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كلية الصيدلة

Development and Validation of Spectrophotometric Method for Determination of Cefixime and Glimepiride by Ternary Complex Formation

A THESIS

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Dedication

To my Parents

To my brothers and sisters

To my wife and my children

With deep love and appreciation I dedicate this effort

Acknowledgement

Foremost, I would like to express my sincere gratitude to my supervisor, Dr. Ihab Almasri, assistant professor of pharmaceutical chemistry, head of chemistry and pharmaceutical chemistry department at Al-Azhar University-Gaza, for the continuous support of my study and research, for his wisdom, motivation, enthusiasm and professionalism. His guidance helped me in all the time of research and writing of this thesis.

I would like to extend my thanks to all members of the faculty of pharmacy at Al-Azhar University-Gaza, my friends and to all those who supported and encouraged me.

Last but not the least, I would like to thank my family for supporting me throughout my life.

Abstract

A simple, accurate and sensitive UV-Visible spectrophotometric method have been developed and validated for the quantitative determination of cefixime and glimepiride in either pure form or in their dosage forms. The method is based on the formation of a ternary complex with copper(II) and eosin. The method does not involve solvent extraction.

Appropriate conditions were examined for the reaction to obtain maximum absorptivity and sensitivity. Under the optimum reaction conditions, linear relationships with good correlation coefficients (0.9997) were found between the concentrations and the absorbance of the formed complexes of the two drugs studied.

The color of the produced complex is measured at 550 and 544 nm with apparent molar absorptivities of 1.49 X 10^4 $Lmol^{-1}cm^{-1}$ and 1.657 X 10^4 $Lmol^{-1}cm^{-1}$ and Sandell's sensitivities of 3.1 X 10^{-2} and 2.9 X 10^{-2} $\mu g/cm^2$ for cefixime and glimepiride, respectively. The method is applicable over concentration range of 4-28 and 5-50 μgmL^{-1} for cefixime and glimepiride, respectively. The effect of excipients on the method was tested with very good recovery percentage. The results of analysis have been validated statistically, and recovery studies confirmed the accuracy of the proposed methods which was carried out by following the ICH guidelines. Furthermore, the developed methods hold their accuracy and precision well when applied to the determination of cefixime and glimepiride in their dosage forms.

ملخص الدراسة

تطوير و التحقق من طريقة تحليل طيفي لتحديد كمية دوائي cefixime و glimepiride عن طريق تكوين مركب ثلاثي.

ملخص البحث:

تطوير و معايرة طريقة بسيطة و حساسة و دقيقة لتحديد كمية دواء cefixime و دواء glimepiride باستخدام التحليل الطيفي بالأشعة فوق البنفسجية سواء كان الدواء في حالته النقية او في الاشكال الصيدلانية. تعتمد هذه الطريقة الجديدة على تكوين مركب ثلاثي من الدواء و النحاس و صبغة eosin و من ثم قياس هذا المركب دون استخلاصه من المحلول باستخدام مذيبات عضوية.

تم فحص الظروف الملائمة للتفاعل للحصول على الحد الاقصى من الامتصاصية و حساسية الطريقة. تحت هذه الظروف المناسبة للتفاعل تم الحصول على علاقة طردية بين تركيز الدواء و الامتصاصية بمعامل ارتباط 0.9997 للدواءين قيد الدراسة.

المركب الثلاثي الناتج اعطى اعلى امتصاصية عند طول موجي 550 و 544 نانومتر بامتصاصية مولرية 14.9×10^3 mol^{-1} cm^{-1} و 16.5×10^3 mol^{-1} cm^{-1} و حساسية Sandell 3.1×10^{-2} و 2.9×10^{-2} و نطاق تركيز من 4-28 و من 5-50 ميكرو جرام/ ملي لتر لدواء cefixime و glimepiride على التوالي.

تم اختبار تأثير الاضافات في الاشكال الصيدلانية على طريقة التحليل و اتضح انها لا تأثر عليها حيث حصلنا على نسبة استرداد عالية للدواءين بعد خلطهما بهذه المواد المضافة.

و قد تم معايرة نتائج التحليل إحصائيا و التأكد من دقة الطريقة عن طريق إتباع مبادئ ICH. كما حافظت الطريقة على دقتها عند فحص المستحضرات الصيدلانية المختلفة.

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List of Abbreviations

Abbreviation	Full Word
A	Absorbance
BNF	British National Formulary
BP	British Pharmacopoeia
DMF	Dimethylformamide
FDNB	1-Fluoro-2,4-dinitrobenzene
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
ICH	International Conference on Harmonisation
LOD	Limit of detection
LOQ	Limit of quantitation
MC	Methylcellulose
MEKC	Micellar electrokinetic chromatography
NQS	1,2-Naphthoquinone-4-sulphonate
QC	Quality Control
RSD	Relative standard deviation
SD	Standard deviation
SLS	Sodium Lauryl Sulfate
TLC	Thin layer chromatography
USP	United states pharmacopoeia
UV	Ultraviolet
Vis	Visible
λ max	Wavelength of maximum absorption

Chapter (1)

1. Introduction

1.1 Overview

Quality control (QC) is integral to pharmaceutical industry as the quality of a product may deviate from the required standards. The term quality control refers to the sum of all procedures undertaken to ensure the identity and purity of a particular pharmaceutical. Such procedures may range from the performance of simple chemical experiments which determine the identity and screening for the presence of particular pharmaceutical substance (thin layer chromatography, infrared spectroscopy, etc.), to more complicated requirements of pharmacopoeial monographs. QC measurements include stability testing of the drug formulation, dissolution testing and analysis of raw materials and formulated finished products. It doesn't only protect the manufacturer against compensation claims, but also guarantees the patient a safe and effective product. Poor quality of medicines is a health hazard because of increasing toxicity or decreasing efficacy and is also a waste of money for both government and consumers, so there is a great need to develop simple and accurate analytical methods for quality control of different manufactured drugs.

1.2 Absorption spectroscopy

Absorption method based on ultraviolet (UV) and visible (vis) radiation find wide spread applications for the identification and determination of myriad organic and inorganic species. Molecular UV/vis absorption methods are perhaps the most widely of all quantitative analysis techniques in chemical and clinical laboratories throughout the world, (Beckett, 2002).

Absorption of light in both UV (190-380 nm) and visible (380-800 nm) of electromagnetic spectrum occurs when the energy of the radiation matches that required to promote electrons from the ground state to an excited state e.g., electronic transition. Molecules with electrons in delocalized aromatic systems often absorb light in the near-UV or the visible region. A spectrum is obtained when the absorption of light is measured as a function of its frequency or wavelength. Ultraviolet (UV) and visible spectrophotometry have been widely used for quantitative analysis of pharmaceuticals. (Brown, 1980).

Molecular absorption spectroscopy is usually performed with molecules dissolved in a transparent solvent, such as in aqueous buffers. The absorbance (A) of a solute depends linearly on its concentration and therefore absorption spectroscopy is ideally suited for quantitative measurements. Absorbance is a measure of the amount of light absorbed by the substance, and it is proportional to the path length (b) through which the radiation passes and the concentration (C) of the substance in solution (Beer-Lambert law). $A = \varepsilon bC$, where "ε" is molar absorptivity if b is expressed in centimeters and C in mole/liter. Spectroscopic measurements are simple, sensitive, nondestructive, and require only small amounts of material for analysis.

Spectrophotometers intended for measuring in the UV and visible range have an optical system capable of producing monochromatic radiation in the range of 200-800 nm and a device to measure absorbance (A) (Fig. 1.1) .

The main components of UV/vis spectrophotometer include:

- Light source: deuterium lamp for UV region from 190-350 nm and tungsten lamp for visible region from 350-800 nm, and optics to split the light to two sample compartments, so blank solution can be used to correct the reading.
- Monochromator: is wavelength selecting device. It consists of an entrance slit, a dispersive device, a collimator and an exit slit.
- Sample cells: are commonly made of quartz. Plastic is available for measuring in the visible region only.
- Detectors: photo emissive and photomultiplier tubes, photovoltaic cells or photodiode array detectors.
- Readout device: convert the electrical signal from the detector to a digital signal.

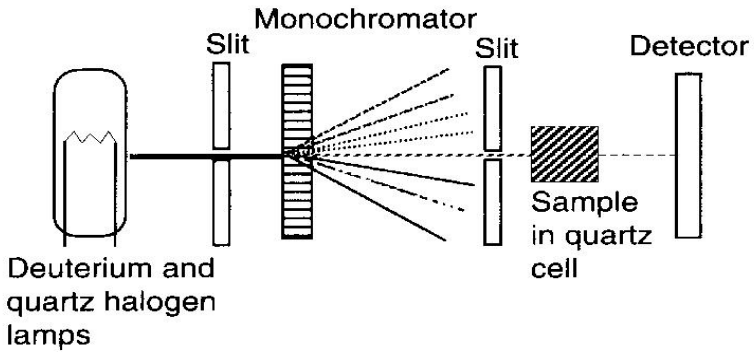

Figure 1.1: Schematic diagram of UV/vis spectrophometer.

Important characteristic of absorption spectroscopy include:

- ♦ Wide applicability of both organic and inorganic system
- ♦ Typical sensitivity of 10^{-4} to 10^{-5} M
- ♦ Moderate to high selectivity
- ♦ Good accuracy
- ♦ Ease of convenience of the data acquisition (Edisbury, 1996)

1.3 Derivatization spectroscopy

The field of absorption spectroscopy can be extended by color reactions which can lead to shifting of absorption to visible range and thus separate the molecule from interfering substances in the UV spectrum. Also these reactions can convert non absorbing molecules to stable derivative with significant absorption. Derivatization reactions should be complete and produce only one product which should be stable.

European Pharmacopoeia (2011) used phenylisothiocyanate and (dimethylamino) azobenzene sulfonyl chloride for precolumn derivatization of amino acids to enable detection by UV and visible spectroscopy, respectively. 1-Fluoro-2,4-dinitrobenzene (FDNB, Sanger Reagent) was used for determination of compounds containing primary or secondary amino groups (Paraskevas et al., 2002). 1,2-Naphthoquinone-4-sulphonate (NQS) also reacts with primary and secondary amino groups under relatively mild conditions (Darwish et al., 2012). Ninhydrin reacts only with primary amines (especially for α-amino acids except for cysteine), giving Ruhemann purple, a blue-violet colored indanic compounds (Rahman and Kashif, 2003). p-chloranilic acid have been used for visible determination of drugs (El-Zeany et al, 2003). Researchers used also 4-aminoantipyrine for spectrophotometric determination of some drugs such as ritodrine and isoxsuprine (Revanasiddappa and Manju, 2000).

Other derivatizing reagents used for visible spectrophotometry, some of them include: chloranil (Bebawy et al, 1999), 2,3-dichloro-5,6-dicyano-p-benzoquinone (Issa et al, 1987), p-dimethyl amino cinnamaldehyde (Saeed et al, 1993), 3-methylbenzothiazolin-2-one hydrazone (Revanasiddappa and Manju, 1999) and 2,6-dichloro quinone chlorimide (Gibb's reagent) (Gadkariem et al, 2012).

Binary ion-pair complex formation between organic dye and organic compounds can be used to increase the sensitivity of determination of these organic compounds but this method is complicated by the extraction process of water-insoluble complexes formed. The addition of a surfactant or water-miscible organic solvent to avoid extraction usually causes decomposition of these complexes, and thus the organic compound will not be determined.

Ternary complex formation between organic compound, organic dye (as eosin) and a metal ion (as Cu^{2+}, Pd^{2+}) increases the determination sensitivity of organic compounds. By the addition of a surfactant or water-miscible organic solvent, the ternary complex will be dissolved and measured directly, so extraction is avoided.

Eosin B (Fig. 1.2) is chemically 4′,5′-Dibromo-3′,6′-dihydroxy-2′,7′-dinitro-1-spiro[isobenzofuran-3,9′-xanthene]one disodium salt.

