# SIMPLE AND HIGHLY SELECTIVE DIRECT STABILITY INDICATING ULTRAVIOLET AND INDIRECT VISIBLE SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF ENROFLOXACIN IN PHARMACEUTICALS [**]

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*Three simple, economic, selective and accurate and precise spectrophotometric methods are developed for determination of enrofloxacin (EFX) in pharmaceuticals. Method A is based on the measurement of absorbance of EFX in 0.1M HOAc at 315 nm. The ketoxime formation reaction has been employed in method B, in which the absorbance measurement of EFX oxime product at 275 nm is described. The third method (Method C) is indirect one and is based on the oxidation of EFX by cerium(IV), reaction of unreacted cerium(IV) with p-toludine (p-TD) and measurement of coloured solution at 540 nm. The Beer's law is obeyed in the concentration ranges of 1.2–24, 1–8, and 1–20 μg/mL EFX in methods A, B, and C, respectively, with the corresponding molar extinction coefficients of $1.52\times10^4$, $3.86\times10^4$, and $6.6\times10^3$ L/mol/cm. The regression coefficients of calibration lines are 0.9996, 0.9913, and –0.9965, in methods A, B, and C, respectively. The limits of detection (LOD) and quantification (LOQ) have also been reported for each method. The methods have been validated to check accuracy, precision, robustness and ruggedness. The application of the methods proposed to determine EFX in tablets has been described and the results have been compared with a standard method. The results of validation and application have been found to be with excellent agreement. The standard addition procedure has been adopted in recovery experiments to further ascertain the accuracy of the methods and the results of the experiments are well satisfied. The stability indicating ability of Method A has been studied by subjecting EFX to acid and alkaline hydrolysis, oxidative, thermal and UV degradation followed by measurement of absorbance of resultant EFX solutions at 315 nm. The results of degradation study indicated unsusceptible nature of EFX to any of the stress conditions.*

***Keywords:*** *enrofloxacin, spectrophotometry, determination, pharmaceuticals, stability indicating.*

# ВЫСОКОСЕЛЕКТИВНЫЕ ПРЯМЫЕ И НЕПРЯМЫЕ СПЕКТРОФОТОМЕТРИЧЕСКИЕ МЕТОДЫ ОПРЕДЕЛЕНИЯ ЭНРОФЛОКСАЦИНА В ФАРМАЦЕВТИЧЕСКИХ ПРЕПАРАТАХ

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*УДК 543.42.062*
*https://doi.org/10.47612/0514-7506-2021-88-6-982-992*

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*(Поступила 7 июля 2020)*

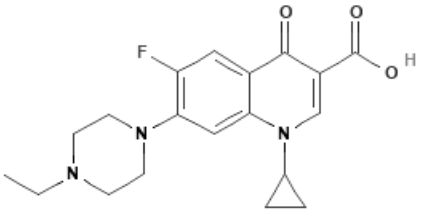
*Для определения энрофлоксацина (EFX) в фармацевтических препаратах разработаны три спектрофотометрических метода. Метод А основан на измерении поглощения EFX в 0.1 М HOAc на длине волны 315 нм. В методе В использованы реакция образования кетоксима и измерение продукта оксима EFX на 275 нм. Метод С является косвенным и основан на окислении EFX церием(IV), реакции непрореагировавшего церия(IV) с р-толудином (p-TD) и измерении поглощения окрашенного раствора на 540 нм. Закон Бера соблюдается в диапазонах концентраций EFX 1.2–24, 1–8 и 1–20 г/мл*

[**] This paper is published in electronic version of ZhPS V. 88, No. 6 (http://www.elibrary.ru/title_about.asp?id=7318; sales@elibrary.ru).

*для методов A, B и C соответственно, с молярными коэффициентами экстинкции $1.52 \cdot 10^4$, $3.86 \cdot 10^4$ и $6.6 \cdot 10^3$ л/моль/см. Коэффициенты регрессии калибровочных линий 0.9996, 0.9913 и –0.9965 для методов A, B и C соответственно. Для каждого метода получены пределы обнаружения и количественного определения, проверены их точность и надежность. Предложенные методы применены для определения EFX в таблетках. Результаты сопоставлены со стандартным методом и установлено их хорошее согласие. В экспериментах по восстановлению использована стандартная процедура добавления для дальнейшего подтверждения точности методов. Результаты экспериментов удовлетворительные. Стабильность, указывающая на потенциал метода A, изучена путем проведения кислотного и щелочного гидролиза, окислительного, термического и ультрафиолетового разложения EFX с последующим измерением поглощения полученных растворов EFX на длине волны 315 нм. Результаты исследования разложения свидетельствуют о невосприимчивости EFX к стрессовым условиям.*

***Ключевые слова:*** *энрофлоксацин, спектрофотометрия, определение, фармацевтический препарат, показатель стабильности.*

**Introduction.** Enrofloxacin (EFX), chemically known as 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid




has a molecular formula of $C_{19}H_{22}FN_3O_3$ and molar mass of 359.4 g/mol. EFX is an antibacterial compound, which will function against Gram-negative and Gram-positive bacteria. EFX is one of the medicaments to treat urinary and respiratory tract, and skin infectious diseases in pets and livestock [1].

EFX is official in United States Pharmacopeia (USP) [2]. The monograph of USP describes the non-aqueous potentiometric titration of EFX in acetic acid medium with 0.1 M perchloric acid. This procedure is applicable for the assay of EFX only to macro-size samples.

