limit of quantitation (LOQ) (0.024 μg ml^{-1}). **Conclusion**: Statistical comparison of the results with those of a reported method revealed good agreement.

***Keywords:** Nepafenac, Terbium; Spectrofluorimetric determination.*

INTRODUCTION

Nepafenac (NEPA) [2-(2-amino 3-benzoyl phenyl) acetamide] (**Figure 1**) is a non- steroidal anti-inflammatory drug with analgesic activity, it is selective Cyclooxygenase-2 (COX-2) inhibitor [1],which is indicated for the treatment of pain and inflammation associated with cataract surgery and the reduction in the risk of postoperative macular edema in diabetic patients[2]. NEPA is a prodrug. After penetrating the cornea, NEPA undergoes rapid bioactivation to amfenac, which is a potent NSAID that uniformly inhibits the COX1 and COX2 activity[3]. Amfenac is thought to inhibit the action of prostaglandin H synthase (cyclooxygenase), an enzyme required for prostaglandin production.

The literature survey reveals that, some visible spectrophotometric[4-6], HPLC [7-11], electrochemical [12,13] and spectrofluorimetric[14] methods had been developed for the estimation of NEPA in pure, pharmaceutical dosage forms and biological fluids.

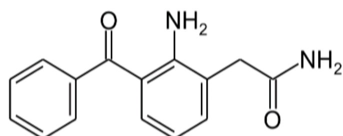

Figure 1. Structure of nepafenac (NEPA)

Lanthanide–sensitized luminescence [15] is a characteristic phenomenon where lanthanide ions Terbium (Tb^{3+}) and Europium (Eu^{3+}) form stable chelates with organic ligands. The luminescenceof these lanthanide chelates is characterized by large stock's shift, narrow emission bands and long fluorescence life times, so it has been used successfully for spectrofluorimetric determination of certain organic analytes as fluoroquinolones [16-18], tetracycline [19], fexofenadine [20], and DNA [21]. Some organic compounds can quench the back ground luminescence of lanthanide ions especially when they are in the form of chloride salts, because the probability of collisions leading to energy transfer is larger for the chloride salts [15]. This quenching phenomenon was reported for the fluorimetric determination of some cephalosporines[22] and some non-steroidal anti-inflammatory drugs[23].

This work provides a new simple method for quantitative spectrofluorimetric measurement of NEPA through quenching the fluorescence of Terbium-Tris complex.

MATERIAL AND METHODS

Apparatus

All the fluorescence spectra were recorded using a JASCO FP-6200 Spectrofluorometer, equipped with 150 W Xenon lamp, grating excitation and emission monochromators, and a recorder. Slit widths for both monochromators were set at 10 nm. A 1.0 cm quartz cell was used. Spectra were evaluated using Spectra Manager FP-6200 Control Driver software, Version 1.54.03 [Build 1], JASCO Corporation.

Samples

Reference samples

Nepafenac pure sample was purchased from Enaltec Labs, Navi Mumbai, India. The purity of NEPA was provided by the company to be 99.7 % and used as supplied.

Market samples

Nevaxal Sterile ophthalmic suspension, was provided by Orchidia Pharmaceutical Industries, Cairo, Egypt. Each 1ml is labeled to contain 1.0 mg of NEPA.

Reagents

All chemicals used were of analytical grade and were used without further purification. Terbium (III) Chloride hexahydrate (Tb^{3+}) 99.9 % (Aldrich, Germany), 2×10^{-3} M methanolic solution was prepared. Tris buffer [Tris (hydroxyl methyl) amino methane] was obtained from (Nice Chemicals, India), 0.1 mol L^{-1}methanolic solution was prepared.

Methanol HPLC grade 99.9 % was obtained from (Sigma, Germany). Sodium hydroxide pellets was obtained from (Winlab, Leicestershire, U.K.). Sodium chloride was obtained from (Winlab, Leicestershire, U.K.). Hydrochloric acid HPLC grade 37 % was obtained from (Rediel –De-Haen, Germany).

Preparation of Sample Solutions

NEPA stock sample solution (1.0 mg ml^{-1}) were prepared by dissolving 25.0 mg of the studied drug in methanol and then completed to volume with the same solvent in 25.0ml volumetric flask. A working sample solution (10.0µg ml^{-1}) of NEPA was freshly prepared by appropriate dilution of the stock solution with methanol. The stock solution were kept in a refrigerator at approximately 4^0C and remained stable for at least 1 month.

