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RESEARCH ARTICLE

Cloud Point Extraction Spectrophotometric Method for Mutual Determination of Norfloxacin and Iron(III) in Human Serum and Drug Formulations

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Abstract: An eco-friendly procedure was developed for dual detection of drug norfloxacin (NOR) and iron(III) ions in biological and pharmaceutical samples via the coupling of cloud-point extraction procedure with molecular spectrophotometry. The drug norfloxacin reacts with Fe(III) ions in dilute acidic medium to form a colored hydrophobic (Fe(III)-NOR) complex which initially extracted into micelles of Triton x-114 as a mediated extractant followed the determination of both NOR and Fe(III) ions individually, by using spectrophotometry at same wavelength maximum of 432 nm. To achieve this goal, all experimental variables for both target analytes were previously optimized. The results have shown that the preconcentration factors of 83 and 71 for NOR and Fe(II) leading to achieve a limit of detection of 0.692 μg mL^{-1} 3.42 ng mL^{-1} with linear range of 2.5-125 μgmL^{-1} (r= 0.9998) and 5-150 ng mL^{-1}(r=0.9997) respectively. The mean recovery percentage of 98.95±1.09% and 98.77±2.29%; the precision (RSD%) ranged between 0.04-0.66% and 0.59-0.97% were obtained for NOR and Fe(III) respectively. The proposed method was used for the determination of NOR in the serum samples of different subjects orally administration for two hours with Norfloxacin tablets BP 400 mg, in addition to drug formulations like Norflox Eye/Ear drops, While iron was determined in four selected drug formulations and in both cases, the experimental values are agreed with the quoted values that stated by the manufacturer company,

Keywords: Norfloxacin, Iron(III), Hydrophobic complex, Human cerum, Cloud coint cxtraction, Spectrophotometry

Introduction

The mutual determination of two analytes or more in the same reaction system by one procedure can be considered one of the charming works in analytical chemistry to simplify analytical methods, which would inevitably result in a reduction in the analyst effort, time and chemicals. From this perspective and the lack of published works in this trend, the authors decided to resolve to engage in this attempt to develop analytical methods based on the existing of chemical reaction systems, to contribute to the opening of new horizons in analytical chemistry. This work was relied on the possibility of the reaction of some drugs

with iron(III) ions to form a colored complex which can be extracted by one of extraction methods; thereby the drug and iron ions are determined by the matching procedure. The complexometric reaction system chosen in this study is the reaction of a norfloxacin (abbreviated as NOR or NF) drug with iron(III) ion in acidic medium to form a soluble Fe(III)-NOR complex. The main reason for selection of this reaction because the drug NOR and Fe(III) have a great importance to humans.

Norfloxacin (Figure 1) is a second generation fluoroquinolone antibacterial and commonly used in the treatment of urinary tract infections, gonococcus urethritis and infectious diarrhea[1-2]. It is chemically known as 1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid, its empirical formula is $C_{16}H_{18}FN_3O_3$, molecular weight of 319.34 g/mole, white or pale yellow crystalline powder, very slightly soluble in water, slightly soluble in acetone and in ethanol[3-4].

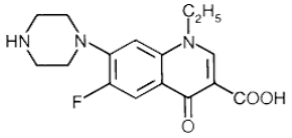

Figure 1. Chemical structure of norfloxacin

Many instrumental methods have been reported in chemical literature revealed that this medicament was determined alone or in combined dosage form including; high performance liquid chromatographic methods[5-8], electroanalytical methods[9-11], electro-generated chemiluminescence[12], atomic absorption spectrometry[13] and spectrophotometry[14-17]. Although of these methods, NOR is the subject of a monograph in most international pharmacopoeias such as the British Pharmacopoeia, BP[3], United States Pharmacopoeia, USP[18] and Indian Pharmacopoeia[19], which have recommended a non aqueous titration for the raw material and HPLC technique for tablets. However, there are only two papers appeared in literature concerning the determination of NOR by using HPLC in complex matrices such as human serum[20] and milk products. Although HPLC has a good detection power, but it does not exist in most laboratories, need an internal standard and sometimes involve more than one extraction step[21]. It was reported that the use of molecular spectrophotometry combined with cloud point extraction (CPE) provides attractive features which drive toward simplification and facilitation of the analytical procedures in routine analyses of organics and metal ions in different matrices instead of using sophisticated and expensive instrumentations[22,23].

To the best of our knowledge, there is no work reported in chemical literatures concerning the determination of NOR drug whether in biological samples or drug formulations utilizing CPE-Spectrophotometric method. Therefore, the present study is an attempt to establish a new procedure for the extraction of NOR drug as well as iron(III) ions based on the complexation reaction system, in human serum and drug formulations using CPE followed their determination by molecular spectrophotometry.

Experimental

The absorption spectra and absorbance measurements of both analytes were carried out by using a Shimadzu double-beam UV-Vis Spectrophotometer model UV-1800 (Kyoto, Japan) equipped with 10 mm optical path cell. A double-beam Atomic Absorption Spectrophotometer AA-6300(Shimadzu, Japan) equipped with a burner unit made of titanium (10 cm slot) air-cooled premixed for air/acetylene flame was used for checking of the residual iron in aqueous solution. A portable pH-meter microprocessor (HANNA, Germany) was used for checking the pH of the solution. Thermostatic water bath (WNB7-45) Experts (England) was used for CPE experiments.