Liquid chromatography [3–19], colorimetry and fluorimetry [20], fluorescence spectroscopy with chemometry [21], capillary electrophoresis [22], flow injection chemiluminescence [23], and luminescence [24] techniques have been employed earlier to determine EFX in pharmaceuticals and in biological materials. But these methods are suffering from some disadvantages such as a need of large sample/reagents, highly skilful operator and expensive equipment. Besides, several spectrophotometric methods [25–34] have also been reported by different workers. The procedures of these spectrophotometric methods involve the use of expensive reagents and stringent experimental conditions such as tedious precipitation, heating or liquid-liquid extraction steps. Hence, there is a need of simple, rapid and cost effective, analytical methods to determine EFX in pure form and in its dosage forms.

Three new spectrophotometric methods have been presented for determination of EFX in pure form and in tablets. The first (Method A) and second (Method B) methods are based on the measurement of EFX in 0.1 M acetic acid and ketoxime of EFX at 315 and 275 nm, respectively. In the third method (Method C), EFX was made to react with the known excess of cerium(IV), after oxidation of EFX is over the surplus cerium(IV) treated with *p*-TD and the coloured species formed thereupon was measured at 540 nm. The stability indicating ability of Method A was assessed by subjecting EFX for acid-, alkaline-, oxidative-, thermal and photocatalytic stress conditions and by using the products of stressed samples for measuring the absorbance at 315 nm. The results of validation and applications of all the three methods along with those of stability studies in method A have been presented in this paper.

**Experimental.** Shimadzu Pharmaspec 1700UV/Visible double beam spectrophotometer (Hyderabad, India) was used in measurement of absorbance.

The used chemicals and reagents were of analytical grade. Distilled water was used throughout the work. The pure EFX (99.8%) was kindly provided by Cipla India Ltd. Ataxin tablets (150 mg EFX /tablet) (Sava Health Care Pvt Ltd., Pune, India) were purchased from local commercial sources. All the reagents and solvents used in the work were manufactured by Merck, Mumbai, India, and they were procured from authenticated suppliers.

The solutions of 0.1 M each of acetic acid (HOAc), sulphuric acid ($H_2SO_4$) and hydrochloric acid (HCl) and 5% (v/v) hydrogen peroxide ($H_2O_2$) were prepared by diluting suitable volumes of glacial acetic acid (98% pure), concentrated sulphuric acid (98% pure), concentrated hydrochloric acid (38% pure) and commercial $H_2O_2$ (98% pure), respectively, with distilled water. The solutions of 0.1 N NaOH, 0.05% (w/v) hydroxylammonium chloride (HAH) and 0.5% (w/v) *p*-toludine (*p*-TD) were prepared by dissolving required weights of pure compounds in water. A solution of 0.01 M cerium(IV) sulphate solution was prepared by dissolving calculated quantity of standard commercial ceric ammonium sulphate in 0.5 M $H_2SO_4$ by heating. The solution was filtered and standardized [35] before use.

*Preparation of a standard EFX solution.* A stock standard 400 μg/mL EFX solution was prepared by dissolving 40 mg of pure drug in either 0.1 M HOAc for UV methods or in 0.1 M $H_2SO_4$ for visible spectrophotometric method. Suitable aliquots were subsequently diluted with respective diluent to a particular volume to obtain 40 μg/mL solution of EFX.

*Procedure for bulk drug. Method A.* Into a series of 10.0 mL volumetric flasks varying aliquots (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mL) of 40 μg/mL standard EFX solutions were placed with the help of a microburet. The volumes were diluted up to the mark with 0.1M HOAc and absorbance of each solution was measured at 315 nm against blank.

*Method B.* Different aliquots (0, 0.5, 1.0, 1.5, and 2.0 mL) of standard 40 μg/mL EFX solution were taken in a series of 10.0 mL volumetric flasks and the volumes were adjusted to 5.0 ml by adding 0.1M HOAc. To each flask 1ml of 0.05% HAH was added, flasks were stoppered, content of each flask was mixed and kept aside for standing for 5 min. The solutions of each flask were brought to the mark with water and mixed well. Finally, the absorbance was measured at 275 nm against reagent blank.

*Method C.* Different aliquots (0, 1.0, …, 5.0 mL) of standard 40 μg/mL EFX solution were taken in a series of 10.0 mL volumetric flasks and the volumes were adjusted to 5.0 mL by adding 0.1M $H_2SO_4$. To each flask 1mL of 0.01 M cerium(IV) solution was added, content was mixed and kept aside for 10 min. Then, a 1 mL of 0.5% *p*-TD was added to each flask, the volumes were brought to the mark with water, contents were mixed and the absorbance was measured at 540 nm against water.

For each method the calibration graph was prepared by plotting the measured absorbance against concentration of EFX, and the concentration of the unknowns were read from the corresponding calibration graph or computed from the curve fitting regression equation derived using the absorbance-concentration data.

*Procedure for tablets.* Ten tablets were weighed accurately and ground into a homogeneous powder. The tablet powder equivalent to 40 mg of EFX was weighed out to a 100 mL volumetric flask, 60 ml of solvent (0.1 M HOAc for Methods A and B, and 0.1 M $H_2SO_4$ for Method C) was added and shaken for about 20 min. The content was diluted to the mark with respective solvent, mixed well and filtered through a Whatman No 41 filter paper. The resulted tablet extract was equivalent to 400 μg/mL in EFX. Suitable volumes of tablet extracts were diluted to 40 μg/mL in EFX using the diluent (solvent) and subjected to assay by following the procedures as described under 'procedure for bulk drug'.