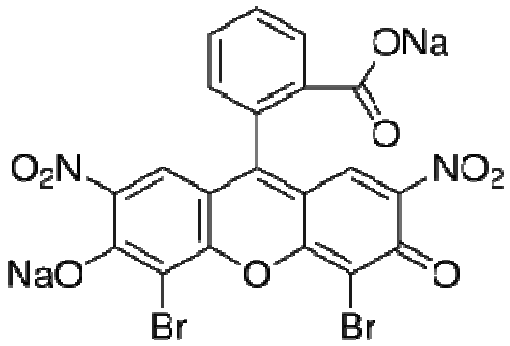

Figure 1.2: Chemical structure of eosin B

1.4 Method Validation

It is the process of proving that an analytical method is acceptable for its intended purpose (International Conference on Harmonisation, ICH, 2005). In pharmaceutical chemistry method validation requirements include studies of method

- Linearity
- Range
- Accuracy
- Precision

- Specificity
- Limit of detection
- Limit of quantitation

a) Linearity:

Linearity measures how well a graph of physical signal response versus concentration (or quantity) of analyte follows a straight line. In another word, linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

The common measure of linearity is the square of the correlation coefficient R^2. Square of correlation coefficient:

$$R^2 = \frac{\text{explained variation}}{\text{total variation}} = \frac{\sum (Y_{est} - \overline{Y})^2}{\sum (Y - \overline{Y})^2}$$

where **Y** are the observed values for the dependent variable, $\overline{\mathbf{Y}}$ is the average of the observed values and $\mathbf{Y_{est}}$ are predicted values for the dependent variable (the predicted values are calculated using the regression equation).

Residual standard deviation: the standard deviation of the residuals (residuals = differences between observed and predicted values). It is calculated as follows:

$$s_{res} = \sqrt{\frac{\sum (Y - Y_{est})^2}{n - 2}}$$

R^2 must be close to 1 to represent a tally linear fit.

b) Range:

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

c) Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the experimental values found. This is sometimes termed trueness. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of the analyte in the sample or as the difference between the mean and the accepted value, together with confidence intervals. The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (i.e. three concentration of three replicate of each concentration).

d) Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. It is the reproducibility of a result observed when the same quantity of one sample is repeatedly introduced in the instruments. Intra assay is evaluated by analyzing aliquots of a homogeneous material several times on the same day with the same instrument. Inter assay is evaluated by analyzing aliquots of homogeneous material several times on different days. The most common way through which precision is usually expressed are (i) Variance (ii) Standard deviation (SD) (iii) Relative standard deviation (RSD).

e) Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

f) Limit of detection (LOD):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated as an exact value.

g) Limit of quantitation (LOQ):

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and

accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

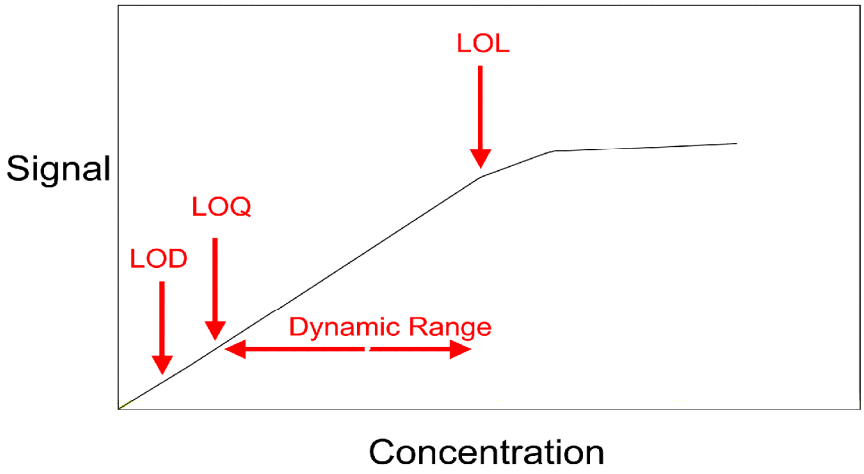

Figure 1.3: Illustration of limit of detection (LOD), limit of quantitation (LOQ) and limit of linearity (LOL).

1.5 Justification

The pharmaceutical product must be safe and effective, so it should be tested for active ingredient concentration to be in acceptable range before it is released to the market and dispensed to the patient.

The determination of the active ingredient in the raw material before manufacturing and in the final pharmaceutical preparation is very important to ensure that it presents in the required concentration to avoid sub-therapeutic or toxic effect if lower or higher concentration is present.

In Gaza strip many medicines are coming from subways in Rafah border and some of them are of unknown source and improper storage and transport conditions, so there is increasing need to analyze these medications for efficacy and safety. Since cefixime (antibacterial) and glimepiride (oral hypoglycemic) are widely used in therapy, quantitative analysis of those drugs is important to be sure that the patient will take the correct dose.

Most methods that have been developed for determination of cefixime and glimepiride are sophisticated, expensive and require modern instruments, so there is a need for simple, accurate and available method for analysis.

Extraction of ternary complex formed is avoided in this study by the addition of surfactant or water miscible organic solvent to help in solubilizing the complex. Extraction step is time consuming and require large volumes of organic solvents and thus the method of analysis will be costly and more hazardous to environment.

1.6 Study objectives

1.6.1 General objective

The objective of this study is to develop and validate a simple and accurate method for the determination of cefixime and glimepiride in raw material and in their pharmaceutical preparations.

1.6.2 Specific objectives

1. To develop a new sensitive and simple method for the quantitative determination of two drugs (cefixime and glimepiride) and optimize the conditions that could affect the method as temperature, pH, type and concentration of surfactant and time of reaction.
2. To validate the method according to ICH guidelines (evaluating accuracy, precision, limit of detection, limit of quantitation and linearity).
3. To determine the stability of the color of the formed complex.
4. To evaluate the specificity by testing the effect of different excipients on the determination of the analyte.
5. To apply the developed method for the recovery of cefixime and glimepiride in their pharmaceutical preparations.

Chapter (2)

2. Literature Review

2.1 Drug profile of cefixime

2.1.1 Physicochemical properties of cefixime

Cefixime, a cephalosporin antibiotic, is white to almost white, slightly hygroscopic powder, it is slightly soluble in water, soluble in methanol, sparingly soluble in anhydrous ethanol, practically insoluble in ethyl acetate, (British Pharmacopoeia (BP), 2011).

Cefixime is (6*R*,7*R*)-7-[[(*Z*)-2-(2-Aminothiazol-4-yl)-2 [(carboxymethoxy)imino]acetyl] amino]-3-ethenyl-8-oxo-5-thia-1 azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid trihydrate (Figure 2.1), (BP, 2011).

Molecular formula of cefixime is $C_{16}H_{15}N_5O_7S_2$, molecular mass is 453.5 and the melting point is 218-225 °C (Clarke, 2011).

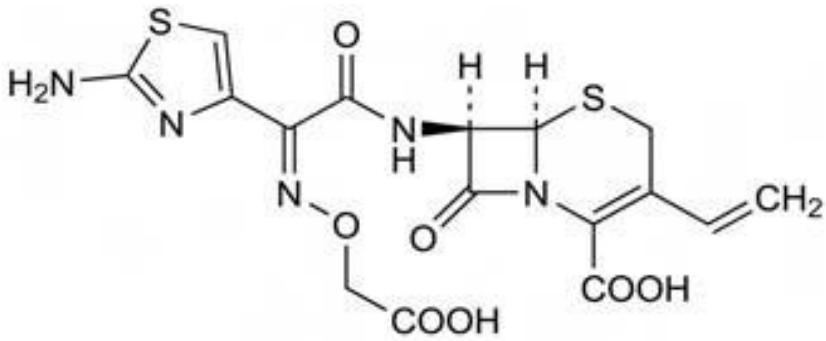

Figure 2.1: Chemical structure of cefixime

Regarding the degradation profile of cefixime, it was observed that 25% of cefixime was degraded on heating at 80°C for 1 h in 0.01 M NaOH. The drug was totally degraded if heated at 80°C for 4 h in 0.1 M NaOH. In acidic conditions, 25% of drug was degraded if heated with 0.01 M HCl at 80°C for 2.5 h. The 100% degradation in 0.1 M HCl was observed at 80°C after 7 h. The degradation was very rapid under oxidative conditions, as

25% drug was degraded if left at 25°C with 1% H_2O_2 in 3.5 h. (Gandhi and Rajput, 2009). The drug should be stored in a dry and cool place and avoid light.

2.1.2 Pharmacological action of cefixime

Cefixime is a cephalosporin antibacterial that inhibits bacterial cell wall synthesis, cephalosporins are semisynthetic antibacterial agent derived from cephalosporin C, a natural antibacterial produced by the mould *Cephalosporium acremonium*.

Cephalosporins are classified to generations according to their antibacterial activity. Succeeding generations generally have increasing activity against Gram-negative bacteria. Cefazolin, cefradine, cefalexin and cefadroxil are commonly used first generation cefalosporins. Second generation cefalosporins have similar activity to first generation against Gram positive bacteria but with greater activity against Gram negative bacteria. Cefaclor and cefuroxime are classified as second generation cefalosporins. The third-generation cephalosporins are more stable to hydrolysis by β-lactamases than the earlier generations of cephalosporins and have a wider spectrum and greater potency of activity against Gram-negative organisms.

Cefixime is a third generation cephalosporin and is effective against the *Enterobacteriaceae* as well as against pathogenic *Neisseria* spp. (including penicillin-resistant gonococci), *Haemophilus influenzae* (including ampicillin-resistant strains), penicillin-susceptible *Streptococcus pneumoniae* and other *Streptococcus species*. Species resistant to cefixime included penicillin-resistant *pneumococci*, *Staphylococcus* spp., *Enterococcus* spp., *Listeria* spp., *Acinetobacter* spp. and *Pseudomonas* spp. Cefixime is given orally in the treatment of susceptible infections including gonorrhoea, otitis media, pharyngitis, lower respiratory tract infections and urinary tract infections (Martindale 36, 2009).

Cefixime dose for adults is 200 to 400 mg daily as a single dose or in two divided doses. The recommended dose in children above 6 months is 8 mg/kg/day as a single dose or in two divided doses (British National Formulary, BNF 61, 2011).

Only 40 to 50% of oral dose of cefixime is absorbed from the gastrointestinal tract, whether taken before or after meals, although food may decrease the rate of absorption. Absorption is fairly slow; peak plasma concentrations have been reported between 2 and 6 hours (Martindale 36, 2009).

2.1.3 Methods of analysis of cefixime

Reviewing the literature revealed that several methods have been used for the determination of cefixime in pure form, pharmaceutical formulations or in biological samples, whether as single active ingredient or in combination with other drugs, these methods include HPLC, high performance thin layer chromatography (HPTLC), spectrophotometric, spectrofluorimetric, electrophoretic and voltammetric methods.

British Pharmacopoeia, (2009) stated HPLC method for the determination of cefixime and related substances on column C-18 (12.5 cm x 4 mm) at 40 °C temperature with tetrabutylammonium hydroxide (adjusted to pH 6.5 with dilute phosphoric acid) and acetonitrile (3:1) as mobile phase, flow rate 1mL/min, and UV detection at 254 nm. United states pharmacopoeia (USP, 2007) recommended a similar HPLC method but the reference standard and the sample were dissolved in pH 7 phosphate buffer.

Several HPLC methods have been developed for the determination of cefixime in bulk and in pharmaceutical dosage forms. All of these methods have used reversed phase C-18 columns but with different dimensions (10, 15 and 25 cm length, 4 and 4.6 mm internal diameter). Different mobile phases have been used (phosphate buffer with acetonitrile, phosphate buffer with methanol, tetrabutylammonium hydroxide with acetonitrile, methanol with water and acetonitrile with methanol with ammonium acetate). In these methods flow rates of 0.8, 1 and 2 mL/min was used and detection was done by UV absorption at different wavelengths (254 nm, 285-287 nm and 295 nm), (Gonzalez-Hernandez et al., 2001, Shah and Pundarikakshudu, 2006, Arshad et al., 2009, Saikrishna et al., 2010, Raj et al., 2010, and Pasha A. et al., 2010).

Several RP-HPLC methods with UV detection were developed and validated for the simultaneous determination of cefixime with other drugs in the same dosage form. For example, Rathinavel et al (2008) have validated a RP-HPLC method for the simultaneous

determination of cefixime and cloxacillin in tablets. They used a C-18 column. The mobile phase was a mixture of phosphate buffer (pH 5), acetonitrile and methanol and flow rate was 2 mL/min with UV detection at 225 nm. The method was found to be linear in the range of 160-240 μg mL^{-1} and 400-600 μg mL^{-1} with retention times 5.6 and 6.2 min for cefixime and cloxacillin, respectively. The authors concluded that the method was rapid and sensitive.

Wankhede et al (2010) developed and validated a similar RP-HPLC method for the simultaneous determination of cefixime and cloxacillin in tablets. They used a C-8 column, acetonitrile and tetrabutylammonium hydroxide as mobile phase, flow rate 1 mL/min with UV detection at 225 nm. The method was found to be linear in the range of 10-50 μg mL^{-1} and 25-125 μg mL^{-1} and retention time 5.75 and 11.9 min for cefixime and cloxacillin respectively.