Construction of Calibration Graphs

1.0 ml of Tris buffer pH 10.0 ± 0.2 was transferred in a series of 10.0ml volumetric flasks followed by1.0 ml of terbium solution (2×10^{-3}M)[23]. Aliquots of NEPA working solutions equivalent to 2.5-100 μg were added. The solutions were mixed well and were completed to volume with methanol. The fluorescence intensity was measured at λ580 nm after excitation at λ 237 nm. The final concentration of the studied drug was plotted versus the calculated fluorescence quenching (F^0/F) to obtain the standard calibration graphs. Alternatively, the corresponding regression equation (Stern-Volmer equation) [24] was derived., where, F^0 is the fluorescence of 0.2 mM solution of Tb^{3+} in Tris buffer and methanol without drug and F is the fluorescence of 0.2 mM solution of Tb^{3+} in Tris buffer and methanol after addition of the drug.

Analysis of Dosage Forms

Nevaxal Ophthalmic suspension

An amount equivalent to 25.0 mg of NEPA was accurately measured and transferred to 25.0ml volumetric flask and made up to volume with methanol. The produced solution (1.0 mg ml^{-1}) of the studied drug was used to proceed as under preparation of sample solution.

Suitable aliquots of the above prepared solution of the pharmaceutical preparation were treated as described under construction of calibration graph. The results were calculated for each determination from the corresponding regression equation.

RESULTS AND DISCUSSION

The present work provides a simple, reliable and selective method for determination of NEPA in pure and pharmaceutical dosage forms. Although, a spectrofluorimetric method[14] for assay of NEPA is reported in the literature, but the proposed method has the advantage of covering wider linearity range (0.25-10.00 µg.ml^{-1}) compared to the reported method (0.2- 1.5 µg.ml^{-1}), furthermore our method is more sensitive with Lower values for LOD (0.008 µg.ml^{-1}) and LOQ (0.028 μg.ml^{-1}) than those of the reported method [LOD(0.032 μg.ml^{-1}) and LOQ (1.08 μg.ml^{-1})].

Fluorescence spectral characteristics

The solution of Terbium chloride (Tb^{3+}) in Tris buffer and methanol shows intense fluorescence, when excited at λ 237 nm, an emission band at λ 580 nm was observed as shown in **Figure 2**. This band is characteristic of terbium due to $^5D_4$-$^7F_6$ and $^7F_5$ transitions respectively[25]. Quantitative measurements are performed after excitation at λ 237 nm, where the fluorescence is measured at λ580 nm. When the studied drug is added to Tb^{3+} it reacts forming stable complex with Tb^{3+} which has no fluorescent properties and results in quenching the fluorescence intensity of methanolic Terbium-Tris complex. This quenching is found to be proportional to the concentration of the added drug.

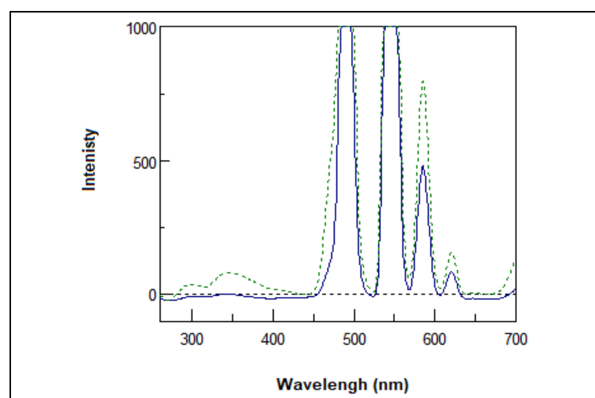

Figure 2. Emission Spectra of 0.2mM Terbium-Tris complex in methanol (…….), 0.2 mM Terbium-Tris complex in methanol + 6μg ml^{-1} of NEPA (___) at λ 580 nm after excitation at λ 237.

Although two other emission bands were observed at λ 480 nm and λ 540 nm for methanolic solution of terbium-tris complex after excitation at λ 237 nm (**Figure 2**). We could not perform quantitative measurements using these emission bands due to sever interference with solvent emission (methanol) as indicated by **Figure 3**.