*Procedure for the analysis of placebo blank and synthetic mixture.* A placebo blank was prepared by mixing and grinding the ingredients, namely, acacia (15 mg), hydroxyl cellulose (10 mg), sodium citrate (10 mg), starch (10 mg), talc (20 mg), magnesium stearate (15 mg), and sodium alginate (10 mg) to form a homogeneous mixture. A 5 mg of the placebo mixture was weighed accurately, its solution was prepared as described under 'tablets', and it was subjected to analysis by following the general procedure of each method.

*Procedure for the analysis of synthetic mixture.* Synthetic mixture was prepared by mixing and grinding an accurately weighed 20 mg of EFX and 80 mg of the placebo mentioned above to get a homogeneous fine powder. The procedure described for analysis of tablets was followed intact for calculated quantity of synthetic mixture to prepare 40 μg/mL EFX solutions. The aliquots of 8, 12, and 16 μg/mL EFX solutions resulting from synthetic mixture extract were subjected to analysis by following the respective general procedure.

*Procedure for forced degradation study.* Four mL triplicate aliquots of the standard 40 μg/mL EFX were taken in three different 10 mL volumetric flasks. The first, second and third flasks were individually mixed with 5 mL of 0.1 N HCl for acid hydrolysis, 0.1 N NaOH for alkaline hydrolysis and 5% $H_2O_2$ for oxidative degradation, respectively. The contents were boiled for 2 h at 80°C on a hot water bath. The solutions were cooled to laboratory temperature and the contents were diluted to the mark with 0.1 M HOAc. For thermal degradation, the pure solid EFX was kept in Petri dish and placed in an oven adjusted at 100°C for 24 h. After cooling to laboratory temperature, a calculated amount of EFX was weighed and the solution was prepared using the respective solvent. For UV degradation study, the stock solution of 40 μg/mL EFX was irradiated to UV light of 254 nm of 1.4 flux intensity for a couple of days in a UV chamber. Finally, the suit-

able aliquots of the resulted solutions (equivalent to 40 μg/mL in EFX) from each were diluted with the working solvent and spectra were recorded. Besides, the absorbance of all the obtained solutions from acid and alkaline hydrolysis, oxidative degradation, thermal and UV degradation were measured at 315 nm against the respective solvent as blank in each case and the recovery of EFX was calculated.

*Procedures for validation. Intra-day and inter-day accuracy and precision.* EFX solutions in three different concentrations within the range of study in each method (Method A: 6.0, 12.0, and 18.0 μg/mL; Method B: 2.0, 4.0, and 6.0 μg/mL; and Method C: 5.0, 10.0, and 15.0 μg/mL) were analysed in seven replicates in the same day to study intra-day variations. However, to study inter-day variations the analyses of triplicate solutions of each concentration was carried out on five consecutive days by following general analytical procedures of each method. The amount of EFX found was calculated for each concentration in each trial and the pooled-standard deviation was calculated using the following formula [36]:

$$S_p = \sqrt{\frac{\sum(X_i - \overline{X}_1)^2 + \sum(X_j - \overline{X}_2)^2 + \sum(X_k - \overline{X}_3)^2}{N - k}},$$

where $X_i$, $X_j$, and $X_k$ are the individual concentrations of EFX found in the analysis of solutions of three different concentrations; $\overline{X}_1$, $\overline{X}_2$, and $\overline{X}_3$ are the mean values of the data sets 1, 2, and 3, for three different concentrations, respectively, and $N$ is the total number of trials from analyses of whole three ($k$) different concentrations of EFX solutions. The $S_p$ values were converted to %RSD.

The accuracy of the analytical procedure was evaluated by calculating the percentage relative error (%RE) using the mathematical formula:

$$\text{RE}(\%) = \frac{(\text{EFX}_{\text{Taken}} - \text{EFX}_{\text{Found}})}{\text{EFX}_{\text{Taken}}} \times 100,$$

where $\text{EFX}_{\text{Taken}}$ and $\text{EFX}_{\text{Found}}$ are in μg/mL.

*Procedure for assessment of accuracy by recovery experiment using standard-addition procedure.* Accurately measured 2 mL aliquots of pre-analysed tablet extract equivalent to 40 μg/mL EFX were taken in a series of 10 mL volumetric flasks and were spiked with 1, 2, and 3 mL of 40 μg/mL EFX pure drug solution in triplicate for Method A. In Method B, the volume of pre-analysed 40 μg/mL EFX from tablet extract was 1 mL and spiking were done with 0.5, 1.0, and 1.5 mL of standard pure EFX solution. Whereas in Method C, 2 mL of tablet extracts equivalent to 40 μg/mL EFX were spiked in triplicates with 1.0, 2.0, and 3.0 mL of standard EFX solutions. Then, the general analytical procedures described under 'general procedures' for each method were followed and the percentage of pure EFX recovered was calculated for each concentration of each method using the formula given below.

$$\%\text{EFX}_{\text{Pure}}\text{recovred} = \frac{(\text{Total EFX}_{\text{Found}} - \text{EFX}_{\text{From tablet}})}{\text{EFX}_{\text{From pure drug}}} \times 100,$$

where Total $\text{EFX}_{\text{Found}}$, $\text{EFX}_{\text{From tablet}}$, and $\text{EFX}_{\text{From pure drug}}$ are in μg/mL.