Kathiresan et al (2009) have developed and validated RP-HPLC method for determination of cefixime and dicloxacillin combination in tablets. C-18 column was used and a mixture of potassium hydroxide buffer and acetonitrile as mobile phase with UV detection at 220 nm and 1 mL/min flow rate. Linearity obtained in the range of 60-140 μgmL^{-1}.

Sudhakar et al (2010) have published a RP-HPLC method for estimation of cefixime and ornidazole combination in tablet dosage forms. C-18 column was used and acetonitrile with 40 mM KH_2PO_4 as mobile phase. Flow rate was 1 mL/min and UV detection at 310 nm. The retention time of cefixime and ornidazole were 2.75 min and 6.67 min, respectively.

Deshpande et al (2010) described a HPLC method for the simultaneous determination of cefixime and ambroxol by using C-18 column and actonitrile with methanol as mobile phase. UV detection was at 254 nm with retention time 1.68 and 3.7 min and linearity ranges from 4-18 and 4-28 μg mL^{-1} for cefixime and ambroxol, respectively.

Raj et al (2010) developed a HPLC method for the estimation of cefixime and cefuroxime axetil in bulk drug and in dosage forms by using C-18 column. A mixture of methanol and water was used as mobile phase with UV detection at 254 nm.

Dhoka et al (2010) described a RP-HPLC method for simultaneous determination of cefixime and erdosteine in dosage forms. C-8 column was used as stationary phase and tetrabutyl ammonium hydroxide (pH adjusted to 6.5) with acetonitrile in a ratio of 2:1 was used as mobile phase. Detection was done by UV at 254 nm, linearity obtained in the range of 2-22 μg mL^{-1} for cefixime and 3-33 μg mL^{-1} for erdosteine, with retention time 10 min for cefixime and 5.4 min for erdosteine.

Kumudhavalli et al (2010) developed a RP-HPLC method for the simultaneous estimation of cefixime and potassium clavulanate in tablets. C-18 column was used and the mobile phase composed of phosphate buffer and methanol and detection was at 220 nm. Results were linear in the range of 20-100 μg mL^{-1} and 12.5-62.5 μg mL^{-1} and retention time 7.3 and 2.4 min for cefixime and potassium clavulanate, respectively.

Basu et al (2011) described another RP-HPLC method for the simultaneous estimation of cefixime and potassium clavulanate in tablets, using a mixture of tetra butyl ammonium hydroxide solution and methanol as mobile at a flow rate of 1 mL/min, at 40ºC. The detection was carried out at 230 nm. The retention time was 4.63 min and 11.89 min for potassium clavulanate and cefixime respectively and linearity was 10-180 μg mL^{-1} for clavulanic acid and 10-360 μg mL^{-1} for cefixime.

Khandagle et al (2011) published a RP-HPLC method for the simultaneous determination of cefixime and ofloxacin in tablets. C-8 neosphere column was used and methanol with potassium dihydrogen phosphate buffer as mobile phase. Detection was done at 290 nm. Linearity was found in range of 1-10 μg mL^{-1} for both drugs.

Natesan et al (2011) have developed a similar method for separation and determination of cefixime and ofloxacin in tablet dosage forms with some differences in column and mobile phase used. The column used was C-18, as mobile phase used was methanol and 25 Mm phosphate buffer (40:60 v/v) and UV detection at 290 nm. Retention time was 2.5 and 7.8 min for cefixime and ofloxacin respectively. The method was linear in range of 5-25 μg mL^{-1} for both drugs.

Moreover, several RP-HPLC methods were developed for the determination of cefixime in biological fluids:

Falkowski et al (1987) published a RP-HPLC method for estimation cefixime in human serum. The method was linear from 0.1 - 30 μg mL^{-1} for serum assay, and from 5 - 100 μg mL^{-1} for the urine assay.

McAteer et al (1987) developed a RP-HPLC method for the determination of five oral cephalosporins in human serum. Mobile phase was a mixture of methanol : monobasic phosphate buffer (20 : 80), flow rate 2 mL/min in C-8 column and detection was at 240 nm. The authors concluded that this method has advantages over previous methods in that the same conditions are used for five drugs and at clinically significant concentrations.

Zendelovska et al (2003) published a RP-HPLC method for the simultaneous determination of cefixime and cefotaxime in plasma, utilizing a C-8 column and mobile phase of KH_2PO_4 (pH 2.2) and methanol (75:25, v/v). pH of buffer adjusted to 2.2 by adding a very small amount of concentrated ortho-phosphoric acid. Detection was carried out by UV and the method was found to be linear over the concentration range 0.2–12 μg mL^{-1} for cefixime and 0.2–50 μg mL^{-1} for cefotaxime in plasma samples.

Pisarev et al (2009) have developed HPLC method with UV detection for determination of cefixime in blood plasma for comparison of two pharmaceutical preparations.

Ali et al (2011) have published a fast HPLC method for analysis of third generation three cephalosporins including cefixime in human plasma. The method was performed on C-18 column with mobile phase composed of phosphate buffer and methanol at flow rate of 1 mL/min and UV detection at 230 nm. The three drugs were separated within 7 minutes.

Meng et al (2005) have developed a liquid chromatographic tandem mass spectrometric (LC-MS) method for cefixime determination in human plasma. The method was performed on a C-8 column coupled with a triple quadrupole tandem mass spectrometer. The mobile phase consisted of acetonitrile: water: formic acid (40:60:0.5, v/v/v). The method was linear in the range of 0.05-8 μg mL^{-1}.

Additionally, several HPTLC methods were developed for the simultaneous determination of cefixime with other drugs in the same dosage form.

Jovanovic et al (1998) have developed a HPTLC method for the determination of ceftriaxone, cefixime and cefotaxime in dosage forms, mobile phase was ethyl acetate-acetone-methanol-water (5:2.5:2.5:1.5 v/v/v/v).

Khandagle et al (2010) have developed and validated HPTLC method for determination of cefixime and ofloxacin in tablets. The mobile phase was methanol: ethyl acetate: ammonia (3.5: 3.5: 1.5 v/v/v) with UV detection at 295 nm. Results were linear in the range of 50-500 ng/band for both cefixime and ofloxacin.

Dhoka et al (2011) described a validated HPTLC method for the estimation of cefixime and erdosteine in bulk and combined preparations. The method was performed on aluminium plates precoated with silica gel 60F-254, and the mobile phase composed of ethyl acetate: acetone: methanol: water (7.5: 2.5: 2.5: 1.5) and densitometric determination at 235 nm. The results were linear in the range of 100-500 ng/ band and 150-750 ng / band for cefixime and erdosteine respectively.

Rao et al (2011) have developed and validated another HPTLC method for simultaneous determination of cefixime and ofloxacin in tablets. Separation was performed on aluminum foil plates precoated with silica gel 60GF-254, with n-butanol: ammonia: water: DMSO (8:3:1:2, v/v/v/v) as mobile phase. Densitometric detection was performed at 297 nm. linearity was in range of 30-180 ng/spot for both drugs.

Deshpande et al (2010) described a HPTLC method for the simultaneous estimation of cefixime trihydrate and ambroxol hydrochloride. The separation was on aluminium sheets of silica gel 60 F254 using acetonitrile: methanol: triethylamine (8.2:1:0.8, v/v/v) as mobile phase. The method was found to be linear in the range of 200-1000 ng/spot for cefixime and ambroxol, with densitometric measurements of their spots at 254 nm.

Devika et al. (2010) published a validated HPTLC densitometric method for the determination of cefixime trihydrate and ornidazole in bulk drug and in tablets. The method was performed on thin layer chromatography (TLC) aluminium sheets covered

with silica gel 60 F254 with n-butanol: methanol: toluene: ammonia 5:2:1:5 (v/v/v/v) as mobile phase. The results were linear in the range of 360-840 ng/band for cefixime and 900-2100 ng/band for ornidazole.

Honda et al (1992) developed a high-performance capillary electrophoretic method for determination of cefixime and its metabolites.

Raj (2010) developed electrophoretic method for the determination of cefixime trihydrate and cefuroxime axetil in bulk drug and pharmaceutical dosage forms.

Spectrophotometric methods have been developed for determination of cefixime in tablet dosage form using different hydrotropic agents as urea, sodium tartarate, sodium acetate, sodium citrate and others and then cefixime was determined by conventional spectrophotometric estimation at 288 nm or by area under curve method, these methods exhibit linearity range of 5-30 μg mL^{-1}. (Maheshwari, 2005, Maheshwari et al., 2010 and Pareek et al., 2010). Pareek et al. (2010), studied also the role of different hydrotropic agents in the determination of cefixime by HPTLC method.

Kasture (2008) developed two spectrophotometric methods for simultaneous determination of cefixime trihydrate and ambroxol hydrochloride in tablet dosage form. The first method based on first derivative spectrophotometry and second method based on Vierordt's method.

Nanda[1] et al. (2009) described two spectrophotometric methods for simultaneous estimation of cefixime and ornidazole in tablet dosage form. The first method is first order derivative spectroscopy, wavelengths selected for quantitation were 311.5 nm for cefixime (zero cross for ornidazole) and 290 nm for ornidazole (zero cross for cefixime). Second method is area under curve (AUC) method. AUC in the range of 285-295 nm for cefixime and 307-317 nm for ornidazole were selected for the analysis.

Nanda[2] et al. (2009) described in another publication two spectrophotometric methods for simultaneous estimation of cefixime and ornidazole in tablets, first method (Vierodt's method) is based on the simultaneous equations obtained by using mean absorptivity values. Wavelengths selected for analysis were 290 nm (λ_{max} of cefixime) and 312 nm

(λ_{max} of ornidazole) respectively, in methanol. Second method is Q analysis method based on absorbance ratio at two selected wavelengths 303 nm (iso-absorptive point) and 312 nm (λ_{max} of ornidazole) and the concentration of each component is calculated from specific equations.

Dube et al. (2011) developed two spectrophotometric methods for simultaneous estimation of cefixime and ofloxacin from tablets, first method involves formation of simultaneous equations at 234 nm (λ_{max} of cefixime) and 296 nm (λ_{max} of ofloxacin); while second method involves formation of absorbance ratio equation at 275 (isoabsorptive point) and 296 nm (λ_{max} of ofloxacin) using methanol as a solvent.

Kumar et al. (2011) developed a colorimetric method for simultaneous determination of ofloxacin and cefixime in the same pharmaceutical formulation. Ofloxacin forms an orange colored product in the presence of ferric chloride solution in acidic medium and the absorbance of orange colored species formed was measured at 435 nm against reagent blank. While cefixime forms a greenish colored product with Fehling solution and the absorbance of greenish colored species formed was measured at 490 nm against reagent blank.

Shah et al. (2011) published a validated spectrofluorimetric method for determination of cefixime in pure form and pharmaceuticals through derivatization with 2-cyanoacetamide. The fluorescent product showed maximum fluorescence intensity at λ 378 nm after excitation at λ 330 nm. The method was linear in range of 0.02-4 μg mL^{-1}.

Elbashir et al (2011) developed a spectrofluorimetric method for determination of cefixime, cephalexin and cefotaxime in pharmaceutical formulations. The method is based on a reaction between cefixime and 1, 2-naphthoquinone-4-sulfonic (NQS) at pH 12.0 to give highly fluorescent derivative extracted with chloroform and then measured at 600 nm after excitation at 520 nm. The method was found to be linear in range of 10–35 ng mL^{-1}.

Ali Ahmed et al (2012) have developed a newer spectrofluorimetric method for determination of cefixime and other two cephalosporins in pharmaceutical formulations. The method based on reaction between cefixime and 8-hydroxy-1,3,6-pyrenetrisulfonic

acid trisodium salt (HPTS) at pH 12.0 to give highly fluorescent derivative extracted with chloroform and then measured at 520 nm after excitation at 480 nm.

Voltammetric methods have also been developed for the determination of cefixime in dosage forms and biological fluids (Reddy et al., 2003, Golcu et al., 2005 and Jain et al., 2010).

2.2 Drug profile of glimepiride

2.2.1 Physicochemical properties of glimepiride

Glimepiride (Fig. 2.2) is sulfonylurea oral hypoglycemic drug used for the treatment of type II diabetes mellitus, (BP, 2011). Glimepiride presents as white or almost white powder, practically insoluble in water, soluble in dimethylformamide (DMF), slightly soluble in methylene chloride, very slightly soluble in methanol, (BP, 2011).

Glimepiride is 1-[[4-[2-(3-Ethyl-4-methyl-2-oxo-3-pyrroline-1 carboxamido)- ethyl] phenyl]sulphonyl]-3-*trans*-(4-methylcyclohexyl)urea (BP, 2011).
Molecular formula of glimepiride is $C_{24}H_{34}N_4O_5S$. Molecular mass is 490.6 g/mole (Clarke, 2011).