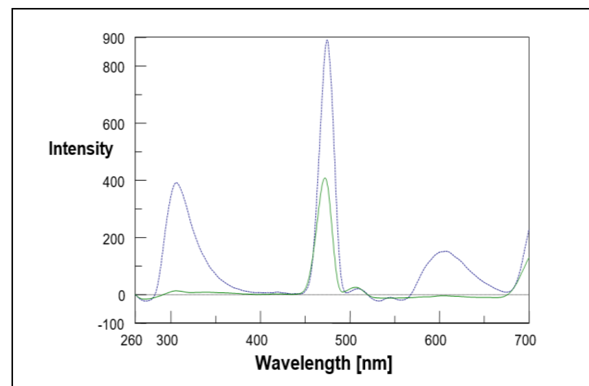

Figure 3. Emission Spectra of 0.002mM Terbium -Tris complex in methanol (——) and methanol as blank (——) after excitation at λ 237.

Optimization of the experimental conditions

Different experimental parameters affecting the fluorescence intensity of Terbium-Tris complex and complex formation between NEPA and terbium were carefully studied and optimized. Such factors were changed individually, while others were kept constant. These factors include Volume of terbium chloride solution, Effect of pH and Effect of time on complex formation.

Volume of terbium chloride solution

The fluorescence of Tb^{3+} in Tris buffer and methanol at λ em580 nm (λ ex 237 nm) was measured with increasing concentration of Tb^{3+} and was found to increase quantitatively with Tb^{3+} concentration as shown in **Figure 4**. It was found that 1.0ml of (2 $\times 10^{-3}$ mol L^{-1}) of Tb^{3+} is appropriate for reasonable fluorescence intensity.

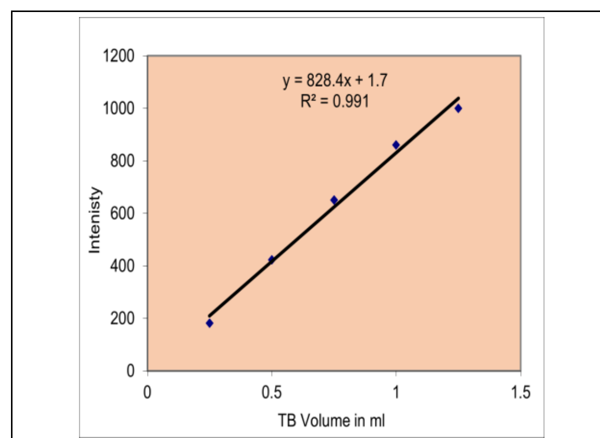

Figure 4. Calibration graph of the fluorescence of Terbium -Tris complex in methanol.

Table 1. Performance data for Nepafenac by the proposed fluorescence quenching method

Parameter	Proposed method
Working range ($\mu g.ml^{-1}$)	0.25- 10.00
Mean % Recovery	99.85
Standard deviation (SD)	0.77
Relative standard deviation (%RSD)	0.77
Regression equation	F0/F = 0.1587C+ 0.9257
Correlation coefficient (r)	0.9996
Standard deviation of the residuals (Sy/x)	0.00038
Percentage error % Er	0.345
LOD* ($\mu g.ml^{-1}$)	0.008
LOQ* ($\mu g.ml^{-1}$)	0.024

F^0: fluorescence of 0.2 m M solution of Tb^{3+} in Tris buffer and methanol without drug
F: fluorescence of 0.2 m M solution of Tb^{3+} in Tris buffer and methanol after addition of the drug C = Concentration in $\mu g\ ml^{-1}$

Effect of pH

The influence of pH on relative fluorescence of Tb^{3+} and complex formation between Tb^{3+} and the studied compound was studied using different pH values of Tris buffer.

The quenching of Tb^{3+} fluorescence after addition of NEPA (1.0 μg ml^{-1} final concentration) was measured in different solutions of Tris buffer pH (8.0, 9.0, and 10.0) ± 0.2. The study revealed that the best fluorescence quenching occurs when Tris buffer of pH 10± 0.2 was used (**Figure5**).