*Determination of limits of detection (LOD) and quantification (LOQ).* The absorbance of either reagent blanks (Method A and B) or water blanks (without drug and oxidant; Method C) was recorded in five replicates at the respective analytical wavelengths and the standard deviation (SD) values for set of data in each method were calculated. The SD values were then used to calculate the limits of detection (LOD) and quantification (LOQ) according to ICH guidelines [37] using the formulae:

$$\text{LOD} = 3.3\text{SD}/\sigma, \quad \text{LOQ} = 10\text{SD}/\sigma,$$

where σ is slope of the calibration curve.

*Robustness. Method A.* The concentration of 0.1 M HOAc was varied by +0.02 and –0.02 M to prepare the standard EFX solution and tablet extracts. Five replicates of 3 mL aliquot of standard 40 μg/mL EFX solutions were prepared using HOAc of 0.08, 0.1, and 0.12 M and measured the absorbance at 315 nm.

*Method B.* Into a series of 10 mL volumetric flasks 1 mL aliquot of standard 40 μg/mL EFX solution was taken, added 1mL of 0.1 M HOAc and the contents were mixed well. Then, 0.9, 1.0, and 1.1 mL of 0.05% HAH solutions were added to the first, second and third series of five flasks each, respectively. The contents were mixed well and after 5 min the volumes were brought to the mark with water. After mixing, the absorbance was recorded at 275 nm against reagent blank prepared in the absence of EFX.

*Method C.* Three mL aliquots of standard 40 μg/mL EFX solution were taken in a series of fifteen 10.0 mL volumetric flasks and the volumes were adjusted to 5.0 mL by adding 0.1M $H_2SO_4$. To each flask 1 mL of 0.01 M cerium(IV) solution was added, content was mixed and kept aside for 10 min. Then, to the

three groups of five flasks each, 0.9, 1.0, and 1.1 mL of 0.5% *p*-TD solutions were added, respectively, and the flasks were shaken for mixing the content. Then, the volumes were brought to the mark with water, again mixed and the absorbance of each solution was measured at 540 nm against water.

The robustness of all the three methods was also checked by varying the wavelength of measurement by 2 nm, i.e. 315±2, 275±2, and 540±2 nm, in Methods A, B and C, respectively. Then, for the resulted absorbance values from all the variations for each concentration of EFX the corresponding RSD values were calculated in each method.

*Ruggedness.* The optimised general procedure was followed and a triplicate analysis was performed by four different analysts, using four different instruments on three different concentrations of EFX (Method A: 6.0, 12.0, and 18.0 μg/mL; Method B: 2.0, 4.0, and 6.0 μg/mL; and Method C: 5.0, 10.0, and 15.0 μg/mL). The regression data were used to calculate the concentration of EFX found in each case. Then, the intermediate variations, expressed as RSD, were calculated.

**Results and discussion.** *Spectral characteristics.* The solution of EFX in 0.1 M HOAc showed an absorption maximum at 315 nm, at which solvent did not show any peak. On the other hand, the ketoxime of EFX was exhibited wavelength of maximum at 275 nm. Therefore, measurement of absorbance of EFX and ketoxime of EFX in 0.1 M HOAc at 315 and 275 nm formed the basis for Methods A and B, respectively. The recorded absorption spectra are presented in Figs. 1a,b. In Method C, the reaction of EFX with a measured excess of cerium(IV) in $H_2SO_4$ medium, the treatment of the unreacted oxidant with *p*-TD and the measurement of absorbance of oxidised *p*-TD at 540 nm are involved. The absorption spectrum of oxidised *p*-TD is presented in Fig. 1c. The linear decrease in absorbance at 540 nm is concomitant with the concentration of EFX, which served as a basis for quantification of the drug.

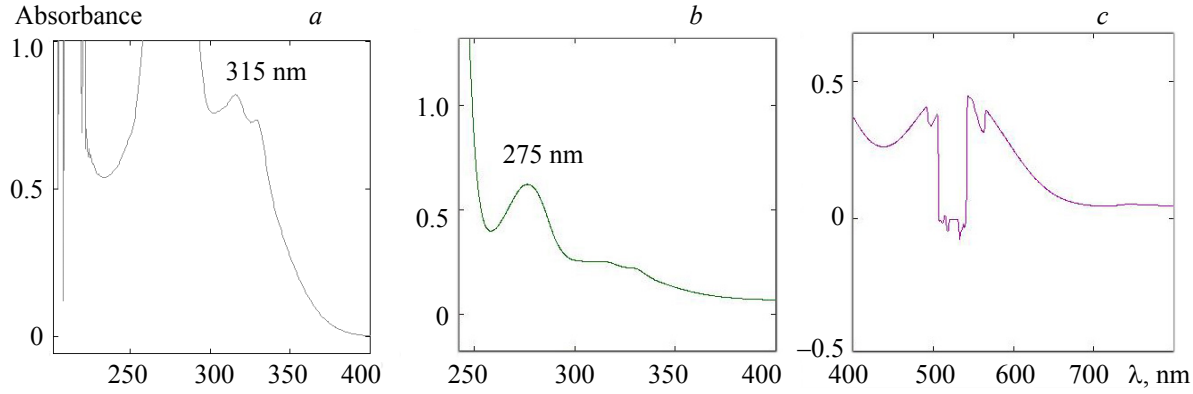
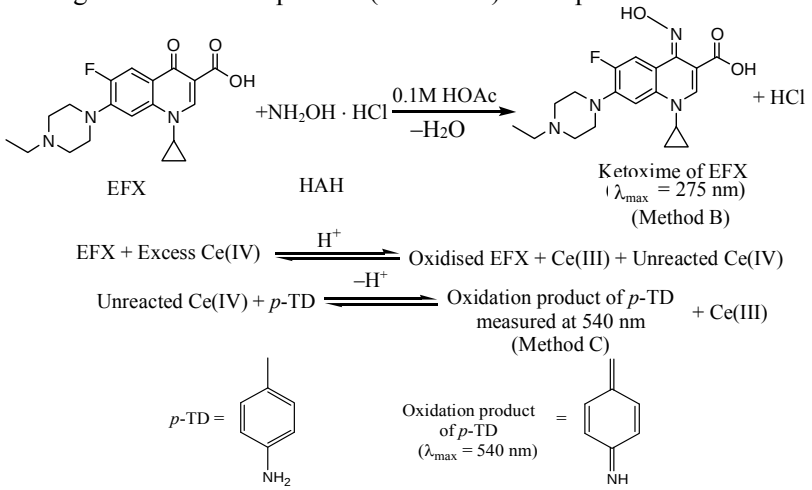