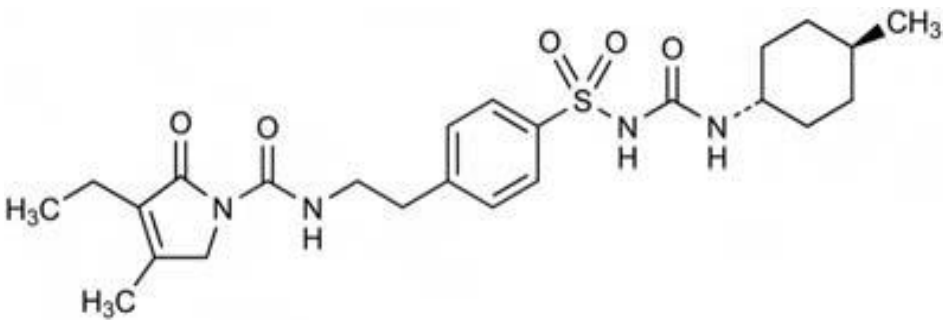

Figure 2.2: Chemical structure of glimepiride

A forced degradation of glimepiride was carried out under condition of acid, neutral, alkaline and oxidative hydrolysis and high temperature (90ºC). It was found that the susceptibility of glimepiride to hydrolytic decomposition increased in following manner: neutral condition < alkaline condition < acid condition < oxidative condition. After 3

hours at 90 °C, degradation of glimepiride was as follows, 41% in neutral solution, 45% in alkaline solution, 60% in acidic solution and 84% under oxidative conditions. (Kovaríková et al., 2004).

2.2.2 Pharmacological action of glimepiride

Glimepiride is a sulfonylurea antidiabetic drug that acts by inhibition of ATP-dependent potassium channels in β-cells of the pancreas and thus increases the release of insulin. It is given orally for the treatment of type 2 (non-insulin-dependent) diabetes mellitus (Martindale 36, 2009). The initial daily dose of glimepiride is 1 mg administered as a single dose and adjusted according to response by 1 mg increments every 1-2 weeks up to 4 mg daily and can be increased occasionally to 6 mg daily (BNF 61, 2011).

Glimepiride is completely absorbed from the gastrointestinal tract and the maximum plasma concentration is achieved at 2-3 hours. It is completely metabolized principally in the liver. In fasting, it is given in the same daily dose but shifted from morning to sunset, it was used in Muslim patients in Ramadan without increasing incidence of hypoglycemia. Adverse effects of glimepiride include hypoglycemia, gastrointestinal effects such as nausea and vomiting, blood dyscrasias as leucopenia, thrombocytopenia, hemolytic anemia, cholestatic jaundice and weight gain (Martindale 36, 2009).

2.2.3 Methods of analysis of glimepiride

Glimepiride was determined by different chromatographic, spectrophotometric and polarographic methods.

USP (2007) and BP (2009) described HPLC method on a C-18 (25 cm x 4 mm) column with sodium phosphate buffer, pH 2.1 to 2.7, and acetonitrile (50:50, v/v) as a mobile phase, acetonitrile and water (4:1) as a diluent, flow rate 1 mL/min, and detection at 228 nm, retention time of glimepiride is about 17 minutes, the method should be carried out at a temperature not exceeding 12°C and the samples should not be stored for more than 15 hours.

Other RP-HPLC methods developed for determination of glimepiride in pharmaceutical formulations using C-18 column of different dimensions (15 and 25 cm length, 3.9 and 4.6mm internal diameter) and different mobile phases (acetonitrile with 0.05 M monobasic potassium phosphate, acetonitrile with 2% formic acid solution and potassium dihydrogen orthophosphate with acetonitrile) and detection done by UV absorption at different wavelengths (210, 220 and 228 nm), (Wanjari and Gaikwad, 2005; Khan et al., 2009; Mishra et al., 2011)

HPLC methods were also developed for simultaneous determination of glimepiride in combination with other drugs (Sane et al., 2004; Kolte et al., 2005; Yao et al., 2007; Jain et al., 2008; Karthic et al., 2008; Pawar et al., 2008; Lakshmi et al., 2009; Kalyankar et al., 2010; Havaldar and Vairal, 2010; Lakshmi and Rajesh, 2011; Mallu et al., 2011; Sharma et al., 2011).

El-Deeb et al. (2006) developed a Fast HPLC method for the determination of glimepiride, glibenclamide, and related substances using monolithic column and flow program.

Khan et al. (2005) published a HPLC method for quantitation of five impurities in glimepiride drug substance.

Different liquid chromatographic methods with mass spectroscopy have been developed for the determination of glimepiride in human plasma (Maurer et al., 2002; Kim et al., 2004; Salem et al., 2004; Dotsikas et al., 2005; Hoizey et al., 2005; Pistos et al., 2005; Yüzüak et al., 2007; Chakradhar et al., 2008; Sengupta et al., 2009).

Furthermore, different HPLC methods with UV detection have been developed for the determination of glimepiride in biological fluids. For example, Lehr and Damm (1990), have developed a RP-HPLC method for the determination of glimepiride and its metabolites in serum and urine. The method involves extraction with diethyl ether, thermolysis of the sulphonylureas at 100 ºC and trapping of the resulting amines with 2,4-dinitrofluorobenzene. The derivatives were quantitated by absorbance at 350 nm.

Song et al (2004) have developed HPLC method with column-switching using UV detection for the determination of glimepiride in human plasma. Plasma samples were deproteinated and extracted with ethanol and acetonitrile. The extract was directly injected into a pre-column to remove proteins and retain drugs using a mixture of acetonitrile and 10 mM phosphate buffer (pH 2.18) (20:80, v/v). The analytes were transferred to an intermediate column and then separated on an analytical column and monitored with UV detection at 228 nm. Retention time of glimepiride was 34.9 min without interference of endogenous substance from plasma. The method was linear in the range of 10-400 $ngmL^{-1}$.

Rabbaa-Khabbaz et al (2005) have described HPLC method for determination of glimepiride in serum. The method involves one step liquid-liquid extraction with dichloromethane in acidified serum. Detection was done at 228 nm and linearity obtained in range of 10-1000 ng mL^{-1}.

Moreover, different HPTLC methods have been developed for the simultaneous determination of glimepiride with other drugs in combination (Sane Menon et al., 2004; Patel et al., 2006; Dhaneshwar et al., 2010; Havele and Dhaneshwar, 2011).

Micellar electrokinetic chromatography (MEKC) methods were developed for the separation and detection of glimepiride with other sulfonylureas (Núnez et al., 1995; Maier et al., 2009).

Second-order derivative UV spectrophotometric methods were developed for the determination of glimepiride in tablets (Altinoz and Tekeli, 2001; Bonfilio et al., 2011).

Lakshmi et al. (2009) published a first derivative UV spectrophotometric method and a HPLC method for the simultaneous determination of metformin, pioglitazone, and glimepiride in tablets.

Khan et al. (2009) have developed a first-order derivative spectrophotometric method for quantitation of glimepiride in tablets. The method was based on the formation of a complex of the drug with 2,3,5-triphenyl-2H-tetrazolium chloride in basic media with detection of the complex at 413 nm from first order derivative spectrum. Methanol was used as a solvent.

Goyal and Singhvi (2007) developed three spectrophotometric methods for simultaneous estimation of rosiglitazone maleate and glimepiride in combined tablet dosage form. Sodium hydroxide solution was used as solvent. Developed methods are based on direct estimation of rosiglitazone at 318 nm (at this wavelength glimepiride has zero absorbance). For estimation of glimepiride first developed method involves formation and solving of simultaneous equation at 238 nm. The second developed method used two wavelength spectroscopy using 244.8 nm and 257.2 nm as the selected wavelengths. The third method is based on first derivative spectroscopy using 252 nm as zero crossing point for estimation of glimepiride.

Ma et al. (2005) described a polarographic method of parallel catalytic hydrogen wave for determination of glimepiride. The authors concluded that the proposed method could be applied to the determination of glimepiride in pharmaceuticals without preliminary separation.

Square-wave voltammetric method have been developed and validated by Suslu and Altinoz (2011).

Chapter (3)

3. Methodology

3.1 Instruments and apparatus

- Double beam scanning UV-Vis spectrophotometer, Shimadzu 1601 with 1 cm matched quartz cell connected to a pc computer with UV-Probe software.
- All weighing were done on electronic analytical balance (Sartorius).
- Digital pH meter (HM-60V from TOA Electronics Ltd).
- Water bath.

3.2 Materials and reagents

3.2.1 Standards

Pure cefixime and glimepiride standards were kindly donated by Pharmacare Pharmaceutical company (Ramallah, Palestine).

3.2.2 Market samples

Commercially available pharmaceutical dosage forms were purchased from the local market. Rizacef® (cefixime) 400 mg capsules manufactured by Pharmacare Pharmaceutical company (Ramallah, Palestine) and Amiran® (glimepiride) 3 mg tablets manufactured by Birzeit Pharmaceutical Company (BPC) (Ramallah, Palestine).

3.2.3 Materials and Reagents

All chemicals were of analytical grade with high purity. Doubly distilled water was used through out all measurements. Methanol was procured from Merck (Germany), DMF from Merck (Germany).

All reagents were prepared using double-distilled water.

1- Eosin (Sigma, Austria), 0.1% w/v, aqueous solution, prepared by dissolving 100 mg of eosin in 100 mL double distilled water.

2- Copper (II) sulfate (Merck, Germany), 0.1% w/v aqueous solution, prepared by dissolving 100 mg of copper (II) sulfate in 100 mL double distilled water.
3- Sodium lauryl sulphate (SLS) (Merck, Germany), 0.3% w/v aqueous solution, prepared by dissolving 300 mg of SLS in 100 mL double distilled water.
4- Methylcellulose (MC) (Sigma-Aldrich, Germany), 0.3% w/v aqueous solution, prepared by dissolving 300 mg of MC in 100 mL double distilled water.
5- Acetate buffer solutions (100 mL) of pH 4, 5 and 5.6 were prepared by mixing different volumes of acetic acid 0.1 M and sodium acetate solution 0.1 M as follows: pH 4 (85 mL acetic acid and 15 mL acetate salt); pH 5 (36 mL acetic acid and 64 mL salt); pH 5.6 (12.4 mL acetic acid and 87.6 mL salt).

3.3 Spectrophotometric estimation of cefixime

3.3.1 Preparation of stock standard solution of cefixime.

Cefixime stock solution (400 μg mL^{-1}) was prepared by dissolving 40 mg of the drug substance in a 100 mL methanol with good shaking for 5 minutes.

3.3.2 General procedure for cefixime determination

Accurately measured aliquots of cefixime stock solution in the range of 4–28 μg mL^{-1} of drug were transferred into separate 10 mL volumetric flasks and then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.6) and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with methanol. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 550 nm against blank prepared in the same manner without addition of the examined drug.

3.3.3 Study of spectral characteristics of cefixime ternary complex in methanol.

After enabling the initial adjustment and blank correction, the absorption of cefixime solution (20 μg mL^{-1}) was scanned in the range from 400 to 600 nm to obtain the wavelength of maximum absorption (λ_{max}).

3.3.4 Optimization of reaction conditions

3.3.4.1 Effect of surfactant

The effect of surfactant was studied by using different surfactants as MC, SLS and methanol as co-solvent.

Procedure: 0.5 mL aliquots of cefixime solution (400 μg mL^{-1}) were transferred to each one of three 10 mL volumetric flasks (to give 20 μg mL^{-1} as final concentration) and then 1 mL copper(II), 1 mL eosin and 1 mL buffer (pH 5.6) solutions were added followed by 1 mL MC in the first flask, 1 mL SLS in the second flask, then the volume was completed with water. The same work was repeated using methanol instead of surfactants and then complete to volume by methanol. The flasks were left for 20 minutes and then the absorbance was measured at 550 nm.

3.3.4.2 Effect of pH

The absorbance of cefixime was measured at different pH values: 4, 5, 5.6 and without buffer (pH 7).

Procedure: To 0.5 mL cefixime standard solution (in 10 mL volumetric flasks), 1 mL of each of copper (II) and eosin solutions were added followed by 1 mL buffer of different pH solutions. The solutions were diluted to 10 mL with methanol and the absorbance was measured at 550 nm after 20 minutes.

3.3.4.3 Effect of reaction time

The absorbance of cefixime was measured after 5, 10, 15, 20 and 25 minutes.

Procedure: 1 mL of copper (II) solution, 1 mL eosin and 1 mL buffer solution pH (5.6) were added to 0.5 mL cefixime standard solution and the mixture was diluted to 10 mL with methanol and the absorbance was measured at 550 nm after 5, 10, 15, 20 and 25 minutes.