Reagents and Materials

Pure grade norfloxacin (active ingredient of ANFlOX GOLD, purity 99.2%) was obtained from the Directorate General of Drug Supervision / Baghdad-Iraq. A stock solution of 1000 μg mL^{-1} for norfloxacin was prepared by dissolving 100 mg in a minimum amount of $5.0x10^{-3}$ M sulfuric acid and dilutes to mark with doubly distilled water in 100 mL volumetric flask. This solution was stored in the refrigerator and working solutions were daily prepared by appropriate dilutions in water. Triton X-114 (purity >99.9%), was available from Acros Organics, New Jersey, USA. A 10% (v/v) of Triton X-114 was prepared by diluting 10 mL in a 100 mL water. A stock solution of Fe^{3+} (1000 mg L^{-1}) was prepared by dissolving 0.8634 g of pre-dried ammonium ferric sulfate (BDH) in 0.050 M sulfuric acid (BDH) in a 100 mL volumetric flask. This solution is used after at least 24 h to guarantee complete dissolution. A 0.1 M sulphuric acid (1 M) solution was prepared by diluted 5.43 mL of 98% H_2SO_4(1.84 g/mL, BDH) with distilled water in 1 L calibrated flask. Ethanol was purchased from (Abo Teeba Co. Iraq). All additives used such as, calcium gluconate, calcium stearte, copper sulphate, dextran, manganese chloride, nicotinamide, potassium nitrate and sodium glycerophosphate were analytical grade and purchased from BDH(UK). Doubly distilled and deionized water was used throughout this work.

General CPE procedure for NOR

In a 10 mL volumetric flask, an amount of norfloxacin standard or sample solution matched within calibration range, l.0 mL ferric ion solution (100 μgmL^{-1}), 0.25 mL of H_2SO_4 ($1x10^{-4}$M) and 1% Triton X-114 (10% v/v) were added, mixed and dilute with to mark with water. The content of the flask was transferred into a 10 mL centrifuging tube and then kept in the thermostatic bath at 65 °C for 20 min. Separation of the phases was conducted by centrifugation at 6000 rpm for 20 min and thereafter cooled down in an ice bath to increase the viscosity of the surfactant-rich phase. The aqueous phase was easily poured by inverting the tube. The surfactant-rich phase that contains the complex was dissolved with suitable amount of ethanol and the absorbance of the complex measured at 432 nm against a reagent blank prepared under similar conditions. The remaining NOR in aqueous solution was determined by traditional spectrophotometry at λ_{max} of 273 nm in order to determine the distribution ratio (D) and extraction efficiency (%E).

General CPE procedure for Fe(III) ions

To an aliquot of 10 mL of a solution containing known amount iron(III) standard or sample solution matched within calibration range, 1.4 mL of $5.0x10^{-4}$ mol L^{-1} NOR reagent solution, $2.5x10^{-4}$ M of H_2SO_4 for NOR complex, 0.6 mL of Triton X-114 (10%) were mixed in a 10 mL standard flask and diluted to mark with distilled water and then followed the same general CPE for NOR. The iron(III) content was measured at λ_{max} of 432 nm. The residual iron in aqueous solution was measured by FAAS at 248.3 nm in order to determine the distribution ratio (D) and extraction efficiency (%E).

Preparation of serum samples for NOR detection

The proposed method was applied to determination of NOR using Fe(III) in blood serum samples for the 10 volunteers whom orally administration with a single tablet of Norfloxacin Tablets BP 400 mg (C.B Pharma Pvt. Ltd., India). None of the volunteers was ill or taking any medication at the time of this administration. After two hours of administration, blood sample (5 mL) was withdrawn from the vein of each subject by using medical syringe and then transferred immediately into centrifuging tubes. The content of each tube was centrifuged

for 15 min to remove the serum from the whole blood and all serum samples were kept freeze in refrigerator until analysis[24]. The serum samples were thawed at ambient temperature and 0.2 mL of each serum sample was transferred into a10 mL volumetric flask and followed the general CPE procedure for NOR. The content in the complex determined spectrophotometrically at λ_{max} 432 nm. Meanwhile, 0.2 mL of each sample was transferred into a 10 mL volumetric flask and NOR content was directly determined by traditional UV-Vis spectrophotometry at λ_{max} of 273 nm, for the purpose of comparison.

Sample preparation of pharmaceuticals for NOR detection

The proposed method was also used for determination of NOR using Fe(III) in the one of the selected pharmaceutical preparations containing NOR such as Norflox /eye drop (Taj Pharmaceuticals Ltd., India) with stated value of 0.3%. The drop eye was analysed for NOR after appropriate dilution with water and subjected to the general CPE procedure and NOR content was determined spectrophotometrically at λ_{max} of 432 nm.