Fig. 1. Absorption spectra of (a) EFX equivalent to 20 μg/mL (Method A); (b) Ketoxime of EFX (equivalent to 20 μg/mL) in 0.1M HOAc (Method B); (c) product of oxidation of *p*-TD (1 mL of 0.5%) with cerium(IV) (1mL of 0.01 M) solution.

*Chemistry.* Tentative reaction pathways showing the formation of the ketoxime of EFX (Method B) and the oxidation of *p*-TD to give red coloured product (Method C) are depicted in Scheme



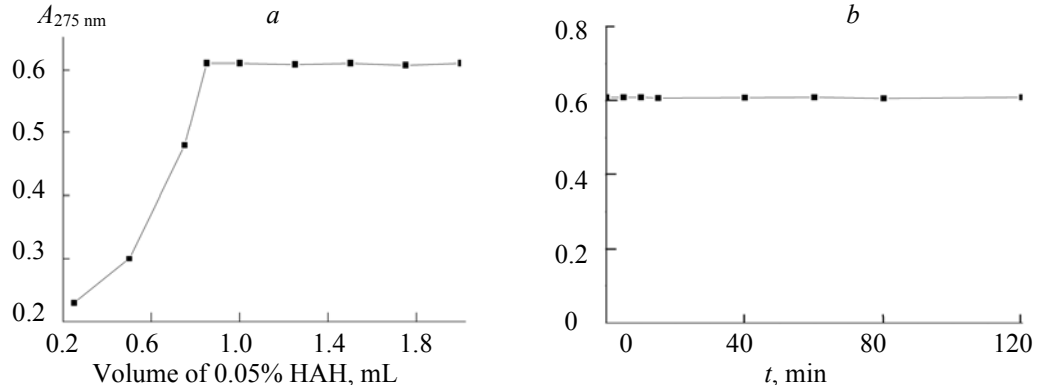



Fig. 2. Effect of (a) different volumes of 0.05% HAH solution on the absorbance of ketoxime of EFX (equivalent to 6 μg/mL EFX) and (b) standing time after adding HAH to EFX (6 μg/mL) solution to form ketoxime of EFX.

*Development and optimization of methods.* Different acids of different concentrations were tried as solvents for preparing standard solutions of EFX and it was revealed by the results that HOAc comes out as best with concentration of 0.1 M. Thus, 0.1M HOAc was used as a solvent in Method A and Method B. In Method B, the amount of HAH required to completely react with EFX was studied using varying quantities of reagent. The results obtained indicated a need of 1 mL of 0.05% HAH (Fig. 2a) in a total volume of 10 mL to get the maximum absorbance at 275 nm and hence the same quantity was fixed as optimum. The reaction between EFX and HAH was found instantaneous to form ketoxime (Fig. 2b). Among 0.1 M HOAc and water as diluents, no significant difference was found in absorbance values. Therefore, after adding HAH, the contents were mixed, diluted to the mark with water and absorbance was measured at 275 nm. The absorbance of final solution was found stable at least for 2 h (Fig. 2b) and this indicated the stability of ketoxime of EFX. Various experimental variables involved in the oxidation of EFX by cerium(IV) such as concentration of acid, oxidant and reagent(s), reaction time, nature of diluents and others on the reaction were studied in Method C. This was done by measuring the absorbance at respective wavelength after each and every variation of each parameter. Different concentrations of different acids were introduced to oxidise EFX with cerium(IV). It was found that 0.1 M $H_2SO_4$ yielded better results and hence, the same was used throughout the investigation. In order to optimize the concentration of oxidant, fixed quantities of EFX were reacted with different concentrations/amounts of cerium(IV) in acid medium. Best results were obtained with up to 1 mM cerium(IV) as in a total volume of 10 mL. The waiting period to add *p*-TD was 10 minutes after adding cerium(IV). The absorbance at 540 nm was found maximum at 1 mL of 0.5% *p*-TD. Hence, the same was maintained as optimum amount. The reaction between cerium(IV) and *p*-TD was found instantaneous. The resulted oxidation product of *p*-TD was found stable for more than 1 h. The effect of quantity of *p*-TD and standing time are presented in Figs. 3a,b, respectively.