3.3.4.4 Effect of concentration of copper (II) and eosin solutions

Different volumes of copper (II) and eosin solutions were examined.

Procedure: To 1 mL cefixime standard solution, 1 mL eosin and 1 mL buffer solution pH (5.6) and different volumes of copper (II) solution (0.5, 1 and 2 mL) were added into different volumetric flasks. The solutions were diluted to 10 mL with methanol and the

absorbance was measured at 550 nm after 20 minutes. The method was repeated by changing eosin concentration.

3.3.4.5 Effect of temperature

The absorbance of cefixime was measured at room temperature and at 40ºC.

Procedure: 1 mL of copper (II) solution, 1 mL eosin and 1 mL buffer solution pH (5.6) were added to 0.5 mL cefixime standard solution and the mixture was diluted to 10 mL with methanol and the absorbance was measured at 550 nm after 20 minutes at RT and the same work was repeated after heating the mixture to 40 ºC for 15 minutes then cool to room temperature before measuring the absorption.

3.3.5 Construction of calibration curve of cefixime

Accurately measured aliquots of cefixime standard solution (0.1, 0.2, 0.3, 0.5 and 0.7 mL) in the range of 4–28 μg mL^{-1} were transferred into separate 10 mL volumetric flasks, then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.6) and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with methanol. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 550 nm against blank prepared in the same manner without addition of the examined drug. Triplicate measurements were done for each concentration. The concentration (μg mL^{-1}) of cefixime against absorbance (mean value) was plotted and least square regression analysis was performed on the obtained data. Correlation coefficient, slope, intercept and molar absorptivity were calculated from the graph.

3.3.6 Analytical method validation

Analytical method validation was performed according the ICH guidelines (ICH, 2005) with respect to accuracy, precision, specificity, linearity, limit of detection and limit of quantitation.

3.3.6.1 Linearity

The linearity of the proposed method was determined by measuring the absorbance of five concentrations covering the range (4, 8, 12, 20 and 24 μg mL^{-1}), each concentration

was measured in triplicate. Then the plot of absorbance against concentration was examined visually and statistically by calculating correlation coefficient.

3.3.6.2 Accuracy

Three concentrations covering the range were measured and each of them was repeated three times to calculate accuracy and precision.

Accuracy of the method was ascertained by performing recovery studies using standard addition method. To a pre-analyzed sample, standard drug aliquots were added at three different levels viz. 4μg mL^{-1}, 12 μg mL^{-1}, 20 μg mL^{-1} to baseline amount of capsule powder equivalent to 4 μg mL^{-1} of cefixime. These mixtures were transferred into separate 10 mL volumetric flasks and then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.6) and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with methanol. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 550 nm against blank prepared in the same manner without addition of the examined drug.

The amount of total drug was calculated and the amount of standard drug aliquots recovered was calculated using following formula, Percent recovery= (T-A/S)* 100. where, T is the total amount of drug estimated, A is the amount contributed by capsule powder (as per amount estimated by proposed method) and S, the amount of pure drug added. The standard linearity curve was prepared with standard solution and which was used in estimation of spiked sample.

3.3.6.3 Precision

Intraday and interday precision were assessed by triplicate analysis of three different concentrations (4, 16 and 24 μg mL^{-1}). Precision of the analytical method is expressed as SD or RSD of series of measurement by replicate estimation of drugs by the proposed method.

3.3.6.4 Method sensitivity (LOD and LOQ)

The values of LOD and LOQ were calculated by using S (standard deviation of response) and *b* (the slope of the calibration curve and by using equations: LOD = 3.3 * S/*b* and LOQ = 10 * S/*b*

S is calculated as the standard deviation of the residuals around the regression line.

and b: Slope of the regression line.

$$s_{res} = \sqrt{\frac{\sum (Y - Y_{est})^2}{n - 2}}$$

3.3.6.5 Specificity

The specificity of the methods was investigated by observing any interference encountered from common excipients of the pharmaceutical formulation such as starch, magnesium stearate, and lactose. To fulfill this purpose, a mixture was prepared by mixing equal amounts of lactose, starch and magnesium stearate and cefixime.

Procedure: A quantity of the prepared mixture containing 40mg cefixime was dissolved in methanol (50 mL) with good shaking, filtered and the volume was completed to 100 mL with methanol. Accurately measured aliquots of cefixime filtrate were transferred into separate 10 mL volumetric flasks and then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.6) and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with methanol. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 550 nm against blank prepared in the same manner without addition of the examined drug.

3.3.7 Structure of the ternary complex of cefixime

The nature of the ternary complex (cefixime–Cu(II)–eosin) was determined using Job's method of continuous variation (Job, 1964). Master equimolar (1×10^{-3} M) aqueous solutions of eosin B and copper (II) sulfate and methanolic solution of cefixime were prepared. Series of 1 mL portions of the master solutions of cefixime and copper (II) sulfate were made up comprising different complementary proportions (0.2:0.8, 0.4:0.6, 0.6:0.4, 0.8:0.2). These 1 ml portions were transferred to 10 mL volumetric flasks, eosin was added to each flask in excess (1.5 mL), then 1 mL of acetate buffer (pH 5.6) was added. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with methanol. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 550 nm against blank prepared in the same manner without addition of the examined drug. A graph of absorbance then plotted versus mole fraction.

The same procedure was repeated by using complementary proportions of cefixime and eosin in presence of excess copper (II) sulfate (1.5 mL) and also repeated by using complementary proportions of eosin and copper (II) sulfate in presence of excess cefixime solution (1.5 mL).

3.3.8 Assay of cefixime pharmaceutical dosage form

The contents of twenty capsules were emptied and triturated and a known weight of the powder equivalent to 40 mg of the drug was dissolved in 60 mL methanol with good shaking for 10 minutes and any remaining residue was removed by filtration. The filtrate solution was then transferred into 100 mL calibrated flask and diluted to 100 mL with methanol. Different volumes (0.1, 0.3 and 0.6 mL) of the filtrate solution were transferred into separate 10 mL volumetric flasks and then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.6) and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with methanol. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 550 nm against blank prepared in the same manner without addition of the examined drug. A triplicate measurements were done for each concentration and the percentage of recovery was calculated from the calibration graph.

3.4 Spectrophotometric estimation of glimepiride

3.4.1 Preparation of standard stock solution of glimepiride

Glimepiride stock solution (500 μg mL^{-1}) was prepared by dissolving 50 mg of the drug substance in a 100 mL DMF with good shaking for 5 minutes.

3.4.2 General procedure for glimepiride determination

Accurately measured aliquots of glimepiride standard solution in the range of 5–50 μg mL^{-1} of drug were transferred into separate 10 mL volumetric flasks and then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.0), 1 mL MC and 1 mL eosin solution. The contents of each flask were

shaken thoroughly and each mixture was diluted to 10 mL with water. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 544 nm against blank prepared in the same manner without addition of the examined drug.

3.4.3 Study of spectral characteristic of glimepiride ternary complex

After enabling the initial adjustment and blank correction. Glimepiride solution (25 µg mL^{-1}) prepared according to the general procedure (section 3.4.2) was scanned in the range from 630 to 400 nm to obtain the wavelength of maximum absorption (λ_{max}).

3.4.4 Optimization of reaction conditions

3.4.4.1 Effect of surfactant

The effect of surfactant was studied by using different surfactants such as MC, SLS and methanol as solvent.

Procedure: 0.5 mL glimepiride solution was transferred in 10 mL volumetric flasks and then 1 mL of copper (II), 1 mL eosin and 1 mL acetate buffer (pH 5) solutions were added followed by either MC or SLS then the volume was completed with water. The same work was repeated using methanol instead of surfactants and then complete to volume by methanol. The flasks were left for 20 minutes and then the absorbance was measured at 544 nm.

3.4.4.2 Effect of pH

The absorbance of cefixime was measured at different pH values: 4, 5, 5.6 and without buffer (pH 7).

Procedure: To 0.5 mL glimepiride standard solution (in 10 mL volumetric flasks), 1 mL of copper (II), 1 mL MC and 1 mL eosin solutions were added followed by 1 mL buffer of different pH solutions. The solutions were diluted to 10 mL with water and the absorbance was measured at 544 nm after 20 minutes.

3.4.4.3 Effect of reaction time

The absorbance of glimepiride was measured after 5, 10, 15, 20 and 25 minutes.

Procedure: 1 mL of copper (II), 1 mL eosin, 1 mL MC and 1 mL acetate buffer solution (pH 5) were added to 0.5 mL glimepiride standard solution and the mixture was diluted to 10 mL with water and the absorbance was measured at 544 nm after 5, 10, 15, 20 and 25 minutes and the time required to reach maximum absorbance was determined.

3.4.4.4 Effect of concentration of Cu(II) and eosin solutions

Different concentrations of Cu(II) and eosin solutions were analyzed in ternary complex formation with glimepiride. Accurately measured aliquots of 1 mL of glimepiride standard solution were transferred into separate 10 mL volumetric flasks and then 1 mL acetate buffer solution (pH 5) was added to each flask, followed by 1 mL MC and 1 mL eosin solution. Different volumes (0.5, 1 and 2 ml) of copper (II) sulfate solution were added to the flasks. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with water. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 544 nm against blank prepared in the same manner without addition of the examined drug. The same procedure was repeated by changing the volume of eosin solution.

3.4.4.5 Effect of temperature

Aliquots of 0.5 mL glimepiride standard solution were transferred into five separate 10 mL volumetric flasks and then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5), 1 mL MC and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with water. One flask was left at room temperature for 20 minutes, other flasks were heated on water bath at 40, 50, 60 and 70ºC for 20 minutes and then the absorbance of the resulting solution was measured after cooling to room temperature at 544 nm against blank prepared in the same manner without addition of the examined drug.

3.4.5 Construction of calibration curve of glimepiride

Accurately measured aliquots of glimepiride standard stock solution (0.1, 0.3, 0.6, 0.8 and 1.0 mL) in the range of 5–50 μg mL^{-1} were transferred into separate 10 mL volumetric

flasks and then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.0), 1 mL MC and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with water. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 544 nm against blank prepared in the same manner without addition of the examined drug. A triplicate measurements were done for each concentration. The concentration (μg mL^{-1}) of glimepiride was plotted against the absorbance (mean value). Least square regression analysis was performed for the obtained data. Correlation coefficient, slope, intercept and molar absorptivity were calculated from the graph.

3.4.6 Method validation

Analytical method validation was performed according the ICH guidelines (ICH, 2005) with respect to accuracy, precision, specificity, linearity and limit of detection and limit of quantitation.

3.4.6.1 Linearity

The linearity of the proposed method was determined by measuring the absorbance of five concentrations covering the range (5, 15, 30, 40 and 50 μg mL^{-1}), each concentration was measured in triplicate. Then the plot of absorbance against concentration was examined visually and statistically by calculating correlation coefficient.

3.4.6.2 Accuracy

Three concentrations covering the range were measured and each of them was repeated three times to calculate accuracy and precision.

Accuracy of the method was ascertained by performing recovery studies using standard addition method. To a pre-analyzed sample, standard drug aliquots were added at three different levels viz. 5 μg mL^{-1}, 20 μg mL^{-1}, 40 μg mL^{-1} to baseline amount of tablet powder equivalent to 5 μg mL^{-1} of glimepiride. These mixtures were transferred into separate 10 mL volumetric flasks and then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.0), 1 mL MC and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted

to 10 mL with water. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 544 nm against blank prepared in the same manner without addition of the examined drug.

The amount of total drug was calculated and the amount of standard drug concentrations recovered were calculated using following formula, Percent recovery= (T-A/S)* 100. where, T is the total amount of drug estimated, A is the amount contributed by capsule powder (as per amount estimated by proposed method) and S, the amount of pure drug added. The standard linearity curve was prepared with standard solution and which was used in estimation of spiked sample.

3.4.6.3 Precision

Intraday and interday precision were assessed by triplicate analysis of three different concentrations (10, 25 and 50 μg mL^{-1}). Precision of the analytical method is expressed as SD or RSD of series of measurement by replicate estimation of drugs by the proposed method.

3.4.6.4 Method sensitivity (LOD and LOQ)

The values of LOD and LOQ were calculated by using S (standard deviation of response) and *b* (the slope of the calibration curve and by using equations:

LOD = 3.3 * S/*b* and LOQ = 10 * S/*b*

S is calculated as the standard deviation of the residuals around the regression line.

and b: Slope of the regression line.

$$s_{res} = \sqrt{\frac{\sum (Y - Y_{est})^2}{n - 2}}$$

3.4.6.5 Specificity

The specificity of the methods was investigated by observing any interference encountered from common excipients of the pharmaceutical formulation such as starch, magnesium stearate, and lactose. To assess unequivocally the analyte in the presence of such excipients a mixture was prepared by mixing equal amounts of lactose, starch and magnesium stearate and glimepiride.