Sample preparation of drug formulations for Fe(III) detection

Iron content using NOR as a chelating agent was determined in four types of drug formulations. These are Iron Dextran (50 mg/mL) injection produced by USP Pharma Roth Wiesbaden (Germany), Iron Dextran (100 mg/2 mL) produced by Cox-pharmaceutical Ltd.,G.B, England (100 mg/2 mL), tot'héma Iron gluconate (50 mg/10 mL) solution produced by laboratories' Innotech International (FRANCE) and Ironorm Syrup (250 mg /5 mL) produced by Wallace Manufacturing Chemists Ltd. (England). Each formulation was analyzed after appropriate dilution with water, subjected to the general CPE procedure for iron and Iron content was determined in Fe-NOR complex spectrophotometrically at λ_{max} of 432 nm.

Results and Discussion

Absorption spectra

Spectrophotometric investigations were carried out to ensure the formation of the complex via the reaction of Fe(III) ions and NOR in acidic medium and in the presence of the surfactant. The absorption spectra of $2.5x10^{-4}$ M NOR solution, $1.25x10^{-4}$ M Fe(III) solution and Fe(III)-NOR complex (10 mL aqueous solution containing $2.5x10^{-4}$ M NOR, $2.5x10^{-4}$ M H_2SO_4, $1.25x10^{-4}$ M Fe(III), $5x10^{-4}$ and 1 mL of 10% TX-114 followed general CPE procedure) were recorded against blank solutions, between 190 and 1000 nm using a Shimadzu model UV-1800 equipped with 1 cm quartz cell. It was observed that there is a remarkable absorption bands with shoulder occurred at 432 nm, indicating the formation of a complex between Fe(III) ions and NOR drug. Whilst pure NOR solution gave two absorption maxima at 273 and 332 nm. The Fe(III) ion solution displays one distinct band at 300 nm. All these spectra are presented in Figure 2. Therefore, the wavelength maximum of 432 nm for Fe(III)-NOR complex was chosen throughout this study.

Optimization of CPE procedure

A series of experiments have been performed for the purpose of studying the effect of some important factors affecting the efficiency of the cloud point extraction using a classical optimization. Classical optimization suggests observing the effect of one factor at a time (OFAT) on the instrumental response while other factors are maintained at constant level. Thus, a satisfactory sensitivity of the measurement can be achieved. In this regard, the factors such as, H_2SO_4 concentration, Fe(III) concentration, Triton X-114 amount, and equilibration temperature and incubation time were investigated.

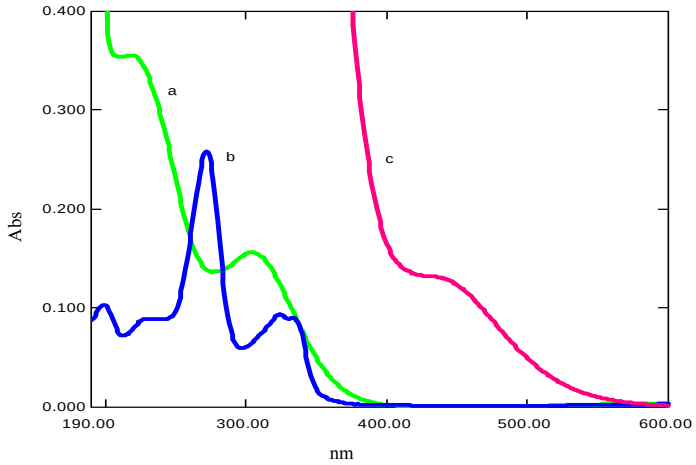

Figure 2. Absorption spectra (a) Fe(III) solution (b) Reagent Norfloxacin and (c) Fe(III)-NOR complex

Effect of H_2SO_4 concentration

It was found that the concentration of H_2SO_4 plays an important role in the complexation reaction between Fe(III) and NOR) drug and it is responsible for the stoichiometry of the complexation[25], in addition, the acidic medium has an important effect in the thermodynamic equilibrium for the formation and stability of chelate extracted by CPE. Therefore, the effect of H_2SO_4 concentration on the formation Fe-NOR complex in Triton X-114 medium was investigated by recording the absorbance signals at λ_{max} of 432 nm over the range of $1x10^{-2}$ -$1x10^{-4}$ M of H_2SO_4. The experiments were performed with 10 μgmL^{-1} Fe(III), 80 μgmL^{-1} drug NOR and 1% of (10%v/v) Triton X-114. The results are presented in Figure 3. It was pointed out that the absorbance increased with increasing H_2SO_4 concentration and reached a maximum at $2.5x10^{-4}$ M H_2SO_4. Thereafter, it suddenly decreases at high concentration which may be expected to result in dissociation of the complex and in incomplete extraction in micelle due to the shifting in the formation reaction toward left. Consequently, a concentration of $2.5x10^{-4}$ M H_2SO_4 which corresponds to ionic strength of $0.75x10^{-3}$ was selected as the optimal for complete formation of Fe(III)-NOR complex.

Effect of Fe(III) ions concentration

The effect of Fe(III) concentration has also a significant role in the thermodynamic equilibrium for the formation and stability of the complex in acidic medium extracted by CPE. Accordingly, the effect of the Fe(III) concentration was studied by measuring the absorbance signals according to the general CPE procedure for the solution containing 80 μgmL^{-1} NOR, $2.5x10^{-4}$ M H_2SO_4 for NOR complex**,** 1% of (10% v/v) of Triton X-114 and varying concentration of the Fe(III) solution ranged from 0.5-12 μgmL^{-1}. The results are illustrated in Figure 4. The results revealed that the best concentration of Fe(III) ions which gave a maximum absorbance for the formation and extraction of the complexes with high efficiency in cloud point layer (CPL) was a 10 μgmL^{-1} for NOR complex. At more excess of ferric ion solution, the responses were decreased and subsequently the extraction efficiency was also reduced due to the deviation of the equilibrium toward the backward reaction because of law of mass action. Whilst at lower concentration of metal ion than

optimum, there is no chance for complex formation completion resulting in less amount of the drug complex extracted into the CPL. Thereafter, 10 µg mL^{-1} was found to be enough for the complex formation Fe(III)-NOR thereby was it used as optimum throughout this study.