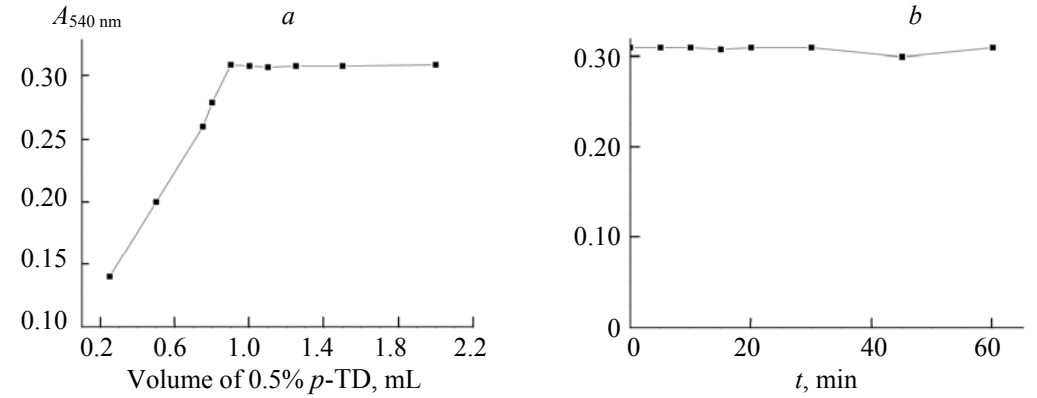



Fig. 3. Effect of (a) volumes of 0.5% *p*-TD and (b) standing time on addition of *p*-TD to the solution composed of EFX (12 μg/mL) and cerium(IV) (1 mM in a total volume of 10 mL) in acidic medium.

*Method validation.* The linearity, accuracy, precision, sensitivity, robustness and ruggedness of the proposed methods were checked according to current ICH guidelines [37].

*Linearity and sensitivity.* The calibration graphs for absorbance at wavelength of maximum against the concentration of EFX in each method were found to be linear (Fig. 4) over the concentration ranges of drug given in Table 1. The linear correlation was found between the two parameters over the calibration or linear range of EFX concentration. The calibration graphs are described by the regression equation:

$$A = b + mX,$$

where $X$ and $A$ are the concentration of EFX in μg/mL and absorbance of 1-cm layer of solution, respectively. The parameters b and m are the intercept and slope of the calibration curve, respectively. The values of $b$ and $m$ for each method are presented in Table 1 along with regression coefficients over the applicable linear concentration ranges.

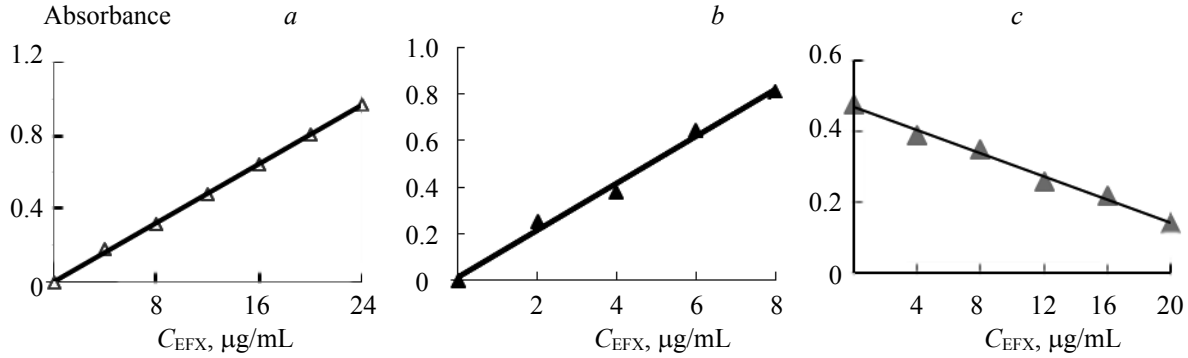



Fig. 4. Calibration curves for (a) Method A, (b) Method B, and (c) Method C.

The Beer's law limits, molar absorptivity and Sandell's sensitivity values were also calculated and presented in Table 1. The calculated LOD and LOQ are also mentioned in Table 1. As it can be seen from the results in Table 1, the high value of ε and low value of Sandell's sensitivity and LOD for each method indicated the satisfactory sensitivity of the proposed methods. Of the three proposed methods, Method B is more sensitive than Method A. However, despite its lower sensitivity, Method C is highly selective one for analysis of EFX. The longer wavelength of maximum of this method makes it free from obvious photometric errors.

TABLE 1. Sensitivity and Regression Parameters of Proposed Analytical Methods

| Parameter | Method A | Method B | Method C |
|---|---|---|---|
| $\lambda_{max}$, nm | 315 | 275 | 540 |
| Linear range, μg/mL | 1.0–4.0 | 1.0–8.0 | 1.0–20.0 |
| Molar absorptivity (ε), L/mol/cm | $1.52\times10^4$ | $3.86\times10^4$ | $6.6\times10^3$ |
| Sandell sensitivity, μg/cm$^2$ | 0.0236 | 0.0093 | 0.0540 |
| Limit of detection (LOD), μg/mL | 0.32 | 0.13 | 0.54 |
| Limit of quantification (LOQ), μg/mL | 0.97 | 0.41 | 1.65 |
| Intercept ($b$) | 0.0088 | 0.0391 | 0.461 |
| Slope ($m$) | 0.0399 | 0.0963 | –0.0158 |
| Regression coefficient ($r$) | 0.9996 | 0.9913 | –0.9965 |

N o t e: $A= b+mX$, where $A$ is the absorbance, $X$ is the concentration in μg/mL, $b$ is the intercept, and $m$ is the slope.