Procedure: A quantity of the prepared mixture containing 25 mg glimepiride was dissolved in DMF (25 mL) with good shaking, filtered and the volume was completed to 50 mL with DMF. Accurately measured aliquots of glimepiride solution were transferred

into separate 10 mL volumetric flasks and then 1 mL copper (II) sulfate solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.0), 1 mL MC and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with water. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 544 nm against blank prepared in the same manner without addition of the examined drug.

3.4.7 Structure of the ternary complex of glimepiride

The nature of the ternary complex (glimepiride–Cu(II)–eosin) was determined using Job's method of continuous variation (Job, 1964). Master equimolar (1×10^{-3} M) aqueous solutions of eosin B and copper (II) sulfate and glimepiride in DMF were prepared. Series of 1 mL portions of the master solutions of glimepiride and copper (II) sulfate were made up comprising different complementary proportions (0.2:0.8, 0.4:0.6, 0.6:0.4, 0.8:0.2). These 1 mL portions were transferred to 10 mL volumetric flasks, eosin was added to each flask in excess (1.5 mL), then 1 mL of acetate buffer (pH 5) and 1 mL of MC solution were added. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with distilled water. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 544 nm against blank prepared in the same manner without addition of the examined drug. A graph of absorbance then plotted versus mole fraction.

The same procedure was repeated by using complementary proportions of glimepiride and eosin in presence of excess copper (II) sulfate (1.5 mL) and also repeated by using complementary proportions of eosin and copper (II) sulfate in presence of excess glimepiride solution (1.5 mL).

3.4.8 Assay of glimepiride pharmaceutical dosage form

The contents of twenty tablets were weighed and powdered and a known weight of the powder equivalent to 25 mg of glimepiride was dissolved in 25 mL DMF with good shaking for 10 minutes and any remaining residue was removed by filtration. The filtrate solution was then transferred into 50 mL calibrated flask and diluted to 50 mL with DMF. Accurately measured aliquots of glimepiride filtrate solution (0.1, 0.3 and 0.6 mL) were transferred into separate 10 mL volumetric flasks and then 1 mL copper (II) sulfate

solution was added to each flask followed by 1 mL acetate buffer solution (pH 5.0), 1 mL MC and 1 mL eosin solution. The contents of each flask were shaken thoroughly and each mixture was diluted to 10 mL with water. The flasks were left at room temperature for 20 minutes and then the absorbance of the resulting solution was measured at 544 nm against blank prepared in the same manner without addition of the examined drug. Triplicate measurements were done for each concentration and the percentage of recovery was calculated from the calibration curve.

Chapter (4)

4. Results and Discussion

The main purpose of this study was to establish simple spectrophotometric methods for the determination of some pharmaceutically interesting compounds in their dosage forms and in their pharmaceutical formulations without prior extraction through ternary complex formation.

Ternary complexes of the general formula ($L_NM_XS_Y$) have been widely used in spectrophotometric analysis of many drugs such as ramipril and perindopril (Abdellatef et al, 1999), amineptine and nortriptyline (Ayad et al, 1999), ramipril and enalapril (Ayad M.M., 2002), ciprofloxacin and norfloxacin (El Walily et al, 1996), astemizole, terfenadine and flunarizine (Kelani et al, 1999), hydrochlorothiazide, indapamide and xipamide (Omar, 2010). The particularity of the ternary complexes in this study is that their main ligands L are the cited drugs, the second ligand S is eosin, and M is copper(II) metal, respectively. These triple complexes are extractable with chloroform, whereas the binary systems (copper–drug and copper–eosin) can not be extracted in the same manner, (Ramadan and Mandil, 2006).

The ternary complex formed between the metal ion, electronegative ligand and organic base, often have higher values of molar extinction coefficient than binary complexes of the same components. The formation of ternary complexes improves not only the sensitivity of the method but also the selectivity as well.

Ternary complex formation had been used for determination of Pd(II) via 1,10 phenanthroline as a cationic component and eosin as an anionic counter ion (Kelani et al, 1999). On the same basis, Fujita et al. (1987) determined a group of drugs by formation of ternary complex with Pd(II) and eosin.

The mechanism of ternary complex formation includes chelate formation between copper ion and the drug molecule through electronegative atoms carrying free electron pairs, and subsequently, a ternary complex is formed by interaction with eosin.

In the present study, Cu(II) was used as complexing ion with the studied drugs. Appropriate conditions were established for the color reaction and for the eosin: Cu(II): drug ratio to reach maximum sensitivity.

To prove the formation of a ternary complex between copper(II) (A), eosin (B) and the drugs (cefixime or glimepiride) (C), the interaction of the three component may be considered as

either **AB + C ↔ AC + B** **(1)**

or **AB + C ↔ ABC** **(2)**

A series of absorption spectra have been obtained for each component, separately, and to their mixtures under the experimental conditions, discussed above (Fig. 4.1 to 4.6). The spectra revealed that aqueous solution of eosin, (B) absorbs in the visible region at λ_{max} 525 nm, while neither copper(II) sulphate (A) nor the drugs (C) have absorbance maximum in the visible region. The mixture (AB) has the same maximum absorbance as that of (B), also the mixture (AC) has the same maximum absorbance as that of (A) and (C), separately. According to these considerations, and to the finding that the complexes formed have absorption maximum at 544 nm and 550 nm for glimepiride and cefixime, respectively, the reaction could not be additive, but it would be a ternary complex system, ABC, having different properties from that of AB or AC. Furthermore, the reaction of copper (II) with cefixime or glimepiride, was studied in the absence of eosin and no visible reaction was observed. However, in the presence of eosin an instantaneous reaction occurs, which indicate the ionic character of the reaction.

4.1 Results of cefixime analysis

4.1.1 Absorption spectra

The absorption spectrum of cefixime was recorded against methanol (Fig. 4.1). It was found that cefixime exhibits a maximum absorption peak (λ_{max}) at 292 nm. Because of the highly blue shifted λ_{max} of cefixime, its determination in the dosage forms based on the direct measurement of its absorption for ultraviolet is susceptible to potential interferences

from the co-extracted common excipients. Therefore derivatization of the drug to a red-shifted light-absorbing derivative was necessary.

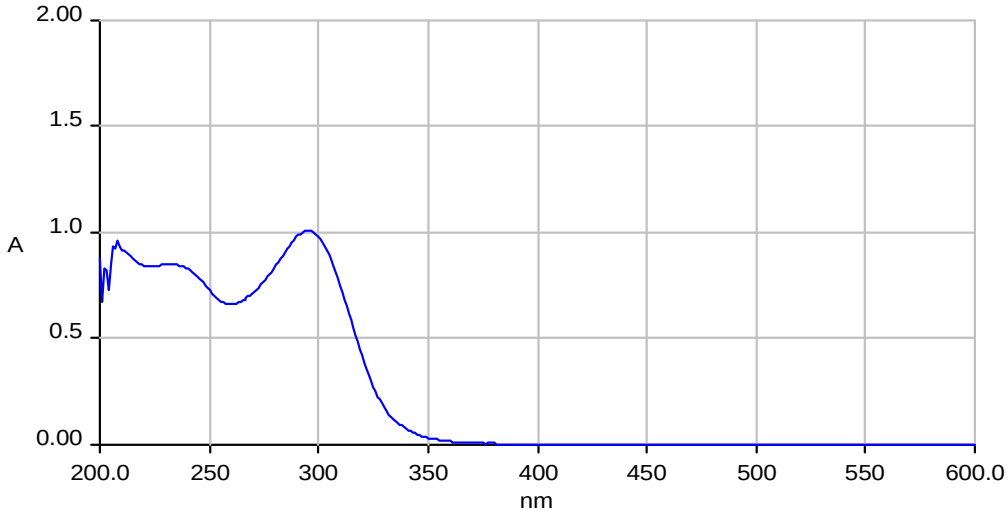

Figure 4.1: Absorption spectrum of cefixime (20µg mL^{-1}) in methanol.

Eosin absorption spectrum showed λ_{max} at 525 nm (Fig. 4.2), copper (II) sulfate have absorption only below 250 nm (Fig. 4.3).

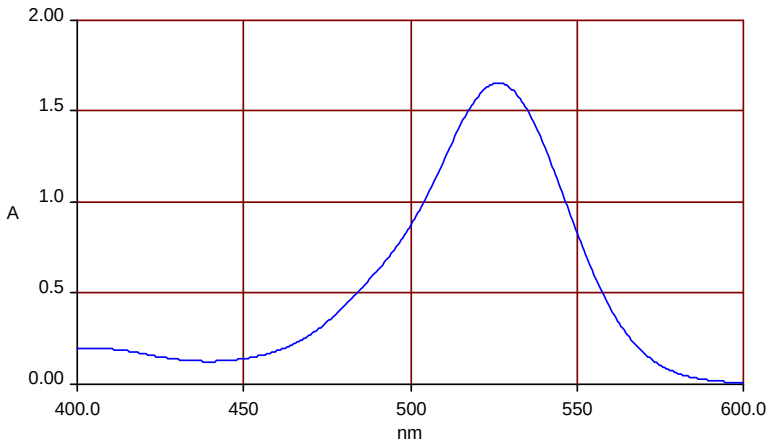

Figure 4.2: Absorption spectrum of eosin (20 µg mL^{-1}) aqueous solution.

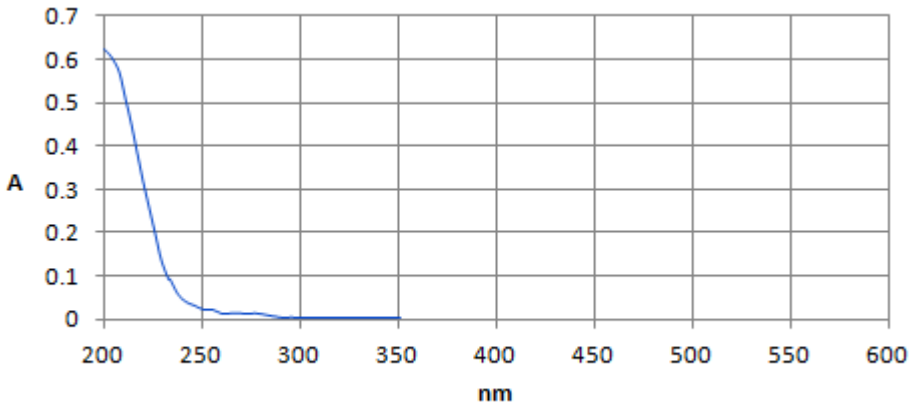

Figure 4.3: Absorption spectrum of copper sulfate (50 µg mL^{-1}) aqueous solution.

The absorption spectra of every two components were examined, none of them have shift in λ_{max} to 550 nm. Eosin with copper (II) sulfate in acidic medium (pH 5) have λ_{max} below 500 nm, (Fig. 4.4). Cefixime added to eosin didn't cause shift in λ_{max} of eosin alone, (Fig. 4.5). Cefixime with Copper (II) sulfate have the same λ_{max} of cefixime alone, and there is no shifting to visible range by changing pH. (Fig. 4.6).

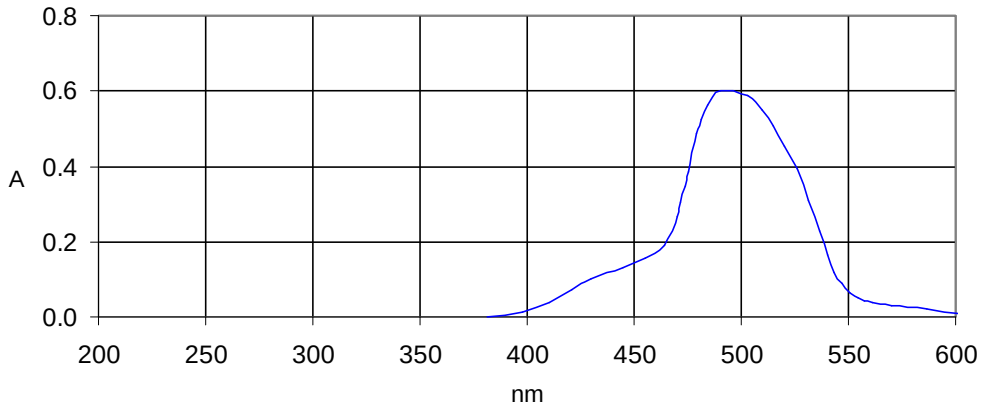

Figure 4.4: Absorption spectrum of eosin-copper(II) solution with acetate buffer.