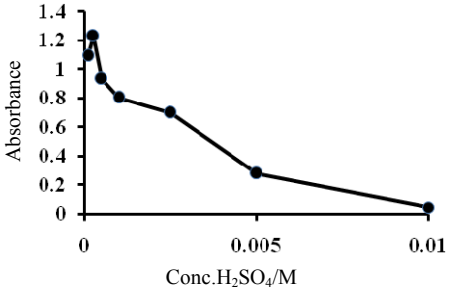

Figure 3. Effect of H_2SO_4 concentration on the formation of Fe(III)-NOR complex

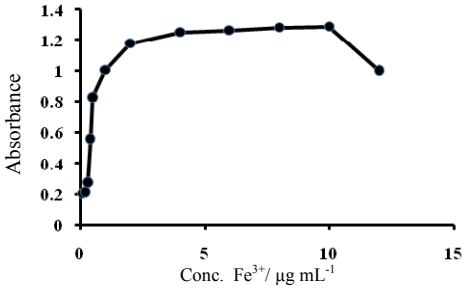

Figure 4. Effect of iron(III) concentration in the formation of Fe-NOR complex by CPE

Effect of triton X-114 amount

The concentration of the surfactant has an important influence in determining the efficiency of the extraction according to the cloud point extraction method, because its important stems from maximizing extraction efficiency by minimizing the phase volume ratio (V_s/V_a), thus improving its perconcentration ability[26]. To illustrate this role, the effect of the triton x-114 concentration on the absorbance of the extracted Fe(III) -NOR complex was studied within the surfactant volume range of 0.1-2 mL of 10% (v/v) triton x-100 and keeping other variables constant. The results are presented in Figure 5. It was noted that the absorbance of the complex increased by increasing the Triton x-114 concentration up to 1.0 mL of 10% (v/v) and then markedly decreased at higher amounts. Thus, a volume of 1.0 mL of 10% Triton x-114 which corresponds to 1.0% of surfactant, representing the optimum amount to reach a state of equilibrium for the extraction process that lead to the formation of point cloud layer with the smaller volume and higher viscosity. For low amount of triton x-114, low sensitivity or extraction efficiency occurs owing to the inadequacy of the assemblies to entrap the hydrophobic complex quantitatively. While at higher amount led to deteriorating the detection signal and thus inefficient extraction. Therefore, 1.0 mL of 10% (v/v) Triton x-114 was used as the optimum amount.

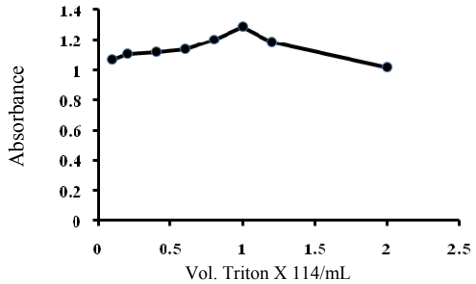

Figure 5. Effect of triton x-114 amount on the analytical signal for Fe(III)-NOR

Effect of temperature and time

To study the effect of temperature on the extraction of Fe(III)-NOR complex, a series of experiments were carried out by taking 10 mL aqueous solution containing all the components

at previously optimized conditions and varying the temperature from 25 to 85 °C at incubation time of 20 min. It was shown that the maximum absorbance for Fe (III)-NOR complex was achieved at 60 ºC (Figure 6). Thereafter, CPE efficiency of the target complex was decreased by increasing temperature. Thus, an equilibration temperature of 60 ºC for maximum extraction of Fe(III)-NOR complex were chosen as optimal. Also, CPE protocol need sufficient time to attain the equilibrium between two phases (*i.e.* surfactant-rich and bulk aqueous phases) via the gathering the surfactant micelles. Accordingly, series of experiments was conducted for extraction of 10 mL solutions containing all reagents at optimum conditions but with varying incubation time from 4 to 40 at 65 ºC. It was noticed that the incubation time of 20 min was enough for the maximum absorbance of Fe(III)-NOR complex as shown in Figure 7. The influence of centrifugation rate and time was also reckoned. It noticed that a centrifugal time of 20 min at 6000 rpm was enough to discriminate between two phases.