*Accuracy and precision.* The intermediate precision and accuracy of the proposed methods were evaluated on intra- and inter-day variation basis. The analysis of EFX was performed in seven replicates of each concentration in intra-day analysis. In the study of inter-day variations, the analysis was performed on five different days. Accuracy was estimated and expressed as percentage relative error (%RE) between the measured and taken amount/concentration of EFX and is presented in Table 2. The precision was assessed by calculating RSD values for each case and each concentration of EFX in each method. The percentages of RE and RSD values of less than four indicated the acceptable accuracy and precision of the proposed methods.

TABLE 2. Results of Accuracy and Precision Studies of Proposed Methods

| Method | EFX taken, μg/mL | Intra-day accuracy and precision, $n = 7$ | | | Inter-day accuracy and precision, $n = 5$ | | |
|---|---|---|---|---|---|---|---|
| | | EFX found±CL, μg/mL | %RE | %RSD | EFX found±CL, μg/mL | %RE | %RSD |
| A | 6.0 | 6.19±0.16 | 3.17 | 2.87 | 5.88±0.16 | 2.00 | 2.22 |
| | 12.0 | 11.73±0.35 | 2.25 | 3.22 | 12.31±0.35 | 2.58 | 2.36 |
| | 18.0 | 18.28±0.36 | 1.56 | 2.11 | 17.43±0.66 | 3.17 | 3.04 |
| B | 2.0 | 2.06±0.04 | 3.00 | 1.87 | 1.96±0.05 | 2.00 | 2.22 |
| | 4.0 | 4.13±0.08 | 3.25 | 2.22 | 4.10±0.11 | 2.50 | 2.26 |
| | 6.0 | 5.85±0.11 | 2.50 | 2.11 | 5.80±0.22 | 3.33 | 3.04 |
| C | 5.0 | 4.88±0.09 | 2.40 | 2.07 | 5.14±0.18 | 2.80 | 2.90 |
| | 10.0 | 10.19±0.27 | 1.90 | 2.83 | 9.77±0.32 | 2.30 | 2.68 |
| | 15.0 | 15.22±0.39 | 1.47 | 2.74 | 15.34±0.46 | 2.27 | 2.45 |

N o t e: %RE. Percent relative error, %RSD. Percent relative standard deviation and CL. Confidence limits were calculated from: $CL = \pm tS/\sqrt{n}$ (the value of $t$ is 2.45 and 2.77 for six and four degrees of freedom, respectively, at 95% confidence level; $S$ = standard deviation and $n$ = number of measurements).

*Selectivity by analyses of placebo and synthetic mixture.* From the analysis of placebo blank, the absorbance values were almost the same as that for blanks in all the three methods. This recommended the inactive role exhibited by the ingredients used to prepare the placebo.

The effect of inactive ingredients in the assay of EFX was also checked using the extract or solution of synthetic mixture containing EFX. The aliquots of the resulted EFX extract at three levels of concentrations in each method were assayed thrice by following the general procedures of each method. The analysis yielded the mean percentage recovery of EFX values ranged from 97.15 to 101.4 and therefore, these results confirmed the non-interference from the matrix added to prepare synthetic mixture in the determination of EFX.

*Robustness and ruggedness.* The robustness of the proposed methods was examined by evaluating the influence of small variation in the optimised concentration of HOAc (Methods A and B), volume of HAH (Method B) and volume of *p*-TD (Method C). The analytical wavelengths in each method were also varied by 2 nm. All other parameters were maintained intact. The resulted absorbance values of each variation were used to evaluate the robustness. It was found that small variation in the concentration of HOAc by 0.02 M, volume of HAH and *p*-TD by ±0.1 mL and wavelengths by ±2 nm did not significantly affect the results. The calculated RSD values for each variation are presented in Table 3. The tabulated values of RSD are within 5% and thus clearly reflected the satisfactory robustness of all the three methods.

TABLE 3. Results of Method Robustness and Ruggedness Expressed as Intermediate Precision (% RSD)

| Method | EFX taken, μg/mL | Robustness | | Ruggedness | |
|---|---|---|---|---|---|
| | | Parameter varied | | Inter-analysts, %RSD ($n = 4$) | Inter-instruments, %RSD ($n = 4$) |
| | | Concentration of HOAc, 0.1±0.02 M | Volume of reagent* | | |
| A | 6.0 | 2.13 | – | 3.23 | 2.22 |
| | 12.0 | 3.22 | | 3.12 | 2.45 |
| | 18.0 | 2.88 | | 2.88 | 2.87 |
| B | 2.0 | 1.45 | 2.32 | 2.34 | 2.78 |
| | 4.0 | 1.78 | 2.27 | 2.65 | 2.45 |
| | 6.0 | 1.89 | 2.87 | 2.44 | 2.82 |
| C | 5.0 | – | 3.16 | 2.12 | 1.99 |
| | 10.0 | | 3.01 | 3.10 | 1.74 |
| | 15.0 | | 2.97 | 2.67 | 2.31 |

*The volumes of 0.05% HAH and 0.5% *p*-TD were varied by 1±0.2 mL in methods B and C, respectively.

Ruggedness was tested by applying the method to the assay of EFX using the optimum experimental conditions, but using four different spectrophotometers. The analysis was also performed by four different analysts and the variations were checked. Results obtained from the above variations were reproducible. The obtained RSD values were ranged between 3.2 and 5.1% (Table 3) and this proved the rugged nature of the three new proposed methods.