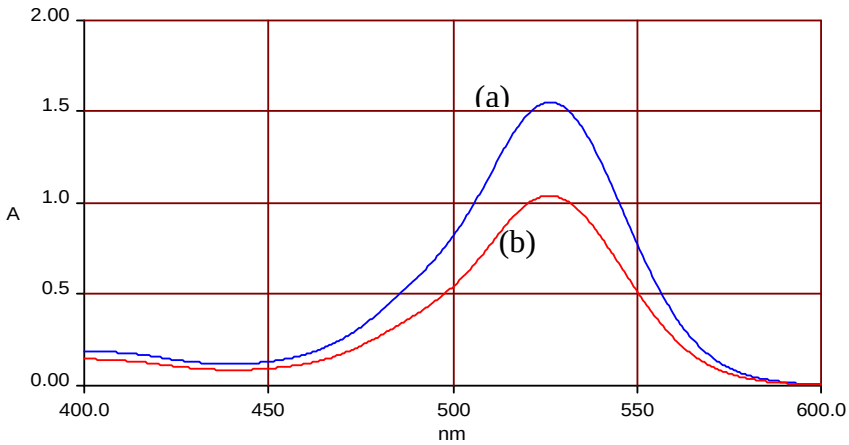

Figure 4.5: Absorption spectrum of eosin (a) versus eosin and cefixime binary mixture (b) (aqueous media)

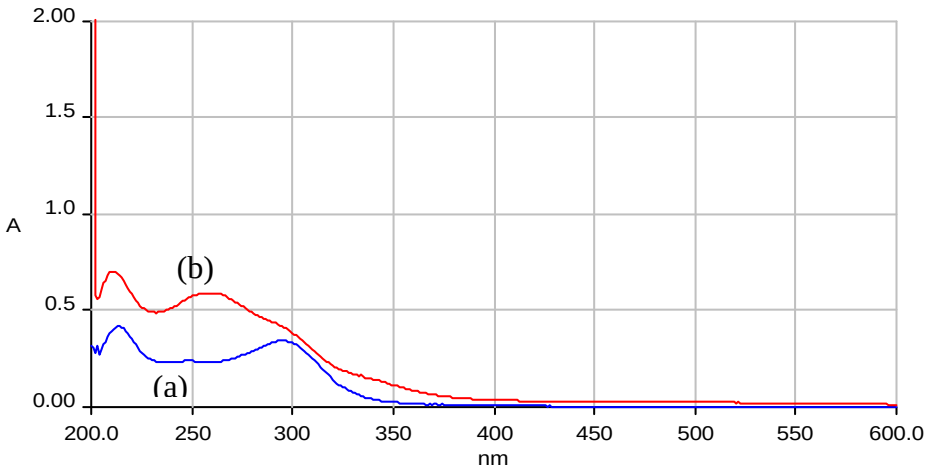

Figure 4.6: Absorption spectrum of cefixime (a) versus Cu (II) and cefixime (b) (aqueous media)

The reaction between cefixime and eosin and copper was performed, and the absorption spectrum of the product was scanned in the range of 400-600 nm against a reagent blank. It was found that the product exhibiting λ_{max} at 550 nm and the absorbance increased directly with cefixime concentration (Fig. 4.7 and 4.8). Obviously, the λ_{max} of the ternary complex (Drug-Cu-Eosin) was red-shifted from the λ_{max} of cefixime alone (292 nm), eliminating any potential interferences. Therefore, the measurements were carried out at 550 nm. The molar absorptivity of the ternary complex is sufficiently high (ε = 1.491 X 10^4 $Lmol^{-1}cm^{-1}$) for acceptable level of sensitivity for the method of analysis.

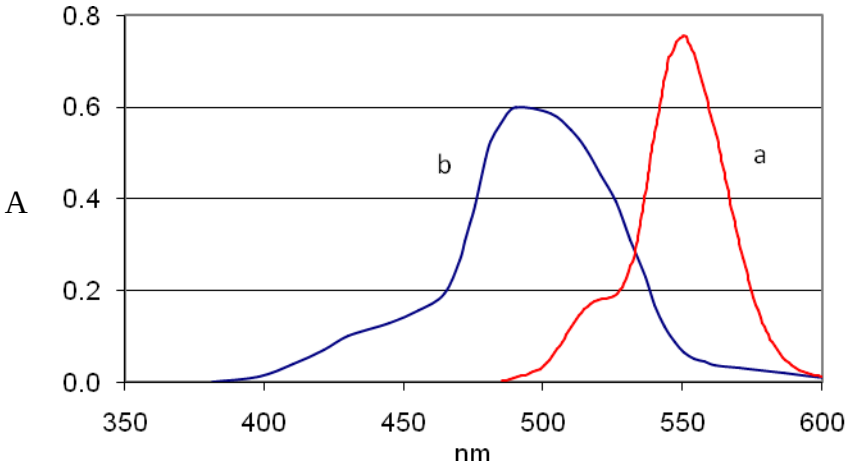

Figure 4.7: Absorption spectrum of ternary complex of cefixime (a) versus binary mixture of Cu(II) plus eosin (b) in acidic medium (pH 5)

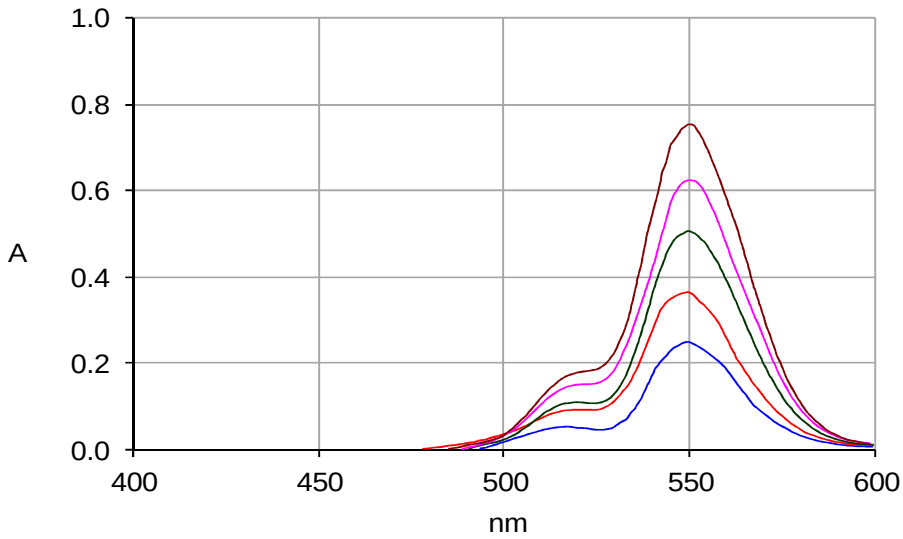

Figure 4.8 Absorption spectrum of ternary complex of cefixime (concentrations from lower to higher curve: 8, 12, 16, 20 and 24 µg mL^{-1}) against reagent blank.

4.1.2 Optimization of reaction conditions

To optimize the assay variables, the effects of surfactant, pH, reaction time, eosin, copper (II) and temperature on the absorbance of the ternary complex formed with respect to maximum sensitivity, adherence to Beer's law and stability were studied through control

experiments. The optimum conditions were established by varying one variable and observing its effect on the absorbance of the colored products.

4.1.2.1 Effect of surfactant

The effect of surfactant on the absorbance of the solution of the complex was studied using various dispersing agents, for example, an ionic surfactant sodium lauryl sulphate (SLS) and the non ionic surfactant methylcellulose (MC). The effect of aqueous surfactant solutions were compared with methanol alone as a solvent. In this study, the use of surfactants and methanol prevented precipitate formation and therefore excluded prior extraction steps. However, the use of methanol alone as a solvent improved the complex stability and produced the highest sensitivity when compared to SLS and MC (Fig. 4.9).

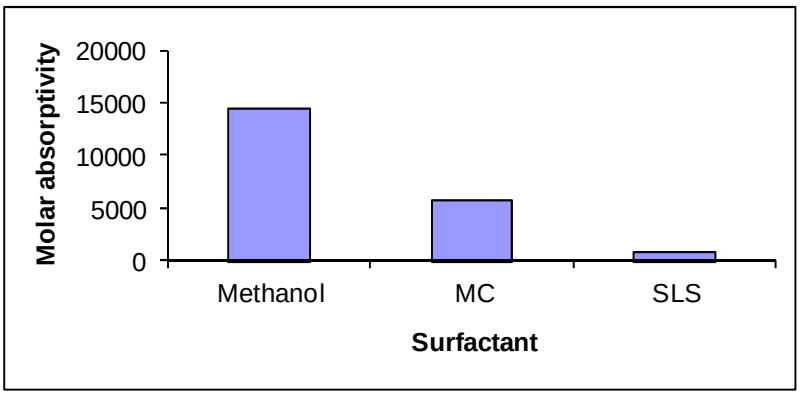

Figure 4.9: Effect of surfactant on absorptivity of cefixime ternary complex.

4.1.2.2 Effect of pH

The effect of pH on the absorbance of ternary complex was studied using acetate buffer of pH values; 4, 5 and 5.6 and also without buffer (pH = 7). Values of pH were examined only in the range of 4-7 because eosin and copper (II) start to precipitate at pH > 9, and also because the color of the ternary complex will disappear at pH < 4.

The optimum absorbance was achieved at pH 5.6 as shown in figure 4.10.

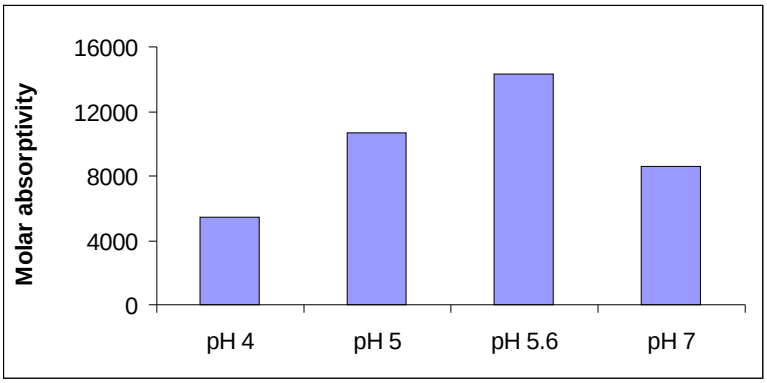

Figure 4.10: Effect of pH on the absorptivity of cefixime-Cu(II)-eosin ternary complex.

4.1.2.3 Effect of temperature and reaction time

In order to examine the effect of temperature and reaction time on the absorbance of the ternary complex, the above mentioned procedure was carried out at room temperature, and 40°C using thermostatic water bath. It was found that the absorbance of ternary complex was not affected by increasing the temperature, so the reaction at room temperature (25 ± 5 ºC) has been selected for ease. The reaction went to completion in 20 minutes after dilution at room temperature (reached maximum and constant absorbance, Fig. 4.11), The color formed under the above mentioned optimum conditions was stable for at least 2 h. Therefore, further experiments were carried out at room temperature (25 ± 5 ºC) for 20 minutes.

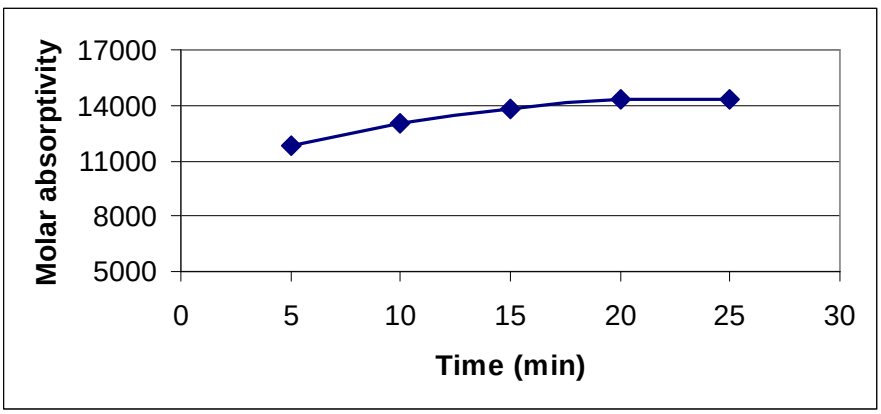

Figure 4.11: Effect of reaction time on the absorptivity of cefixime (20 µg mL^{-1}) ternary complex at room temperature.