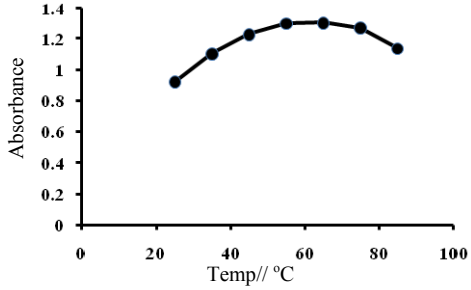

Figure 6. Effect of equilibration temperature on the CPE of Fe (III)-NOR complex

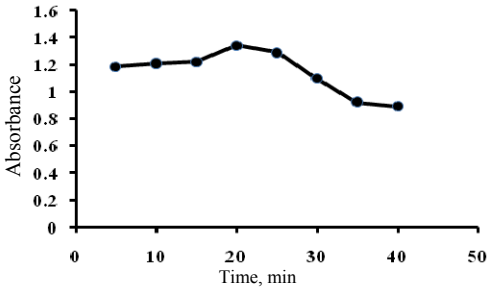

Figure 7. Effect of incubation time on the CPE of the Fe (III)-NOR Complex

Optimization parameters for Fe(III) using NOR

The constant parameters as with NOR determination were optimized, but a discrete variable here is the concentration of NOR reagent. At constant and ultra trace concentration of Fe(III) of 50 ngmL^{-1}, the optimum conditions of most parameters were similar to that obtained in the case NOR optimization, except the amount of Triton x-114 was of 0.6% (v/v) and the optimum concentration of NOR needed for complexation with Fe (III) at nanogram amount was of 7x10^{-5} which correspond to 22.3 µgmL^{-1} of NOR concentration.

Stoichiometry of the Fe(III)-NOR complex

In a preliminary study, it was shown that a deep yellow color is produced instantly when Fe(III) ion solution is added to a certain amount of NOR drug in the presence of a wide concentration of sulphuric acid, indicative the complex formation. But, in this study the optimum concentration of H_2SO_4 of 2.5x10^{-4} M was sufficient to form the coloured complex which appeared as a shoulder peak extended to the visible region with maximum absorbance at 432 nm. Consequently, mole ratio method was used to measure the stoichiometry of Fe: NOR ratio at optimum concentration of sulphuric acid medium. This method was conducted at constant amount of Fe(III) with varying amount of NOR at optimum conditions as showed in Figure 8. Obviously, the plotted curve exhibits a maximum for a mole ratio of Fe(III)-NOR complex via the point of intersection of the two lines which approach to 2.3, indicating that the expected ratio of Fe: NOR in the complex was of about 1:2.

The Job plot (continuous variation method) was also confirmed similar result to molar ratio method which exhibited that the mole fraction close to 0.50, indicating again that the ratio of iron(III):drug in the complex is 1:2 as displayed in Figure 9.

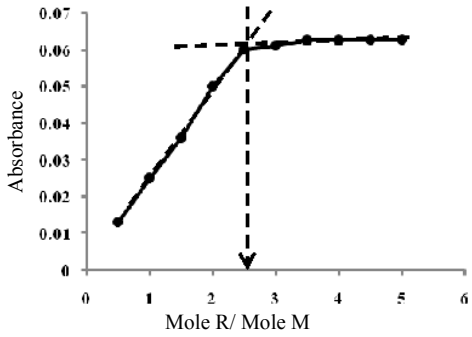

Figure 8. Mole ratio method for Fe(III)–NOR complex

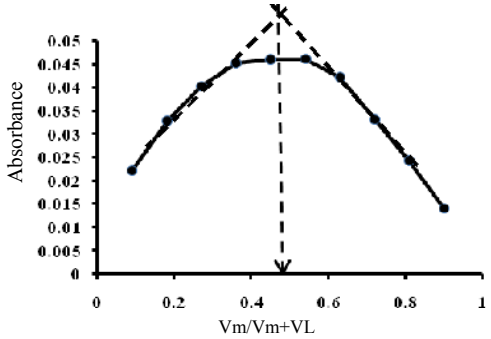

Figure 9. Continuous variation method for Fe(III)–NOR complex

Our finding was in agreement with results obtained by Idrees *et al*.,[27], Suliman and Sultan[25], but they do not agree with the results achieved by other workers[28] where a 1: 1 complex was obtained, when iron(III) was complexed with ciprofloxacin or norfloxacin at acidities higher than 0.025 M. By assuming only a single complex is present, the stability constant (K_f) of the Fe(III)-NOR complex can be determined according to the procedure adopted elsewhere[29] by using the above data and found to be of 2.12×10^{10} at 432 nm. On the basis of above results, the most probable structure of the complex formed between Fe(III) and NOR in dilute acidic medium is displayed in Figure 10.

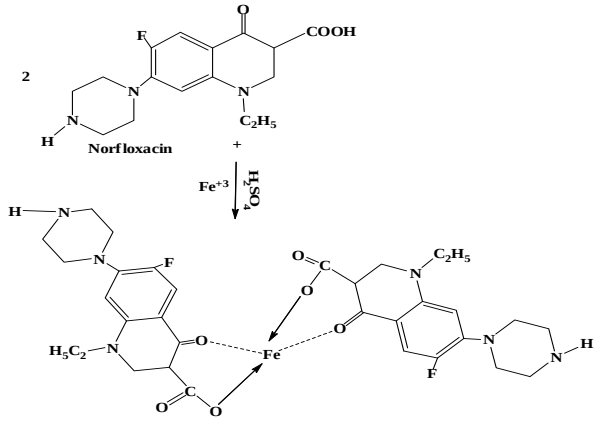

Figure 10. The probable structure of the complex formed between Fe(III) and NOR in acidic medium

From thermodynamic point of view[30], the standard free energy (ΔG°) of complexation is related to the formation constant by the following equation;

$$\Delta G^{o} = -2.303\ RTK$$

Where R is the gas constant (1.987 cal mol^{-1} $degree^{-1}$), T is the optimum temperature in Kelvin and K is the formation constant of Fe-NOR complex (L mol^{-1}). The standard free energy ΔG° for the complexation reaction of between Fe(III) ions NOR drug was of a negative value and found to be of (-15.97) kcal mol^{-1}, indicating that the complexation reaction is a spontaneous.