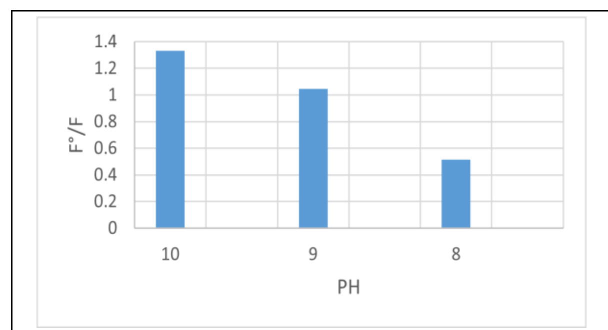

Figure 5. Effect of pH on complex formation between Terbium and 1.0 μg ml^{-1} NEPA

Effect of time on complex formation

The fluorescence of Terbium–Tris solution after addition of NEPA (1.0 μg ml^{-1} *final Concentration) was monitored after several* time intervals. It was found that the complex was immediately formed and remained stable for fifteen minutes as shown in **Figure 6**. The fluorescence all over this study was measured within 15 minutes from starting time of the reaction at room temperature.

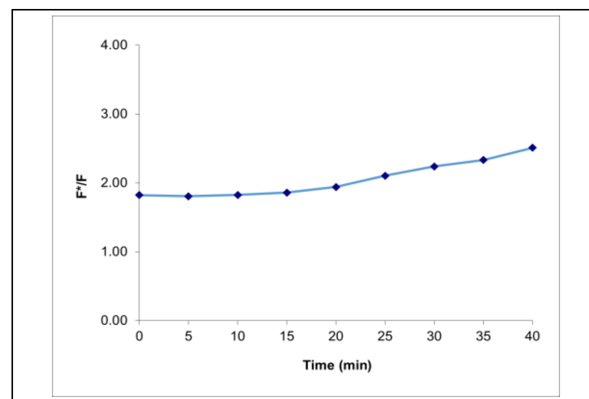

Figure 6. Effect of time on complex formation between Terbium and NEPA (1.0μg ml^{-1}) final concentration in Tris buffer.

Validation of the method

Linearity and range

Validation of the proposed method was assessed according to the ICH Q2 (R1) recommendation[26]. The proposed method was tested for linearity-range ,limit of quantification, limit of detection, specificity, accuracy and precision.

Table 2. Statistical analysis of the results obtained by determination of pure sample of Nepafenac by the proposed spectrofluorimetric method and those obtained by the reported method

Parameter	Proposed method			Reported method**
	Concentration taken ($\mu g.ml^{-1}$)	Concentration found ($\mu g.ml^{-1}$)	% Recovery	% Recovery
	0.5	0.499	99.85	98.59
	2	2.019	100.97	98.58
	4	4.005	100.13	100.84
	6	5.946	99.09	99.15
	8	7.935	99.19	
Mean ($\bar{X}$)		99.87		99.29
Variance		0.59		1.13
Replicates (n)		5		4
F-test(6.59)*		0.95		
Student' s t-test (1.895)*		1.91		

**Figures in parenthesis are corresponding theoretical t- and F- values at p=0.05[27]*
*** Reported method is HPLC method[9].*

Table 3. Precision data for the studied drug by the proposed fluorescence quenching method.

Parameter	Intra-day precision (Repeatability*)			Inter-day precision (Intermediate precision**)		
Concentration taken ($\mu g.ml^{-1}$)	1.0	5.0	9.0	1.0	5.0	9.0
	100.67	100.46	101.61	101.29	101.23	101.47
Recovery %	99.01	99.62	98.13	99.01	99.62	98.13
	98.18	100.88	100.29	98.2	102.63	99.4
Mean ($\bar{X}$)	99.29	100.32	100.01	99.5	101.16	99.67
± SD	0.53	0.64	1.76	1.60	1.51	1.69
RSD %	0.53	0.64	1.76	1.61	1.49	1.69
% Er	0.31	0.37	1.01	0.93	0.86	0.98

**The intraday (n = 9), average of three different concentrations repeated three times within day.*
***The interday (n = 9), average of three different concentrations repeated three times in three successive days.*

Table 4. Determination of Nepafenac in pharmaceutical preparation by the proposed spectrofluorimetric method