*Application to tablet analysis.* Commercial EFX tablets were analysed using the developed methods and the results were compared with USP method [2]. The USP method describes the non-aqueous potentiometric titration of EFX in acetic acid medium with 0.1 M $HClO_4$. The statistical tests were carried out for results of the proposed and reference methods by applying Student's *t*- and *F*- tests. The mean recovery of EFX from the proposed and reference methods were 97.3 and 98.6%, respectively, with the corresponding RSD values of 1.21 and 0.87%. The calculated *t*- and *F*-values did not exceed the tabulated values at 95% confidence level ($t_{crit}$ = 2.77 and $F_{crit}$ = 6.39). These values have the outcome that there were no significant differences between the results of the proposed and reference methods with respect to accuracy and precision. This also meant that the proposed analytical procedures are as accurate and precise as that of the reference method. The detailed results are presented in Table 4.

TABLE 4. Results of Proposed Methods for Analysis of Tablets Ataxin (150) and Statistical Comparison with the Reference Method

| Percent (of label claim) EFX found* ± SD | | | |
|---|---|---|---|
| USP method | Method A | Method B | Method C |
| 98.6±1.48 | 97.3±1.25<br>*t* = 1.50<br>*F* = 1.40 | 96.7±1.18<br>*t* = 2.26<br>*F* = 1.57 | 97.9±0.68<br>*t* = 1.02<br>*F* = 4.74 |

*Mean value of five determinations.

*Recovery study.* The accuracy and reliability of the methods were further ascertained by recovery experiments *via* standard addition procedure. Pre-analysed tablet extract was spiked at three different concentration levels of pure EFX solution and the triplicate contents were subjected to analysis by following the general recommended procedures of the proposed methods. The total EFX found was calculated. In all cases, the percentage recovery of pure EFX out of total found was ranged from 97.0 to 102.4 with standard deviation values of 1.13 and 2.36% and this indicated the absence of interference from excipients of tablets in the determination. Also, the recovery values for EFX were highly satisfactory. The results of recovery experiments are summarised in Table 5. Tabulated *t*- and *F*-values at the 95% confidence level and for four degrees of freedom are 2.77 and 6.39, respectively.

TABLE 5. Results of Recovery Experiments Performed by Following Standard-Addition Procedure using Atarax (15 mg EFX/tablets) Tablets

| Method | EFX in tablet, μg/mL | Pure EFX added, μg/mL | Total found, μg/mL | Pure EFX recovered, %*±SD |
|---|---|---|---|---|
| A | 6.0 | 3.0 | 8.97 | 99.00±2.23 |
| | 6.0 | 6.0 | 11.82 | 97.00±2.31 |
| | 6.0 | 9.0 | 14.84 | 98.22±2.13 |
| B | 2.0 | 1.0 | 3.01 | 101.00±1.93 |
| | 2.0 | 2.0 | 3.99 | 99.50±1.72 |
| | 2.0 | 3.0 | 4.95 | 98.33±2.29 |
| C | 5.0 | 2.5 | 7.56 | 102.40±1.13 |
| | 5.0 | 5.0 | 9.89 | 97.80±1.76 |
| | 5.0 | 7.5 | 12.39 | 98.53±2.36 |

*Mean value of three determinations.

*Stability indicating ability.* The stability indicating property of EFX was studied by inducing the drug to acid, alkali, oxidative, photocatalytic, and thermal degradations. The study was based on the measurement of the absorbance of EFX solutions after subjecting to forced degradation and calculation of the percentage recovery of drug. It was clear from the absorption spectra of EFX resulting from acid and alkaline hydrolysis, hydrogen peroxide induced, thermal and UV degradation that no significant degradation occurred. Therefore, it is confirmed that EFX was not susceptible to acid and alkali to undergo degradation. Besides, EFX was also found intact after subjecting to oxidative, photo and thermal degradations. The results of stress studies for EFX are presented in Table 6.

TABLE 6. Results from Stability Studies for EFX after Inducing to Acid, Alkali, Oxidative, Photocatalytic/uv and Thermal Conditions in Method A

| Stress condition | EFX taken, μg/mL | EFX found*, μg/mL | %Recovery of EFX±SD |
|---|---|---|---|
| Acid | 12.00 | 11.87 | 98.92±1.02 |
| Alkaline | 12.00 | 11.79 | 98.25±1.93 |
| Oxidative | 12.00 | 11.90 | 99.17±2.15 |
| Thermal | 12.00 | 11.89 | 99.08±2.03 |
| Photocatalytic/UV | 12.00 | 11.67 | 97.25±1.59 |

*Average of five determinations.

**Conclusions.** Three new spectrophotometric methods were developed and validated for determination of EFX. The methods are highly selective to determine EFX in pure form and in tableted form. The methods are simpler and economic when compared to published methods with respect to experimental conditions and instrumental setup. The proposed methods are free from using expensive chemicals and reagents. Methods are rapid and allow determining drug in tablets without any interference from the common tablet excipients. The experimental conditions are very simple to adopt and there is no difficulty in variations of factors. Using these methods, a small concentration of drug can be determined with confidence and with a fair degree of accuracy and precision. Besides, the stability results are easily derived for EFX with Method A and the property of drug was seen to remain intact for acid, alkali, oxidative, thermal and photocatalytic or UV degradations. Hence, it would be possible to recommend these methods as routine quality control procedures for determining EFX.

**Acknowledgements.** The author thanks Cipla India Ltd, India, for gifting pure enrofloxacin drug for analyses. The author is very grateful to the Principal of JSS College of Arts, Commerce and Science, Ooty Road, Mysuru, India, and JSS Mahavidyapeetha, Mysuru, India for providing the facilities to pursue this work.

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