Calibration graphs for NOR drug and iron(III) ions

Under optimum conditions established, two calibration curves were constructed, once for NOR drug using Fe(III) and another for Fe(II) ions using NOR drug. Tables 1 and 2 are summarized the statistical evaluation of the two curves showing all figures of merit for target analytes.

The calibration curve of the studied NOR drug using Fe(III) after CPE was linear over the concentration range of 2.5-120 μgmL^{-1} (r=0.9998, 10 points). The preconcentration factor achieved was of 50 fold led to obtaining the limit of detection (LOD) of 0.21 μgmL^{-1} (Table 1). Concerning LOD, our findings was better than with the results obtained by Singh *et al*.,[6], Wagh *et al*.,[9], Shinde *et al.,*[14], Abou-Taleb *et al.,*[15] and Pant *et al.,*[16]. But, it was in agreement with works of Patel *et al.,*[7] and Hanwen *et al*.,[10]. However, it was worse than those obtained by Sher *et al.,*[5], Ahmad *et al*.,[20] used HPLC with UV and fluorescent detectors. The calibration curve of Fe(III) ions using NOR drug as chelating agent was linear over the range of 5-150 $ngmL^{-1}$ (r=0.9997, 10 points). The preconcentration factor obtained was of 83 fold resulted in achieving the limit of detection (LOD) of 3.42 $ngmL^{-1}$ (Table 2). Concerning LOD, our findings was 5 fold more better than obtained by Giokas *et al*.,[31] who used CPE-FI-AAS , but about 2 fold less than those obtained by Ohashi *et al*.,[32] and Shakerian *et al*.,[33] whom used CPE-GFAAS and CPE-FI-AAS. The molar absorptivities, Sandell's sensitivities, extraction efficiencies for NOR and Fe(III) were also given in Tables 1 and 2, indicative the proposed method has a good sensitivity and extractability.

Table 1. The statistical data and analytical figures of merits for NOR determination using Fe(III) by CPE- spectrophotometry

Parameter	value
λ_{max} nm	432
Regression equation with CPE procedure	y = 0.0188x+ 0.0101
Correlation coefficient(r)	0.9998
Coefficient of determination (R^2)	99.98%
C.L. for the slope ($b\pm ts_b$) at 95%	0.0188±0.000243
C.L. for the intercept ($a\pm ts_a$) at 95%	0.0101±0.016512
Concentration range, μgmL^{-1}	2.5-120
Limit of Detection, μgmL^{-1}	0.21
Limit of Quantitation, μgmL^{-1}	0.69
Sandell's sensitivity (mg cm^{-2}/0.001A.U)	0.054
Molar absorptivity, $L.mol^{-1}.cm^{-1}$	$1.3x10^4$
Compsition of complex (Fe-NOR)*	1:2
RSD% (n=3) at 80 μgmL^{-1}	0.46
Preconcentration factor	50
Enrichment factor	280.6
Distribution ratio(D)	28.57
Extraction efficiency(%E)	96.62

**Job's and mole ratio methods*

Table 2. The statistical data and analytical figures of merit for Fe(III) determination using NOR drug by CPE-spectrophotometry

Parameter	value
λ_{max}, nm	432
Regression equation with CPE procedure	y=0.00142x+0.0215
Std. deviation of regression line ($S_{y/x}$)	0.001625
Correlation coefficient(r)	0.9997
Coefficient of determination (R^2)	99.95%
C.L. for the slope (b± ts_b) at 95%	0.00142x±2.5x10^{-5}
C.L. for the intercept (a± ts_a) at 95%	0.0215±0.00226
Concentration range, ngmL^{-1}	5-150
Limit of Detection, ngmL^{-1}	3.42
Limit of Quantitation, ngmL^{-1}	11.40
Sandell's sensitivity, mg cm^{-2}/0.001A.U	7.2x10^{-4}
Molar absorptivity, L.mol^{-1}.cm^{-1}	1.1x10^{6}
Composition of complex (Fe-CIPRO)*	1:2
RSD% (n=7) at 50 ngmL^{-1}	1.96
RSD%(n=7) at100 ngmL^{-1}	0.76
Preconcentration factor	83
Distribution ratio(D)	82.3
Extraction efficiency(%E)	98.7