Preparation	Concentration taken($\mu g.ml^{-1}$)	Concentration found($\mu g.ml^{-1}$)	% Recovery*
Nevaxal ophthalmic suspension**	2.00	2.02	101.10
	4.00	3.99	99.73
	6.00	5.94	99.03
	8.00	7.96	99.50
Mean ± SD	**99.84 ± 0.89**		
% RSD	**0.89**		

**Average of triplicate determinations*

***Batch# 1017190*

After optimizing the conditions, the calibration graph of NEPA was constructed by plotting the calculated fluorescence quenching of terbium solution (F^0/F) versus final concentration in μg ml^{-1}. The regression plot showed linear dependence of fluorescence quenching of terbium solution on concentration of the studied drug over the range cited in **Table 1**. The small values of the %RSD and %Er point out to the low scattering of the points around the calibration curve and high accuracy and precision of the proposed method.

Analysis of the data gave the following regression equation:

$$F^0/F = 0.1587C + 0.9257 \quad (r = 0.9996)$$

Where F^0 is the fluorescence of 0.2 m M solution of Tb^{3+} in Tris buffer and methanol without drug, F is the fluorescence of 0.2 m M solution of Tb^{3+} in Tris buffer and methanol after addition of the drug, C is the concentration of the studied drug in (μg ml^{-1}) and (r) is the correlation coefficient, as shown in **Figure7**.

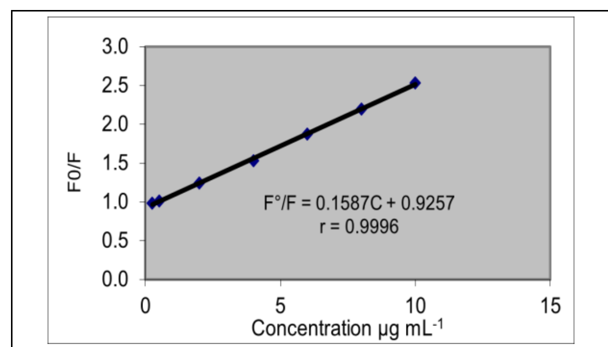

Figure 7. linear relationship between the concentration of the studied drug and the quenching of the fluorescence of Terbium-Tris complex.

The limit of quantification (LOQ) was determined by establishing the lowest concentration that can be measured according to ICH Q2 (R1) recommendations [26], below which the calibration graph is nonlinear, while the limit of detection (LOD) was determined by evaluating the lowest concentration of the analyte that can be readily detected. The results of LOD and LOQ of the studied drug by the proposed method are abridged in **Table 1**.

Accuracy and precision

The proposed method was evaluated by studying the accuracy as percent relative error and precision as percent relative standard deviation. The results are abridged in **Table 1**. Statistical analysis[27] of the results obtained by the proposed method and the reported HPLC method for NEPA[9] using Student's t-test and variance ratio F-test, shows no significant difference between the performance of the proposed and reported methods regarding the accuracy and precision, respectively (**Table 2**).The separation in the reported method was performed using C18 column and elution was done with a mobile phase consisting of acetonitrile: Water (40: 60 v/v) at a flow rate of 1ml/min and at a wavelength of 254 nm.

The intra-day Precision

It was evaluated through analysis of the studied drug in pure form using different concentrations (1.0, 5.0, 9.0 μg ml^{-1}) and each concentration was measured three times within in a day.

The inter-day Precision

It was evaluated through replicate analysis of the studied drug in pure form using different concentrations (1.0, 5.0, 9.0 μg ml^{-1}) and each concentration was measured once a day for three consecutive days.

The results of the inter-day and intra-day precision of the proposed method have been summarized in **Table 3**. The precision of the proposed method was fairly high, as indicated by the low values of SD and %RSD, respectively. Also accuracy was proved by the low values of % Er.

Application

Analysis of pharmaceutical formulation

The proposed method was successfully applied for determination of NEPA in ophthalmic suspension with good accuracy and precision as indicated by recovery and relative standard deviation values. The results are shown in **Table 4**.

Proposed mechanism of the reaction

In lanthanide-sensitized luminescence the intense luminescence originates from an intra molecular energy transfer through the excited triplet state of the ligand to the emitting resonance level of the ion followed by radiative emission from the cation. The efficiency of the energy transfer depends on the matching between the triplet level of the organic compound and the resonance level of the ion. The energy of the triplet level should be close to but higher than, that of the resonance level of the ion. In some instances, when the organic compound has a triplet state level below the excited state level of the lanthanide ion, the organic compound can quench the back ground luminescence of the ion[15]. The suggested mechanism of reaction between NEPA and $Tb^{3+}$is that NEPA coordinated to the metal ion via the2-amino group and Carbonyl-O groups of the benzoyl moiety. The structure of the complex formed between the studied drug and Tb^{3+} is proposed to be as represented in **Figure 8**.

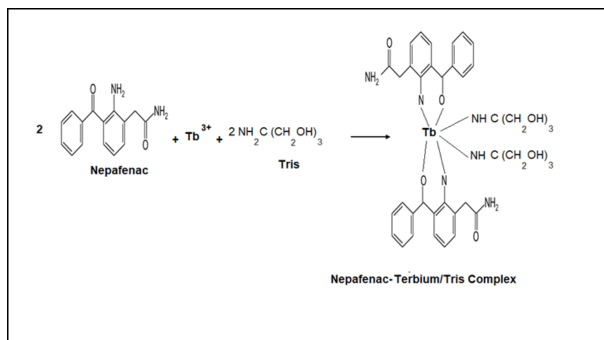

Figure 8. The proposed structure of the complex formed between NEPA and Tb^{3+}in presence of Tris buffer.

CONCLUSION

A simple and rapid spectrofluorimetric method has been developed in this work for quantitative determination of NEPA in pure form and in ophthalmic suspension. The results obtained by this method are indicative for high sensitivity and reasonable selectivity. As the proposed method is rapid, economic, safe and easy to handle it is very useful for accurate determination of NEPA in quality control laboratories.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

1. O'Neil, M. J. The Merck Index, Fourteenth Edition, MERK & CO., INC., Whitehouse station, NJ, USA, P.**2006**
2. Sweetman SC. Martindale the complete drug reference, 37th ed. London, UK: The Pharmaceutical Press, **2011**.
3. Gamache, D. A.; Graff G.; Brady, M. T.; Spellman, J. M. and Yanni, J. M. Nepafenac, a unique nonsteroidal prodrug with potential utility in the treatment of trauma-induced ocular inflammation: I. Assessment of anti-inflammatory efficacy. *Inflammation*. **2000**, *24* (4), 357-370.
4. Hari Prasad, Ch.; Anupama, B.; Jagathi, V.; Baskara Rao, P.; Devala Rao, G. Two visible spectrophotometric methods for determination of nepafenac in ophthalmic suspension. *Int. J. Chem. Sci.* **2010**, *8* (4), 2358-2362.
5. Yadav, S. J.; Doshi,M. N.; Panchori, H. P.; Bhalerao, S. S. A simple and rapid spectrophotometric method for the determination of nepafenac in pharmaceuticals. *J. Pharm. Res.* **2012**,*5* (8), 4292-4294.
6. Wagh, D.; Shahi, S. R.; Dabir, P. D.; Deore, S. Spectrophotometric method for the determination of nepafenac in opthalmic formulation. *World J. Pharm. Res*. **2017**, *6* (6), 623-630.
7. Lipiec-Abramska, E.; Jedynak, Ł.; Formela, A.; Roszczyński, J.; Cybulski, M.; Puchalska, M.; Zagrodzka, J. Development and validation of high performance liquid chromatography with a spectrophotometric detection method for the chemical purity and assay of nepafenac. *J. Pharm and Biomed Anal*. **2014**, (91 C), 1-6.
8. Shrimali, C.; Baghel, M.; Rajput, S. development and validation of stability indicating RP-HPLC method of nepafenac and its degradation products: application to degradation kinetic. *Int. J. Pharm. Pharm. Sci* .**2014**, *6* (9), 387-393.
9. Usman, S.; Akram, M.; Aziz, A.; Ramesh, V.; Sarheed. O. A. Development and validation of HPLC analytical method for nepafenac in ophthalmic dosage form (suspension). *Pak J. Pharm. Sci.* **2014**, *27*, 1541-1546.
10. Rajput, S.; Shrimali, C.; Baghel, M. Development and validation of uv and rp-hplc method for estimation of nepafenac in bulk drug and opthalmic formulation. *J. Adv. Pharm. Educ. Res*. **2015**, *5* (1), 15-20.
11. Pranyusha, V.; Damireddy, S.; Sudireddy, M. Method development and validation of stability indicating rp hplc method for estimating degraded products of nepafenac. *AJPAMC* **2016**, *4*(2), 58 - 68.
12. Nigović, B.; Jurić, S.; Mornar, A. Electrochemical determination of nepafenac topically applied nonsteroidal anti-inflammatory drug using graphene nanoplatelets-carbon nano-fibers modified glassy carbon electrode. *J. Electroanal. Chem*. **2018**, (817), 30-35.
13. Daabees, HG.; Talaat, W.; El-Shaal M.; Ahmed, K. Highly sensitive square wave voltammetric technique for determination of nepafenac in bulk powder, pharmaceutical dosage form and blood serum. *Int. J. Chem. Sci*. **2018**, *16* (1), 1-15.
14. Ragab, M. A.A.; El-Kimary, E. I. Investigation of the spectrofluorimetric behavior of azelastine and nepafenac: determination in ophthalmic dosage forms. *Spectrochim Acta A* .**2018**, *204*, 260-266.
15. George, J. Lanthanide-sensitized luminescence and applications to the determination of organic analytes. *Analyst*. 1993, *118*, 1481.
16. Rizk, M.; Belal F.; Aly, F. A. and El-Enany, N. M. Fluorimetric determination of nalidixic acid in formulations and biological fluids through ternary complex formation. *Anal. Lett.* **1997**, *30 (10),* 1897.
17. Rizk, M.; Habib, I. H. I.; Mohamed. D.; Mowaka, S.; El-Eryan. R. T. Lanthanide-DNA probe for spectrofluorimetric determination of some 6-fluoroquinolones in eye-ear pharmaceutical preparations. *Microchem. J.* **2019**,*150*, 104138.