**Job's and mole ratio methods used*

Accuracy and precision

To test the proposed method in term of freedom from systematic errors for the detection of NOR drug, accuracy was determined via the spiking three blank serum samples with 15, 20 and 25 µg mL^{-1} of NOR taking from the drug Norflox (3000 µgmL^{-1}) solution produced by Mumbai Central Pharmaceutical Industry –India, and containing only chloride and water as additives. The three spiked sample were subjected to the general CPE procedure for NOR. The results were displays in Table 3, revealed that a good accuracy in term of percent recovery can be achieved within the range of 98.95±1.09%, due to the absence of the systematic errors, concluding the presence of serum constituents and drug matrix have no effect on the determination of NOR drug using Fe(III). Meanwhile, each spiked sample was repeated five times for precision testing in term of %RSD and found in the range between 0.04 and 0.661%, indicative a good precision. For Fe(III) using NOR drug as chelating agent, three Fe(III) standard solutions at concentration of 30, 50 and 70 ngmL^{-1} were sipked with the drug Iron Gluconate produced by laboratories' Inno Tech International (France), containing iron at 50 mg/10 mL with additives such as manganese and copper gluconate, at appropriate concentration so that they do not exceed the established linear curve. Each spiked solution was subjected to the general CPE procedure for iron and iron was determined in Fe(III)-NOR complex by the established method with three replicate measurements. The findings presented in Table 4 explicates that a good recovery within the range of 98.77±2.29%, indicative the proposed method with good accuracy and precision.

Table 3. The accuracy and precision of the proposed method for the determination of NOR using Fe(III) in blood serum

Amount of NOR taken, µgmL^{-1}	Amount of NOR found, µg mL^{-1}	Recovery %	E_{rel} %	Mean Rec.±ts/√n, %	RSD % (n=5)
15	14.88	99.20	-0.800	98.95±1.09	0.661
20	19.84	99.20	-0.800		0.330
25	24.61	98.44	-1.56		0.040

Table 4. The accuracy and precision of the proposed method for the determination of Fe(III) using NOR in drug formulation

Amount Fe(III) ion taken, ng mL^{-1}	Amount Fe(III) ion found, ng mL^{-1}	Recovery %	E_{rel} %	Mean Rec.±t.s/√n, %	RSD, % (n=3)
30	29.33	97.76	-2.23		0.96
50	49.48	98.96	-1.04	98.77±2.29	0.73
70	69.71	99.58	-0.41		0.59

Interference study

To test the selectivity of the suggested method for Fe(III) determination using NOR drug, the interference study was conducted by the addition of 100, 1000 and 10000 fold more of each excipient to 80 $ngmL^{-1}$ Fe(III) standard solution. The results are listed in Table 5.

It can be seen from Tables 5, there is no appreciable effect of most additives in the drug formulations in the determination of iron using NOR drug as chelating agent (%E_{rel} less than ±5%)[34], except of sodium glycerophosphate and nicotinamide causes severe interferences on the absorbance of iron. Therefore, these two interfering components should be removed or masked before determination of iron, or added to standard Fe(III) solutions before the construction of the calibration curve.

Table 5. Effect of excipients on the absorption signal of Fe(III) ions (80 $ngmL^{-1}$ and 0.137 absorbance unit) by CPE-spectrophotometry using NOR.

Interferent	Interferent/ Fe(III) ratio	A	ΔA	E_{rel} %
Cu(II) as Sulphate	100	0.141	0.004	2.91
	1000	0.142	0.005	3.60
	10000	0.142	0.005	3.60
Mn (II) as Chloride	100	0.136	-0.001	-0.72
	1000	0.135	-0.002	-1.45
	10000	0.136	-0.001	-0.72
Ca(II) as Stearte	100	0.139	0.002	1.45
	1000	0.139	0.002	1.45
	10000	0.140	0.003	2.18
Ca(II) Gluconate	100	0.140	0.003	2.18
	1000	0.143	0.006	4.37
	10000	0.143	0.006	4.37
Dextran	100	0.137	0.000	0.00
	1000	0.137	0.000	0.00
	10000	0.136	-0.001	-2.72
Sodium	100	0.143	0.006	4.47
Glycerophosphate	1000	0.159	0.022	16.05
	10000	0.163	0.026	18.97
Nicotinamide	100	0.166	0.029	21.16
	1000	0.193	0.056	40.87
	10000	0.219	0.082	59.85
K(I) as Nitrate	100	0.138	0.001	0.72
	1000	0.138	0.001	0.72
	10000	0.140	0.003	2.18

Determination of NOR in serum samples and pharmaceuticals

The proposed method was applied for the determination of NOR in blood serum samples for ten volunteers whom orally administration with a single tablet of Norfloxacin Tablets BP 400 mg. The results of the combined CPE-Spectrophotometry were compared with traditional spectrophotometric method in our laboratory as displayed in Table 6. The paired *t*-test was applied to evaluate the significance level of results obtained between the proposed method and the classical spectrophotometry for further checking of the applicability and reliability of CPE-spectrophotometric methodology.