18. Wang, L.; Guo, C.; Chu, Z.; Jiang, W. Luminescence enhancement effect for the determination of balofloxacin with balofloxacin–europium (III)–sodium dodecylbenzene sulfonate system. *J. Lumin.* **2009**, *129* (1), 90.
19. Tan, H.; Ma, C.; Song, Y.; Xu, F.; Wang, L.; Determination of tetracycline in milk by using nucleotide/lanthanide coordination polymer-based ternary complex. *Biosens. Bioelectron*. **2013**, *5015* 447.
20. Al-Kindy, S. M. Z.; Al-Shamalani, K.; Suliman, F. O.; Al-Lawat H. A. J. Terbium sensitized luminescence for the determination of fexofenadine in pharmaceutical formulations. Arab. J. Chem. **2019**,*12(8)*, 2457.
21. Yegorova, A. V.; Scripinets, Y. V.; Duerkop, A.;Karasyov, A. A.; Atonovich, V. P. and Wolfbeis, O. S. Sensetive luminescent determination of DNA using the Tb (III)-difloxacin complex. *Anal. Chem. Acta.* **2007**, *584* (2): 260
22. Bebawy, L. I.; El-Kelani, K. and Abdel-Fattah, L. Fluorimetric determination of some antibiotics in raw material and dosage forms through ternary complex formation with terbium ion . *J. Pharm. Biomed. Anal.***2003**, *32*, 1219.
23. Rizk M. S.; Toubar, S. S.; Elshahed, M. S. Determination of some non–steroidal anti-inflammatory drugs through quenching the fluorescence of lanthanide tris complex. *Eurasian J Anal Chem*. **2012**, *7* (1), 13-27.
24. Joseph, R. Lakowicz. Principles of fluorescence spectroscopy 3rd edition. springer science+business media, LLC, USA. **2006**, 282.
25. Dagnall, R. M.; Smith, R. and West, T. S. A specific spectrofluorimetric determination of terbium as its EDTA-Sulphosalicylic acid complex. *Analyst.***1967**, *92*, 358.
26. ICH Harmonized Tripartite Guideline, Validation of analytical procedures: Text and methodology, Q2(R1), Current Step 4 Version, Parent Guid lines on Methodology Dated November 6, 1996, Incorporated in November **2005**. http://www.Ich.org/ LOB/ media/MEDIA417.pdf.
27. Miller, JC.; Miller, JN. Statistics and chemometrics for analytical chemistry, 5th Edition. Prentice Hall, U.K.**2005.**