Table 6. Determination of NOR in human serums by the proposed method and traditional UV-Vis spectrophotometric method

Serum Sample No.	Proposed method μg mL^{-1}	direct UV-Vis method μg mL^{-1}	Paired t- test
1	39.81	39.22	
2	38.94	36.52	$\overline{X}_d$ = 0.529
3	57.90	57.25	S_d=0.82868
4	35.76	36.11	$t_{cal(n=10)}$=2.02
5	48.67	48.37	$t_{crit.}$at 95%
6	39.85	39.92	df;9=2.262
7	38.40	38.40	P-value=0.074
8	46.21	46.33	
9	39.67	39.41	
10	43.58	43.39	

The statistical analysis of the results for NOR drug revealed that the calculated experimental values $|t|$ were 2.02 and this value is less than the critical value of 2.26 (α=0.05. dof=9, two-tailed) for NOR and supported by p value [P(T<t) which was 0.074, indicating acceptance of null hypothesis (Ho) which specified that there appears insufficient evidence to suggest the accuracy of the established CPE- spectrophotometry differs with that of traditional UV-Vis method (*i.e.* there is a good agreement between the results obtained by the two methods). It can be seen from Table 6 that the discrepancy in the amount of norfloxacin remaining and measured in the blood serum between volunteers may be attributed to the different amounts of drug absorbed from one person to another (*i.e.* depends on the nature of the metabolism between each volunteer) or to how discharge medication by the body; is that the remaining drug distracted by the kidneys. As it, this is the reason that there is a difference in the proportion of drug in the blood because of the difference in kidney function among the volunteers[21]. The developed method was also applied for the detection of NOR content in one of the selected pharmaceutical preparation containing NOR such as Norflox (eye drop) with stated value of 0.3%. The results are tabulated in Table 7. To validate the applicability of the proposed method, the result was compared statistically with the quoted value claimed by the manufacturer. As can be seen from Table 7 that the calculated t-value was less than the critical (t=4.303) at 95% confidence level and (n-1) degree of freedom, indicating the acceptance of manufacturer's claim (Ho=0.3%), so the null hypothesis H_o is maintained and concluding there is no evidence for systematic and random errors at the 95% confidence level.

Determination of Fe(III) in pharmaceuticals

The developed method was applied to the iron determination in four pharmaceutical drugs containing iron as an active ingredient. Results of the combined CPE-spectrophotometry are displayed in Table 8.

Table 7. Determination of NOR in pharmaceutical formulation by the proposed method and statistical comparison with quoted values

Commercial name and content	Practical content (proposed* method) (x^* ±ts/√n) at 95% C.I.	t=(x* -μ)√n/s Proposed method* *versus* claimed value at 95% C.I.	E_{rel} %	RSD %
Norflox Eye/Ear drops (0.3% w/v) 3000 μg NOR mL^{-1}	0.31 0.29 0.31 Ave: 0.303±0.029%	t_{cal}=0.449 0.449<4.303	1.00	1.3

**Mean of three determinations*

The results in Table 8 revealed that the calculated *t*-values for iron determination using NOR drug as the chelating agent are less than t-tabulated (4.303) at 95% confidence interval and (n-1) degrees of freedom ,indicative the acceptance of manufacturer's claims, so the null hypothesis H_o is maintained and concluding there is no evidence for systematic and random errors at the 95% confidence level. However, the latter medication, which contains 250 mg/5 mL of iron may deviate from the norm as the calculated t was higher the critical value at 95% confidence level, so the null hypothesis (H_o) should be rejected and accepting the alternative hypothesis (H_1), indicating there is evidence for the occurrence of systematic error due to the potential interferences caused by the presence of additives in the drug formulation.

Table 8. Analysis of Fe(III) using NOR in pharmaceutical samples by the combined CPE-spectrophotometry

Pharmaceutical formulations	Quoted value (μ) of iron content	Found content by proposed method $\overline{x}$±ts/√n at (α=0.05)	$t=\frac{(\overline{x}-\mu)\sqrt{n}}{s_d}$ s_d proposed method *vs*. Quoted value at (α=0.05)	% mean recovery at (α=0.05)
Iron Dextran injection USP pharma- roth Wiesbaden, Germany.	50 mg /mL	49.48 49.03 49.76 Ave. **49.42±0.91**	t=2.72 2.72<4.303	98.84± 1.83
Iron Dextran Cox Pharmaceutical Ltd., G.B, England	100 mg/ 2mL	97.55 98.60 99.91 Ave. **98.68±2.92**	t=1.94 1.94<4.303	99.68±2.68
Tot'héma®Iron gluconate solution laboratories innotech international (France)	50 mg/10 mL	48.75 50.07 49.91 Ave. **49.56±0.46**	t=1.06 1.06<4.303	99.15±3.57
Ironorm Syrup Wallace manufacturing chemists Ltd.,England	250 mg/5 mL	263.37 266.87 267.78 Ave. **266.01±5.78**	t=11.90 11.90>4.303	106.40±2.31

Conclusion

This study has shown that it is possible to mutual estimate of the reactants that involved in any chemical reaction by using a combined CPE-spectrophotometry with high sensitivity, better recovery and extraction efficiency.The determination of NOR drug using Fe(III) ions showed minimal detection limits and moderate sensitivity compared with other reported methods in the chemical literatures. Also, the analytical results obtained for the estimation of Fe(III) ions in drug formulations using NOR as a chelating agent was highly sensitive and provides a superior detection limit compared with other analytical methods reported, including those methods with CPE which used the synthesized ligands or commercial organic reagents. The analytical data of NOR and Fe(III) in analyzing drug formulation showed a convergence between them and the result that are mounted on the drug package, allowing to adopt the proposed methods for the purposes of quality control and follow-up product after storage. It became clear from this study, it is possible to consider the NOR drug as the chemical organic reagent of cheaply available, and thereby it can be used in complexation of metal ions other than iron ions.

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