4.1.2.4 Effect of concentration of copper (II) and eosin solutions

Concerning the effect of eosin concentration on the absorbance of the complex formed, the optimum results i.e., maximum and constant absorbance were obtained using 1 mL of 0.1% w/v eosin. Also the effect of Cu(II) concentration was studied by keeping the concentration of eosin constant and varying the metal concentration. Optimum results were obtained by using 1 mL of 0.1% w/v copper sulfate. Higher concentration of reagents did not affect the color intensity, therefore, further experiments were carried out using 1 mL of 1% solution of each of Cu (II) and eosin.

4.1.3 Validation of the proposed method

4.1.3.1 Linearity

Under the above experimental conditions, the calibration curve was constructed by plotting concentration of cefixime versus absorbance (Fig. 4.12). A linear calibration graph was found between absorbance and concentration in the range of 4-28 µg mL^{-1}. The correlation coefficient, intercept and slope for the calibration data for cefixime were calculated using the least squares method (Table 4.1).

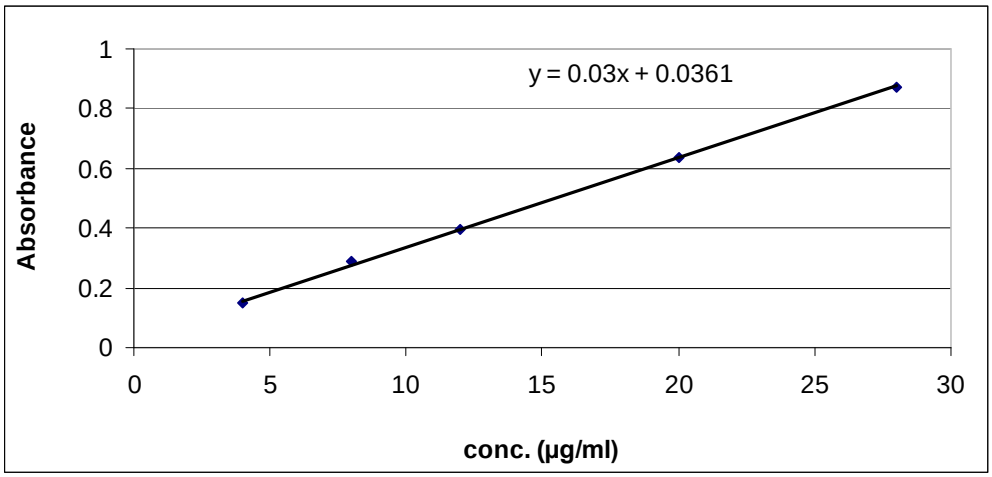

Figure 4.12: Calibration curve for cefixime determination.

Table 4.1: Optical characteristics and statistical data of the regression equation for the ternary complex formation with cefixime.

Parameters	**Cefixime Spectral Data**
λ_{max} nm	550
Beer's law limits, μg mL^{-1}	4 – 28
Molar absorptivity, L mol^{-1} cm^{-1}	1.491 X 10^4
Sandell's sensitivity, μg/cm^2	0.030
Limit of detection, μg mL^{-1} (LOD)	0.900
Limit of quantitation, μg mL^{-1} (LOQ)	2.70
Regression equation*	**Y = 0.0361 + 0.03X**
Intercept (a)	0.036
Slope (b)	0.030
Correlation coefficient (*r*)	0.9997

*Y= a + bX, where Y is the absorbance, a intercept, b slope and X concentration in μgmL^{-1}.

4.1.3.2 Sensitivity (LOD, LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) for the proposed method were calculated using the following equations:

LOD = 3.3 SD/S LOQ = 10 SD/S

SD is calculated as the standard deviation of the residuals around the regression line, S is the slope of calibration curve. LOQs and LODs for cefixime are listed in Table 4.1.

Sandell's sensitivity is the concentration of the drug in μg mL^{-1} which will exhibit an absorbance of 0.001 in 1 cm cell, and is expressed as μg cm^{-1}.

Sandell's sensitivity is calculated from the molecular weight and molar absorptivity according to the equation:

$$\text{Sandell's sensitivity} = \frac{\text{Molecular weight}}{\text{Molar absorptivity}}$$ (Rohitas et al, 2010).

4.1.3.3 Precision

The precision and accuracy of the proposed method were tested by means of replicate measurements of the tested drug within Beer's law limits. The precision of the analytical

procedure is usually expressed as the standard deviation of a series of measurements. Intraday and interday precision were assessed using three concentration and three replicates of each concentration.

The calculated relative standard deviation values were found to be very small (< 2%) indicating good repeatability and reliability of the proposed methods. The results and their statistical analysis were summarized in Table 4.2.

Table 4.2: Evaluation of precision of the analytical procedure of cefixime

Statistical Parameters		**4 µg mL^{-1}**	**16 µg mL^{-1}**	**24 µgmL^{-1}**
Intraday				
	1	4.057	16.08	24.297
	2	4.068	15.835	24.152
	3	4.022	15.807	24.177
	Mean recovery	4.049	15.907	24.209
	Mean % recovery	101.22	99.42	100.87
	S.D.	0.024	0.15	0.0775
	R.S.D. (%)	0.59	0.94	0.320
	Relative error	1.22%	0.58%	0.87%
Interday	1	4.050	15.907	24.210
	2	4.065	15.914	24.220
	3	4.024	16.070	23.921
	Mean recovery	4.046	15.96	24.117
	Mean % recovery	101.15	99.75	100.5
	S.D.	0.021	0.092	0.170
	R.S.D. (%)	0.51	0.58	0.705
	Relative error	1.15%	0.25%	0.488

S.D. = Standard Deviation, R.S.D. = Relative Standard Deviation

4.1.3.4 Accuracy

Accuracy of the proposed method was further confirmed by performing recovery studies using standard addition method. A fixed amount of drug from dosage form was taken and pure standard drug at three different concentrations within Beer's range was added. The total concentration was found by the proposed method. The determination with each concentration was repeated three times and average percent recovery of the added standard was calculated and results are tabulated in Table 4.3. The results obtained in Tables 4.2 and 4.3 showed excellent mean recovery percent values, close to 100 %, and low standard deviation values (S.D. < 1.0) which indicate high accuracy of the proposed analytical methods.

Table 4.3: Results of recovery study for cefixime

Base level ($\mu g\ mL^{-1}$)	**Amount spiked ($\mu g\ mL^{-1}$)**	**Amount recovered* ($\mu g\ mL^{-1}$)**	**% Recovery ± SD**
4	4.00	4.036	100.9±0.273
4	12.0	12.078	100.66±0.302
4	20.0	19.92	99.6±0.64

* Mean value of three determinations

4.1.3.5 Specificity

The commonly used additives and excipients in the preparation of capsules (such as starch, lactose, talc, and magnesium stearate) were found not to interfere in the analysis (Table 4.4).

Table 4.4: Results of specificity study for cefixime

Concentration ($\mu g\ mL^{-1}$)	**Amount recovered* ($\mu g\ mL^{-1}$)**	**% Recovery ± SD**
4.00	4.033	100.8±0.373
16.0	16.06	100.4±0.22
24.0	23.92	99.7±0.74

*****Mean value of three determinations

4.1.4 Structure of the ternary complex of cefixime

The nature of the ternary complex (cefixime–Cu(II)–eosin) was determined using Job's method of continuous variation. The result of applying this method can be summarized as follows: the [cefixime:copper(II)] ratio in presence of excess eosin was 1:1 (Fig. 4.13a), the [cefixime:eosin] ratio in presence of excess Cu(II) sulfate was 1:1 (Fig. 4.13b), and the [eosin:copper(II)] ratio in presence of excess cefixime was 1:1 (Fig. 4.13c). Hence the composition of the ternary complex formed may be expressed as cefixime–copper(II)–eosin (1:1:1).

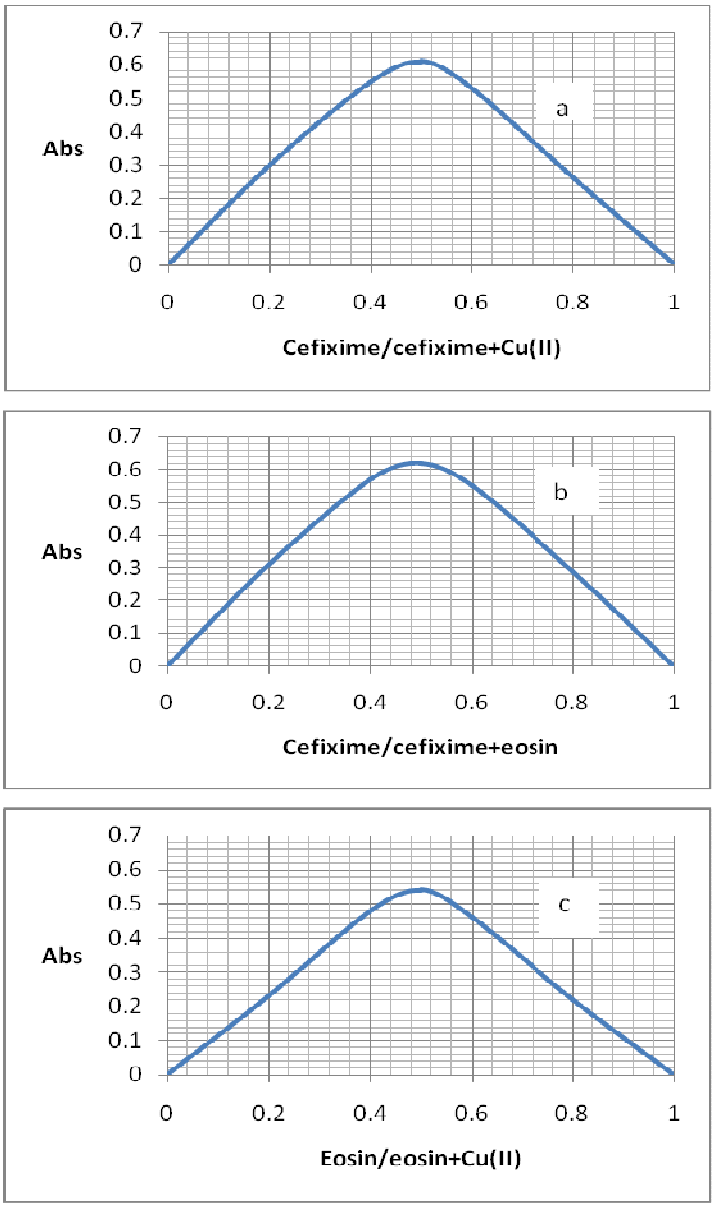

Figure 4.13: (a) Continuous variation plots for cefixime: Cu(II) in the presence of excess eosin. (b) Continuous variation plots for cefixime: eosin in the presence of excess Cu(II). (c) Continuous variation plots for eosin: Cu(II) in the presence of excess cefixime.

According to results of Job's method of continuous variation, a proposed structure of cefixime ternary complex is shown in figure 4.14.

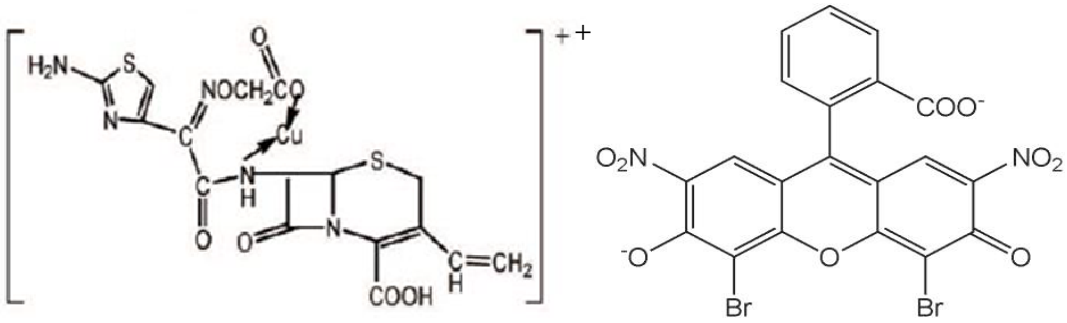

Figure 4.14: proposed structure of cefixime-Cu-eosin ternary complex.

4.1.5 Application to pharmaceutical dosage form (capsules)

The assay for the marketed capsules of cefixime was established using the present optimized spectrophotometric conditions and it was found to be accurate and reliable. The results are shown in Table 4.5. The assay values of cefixime for capsule formulation ranged from 98.30 % to 98.9 %, with relative standard deviation of not more than 1.41. The assay values for the formulations were very close as mentioned in the label claim, indicating that the interference of excipient matrix is insignificant in the estimation of cefixime by the proposed analytical method. Recovery percentage obtained by the proposed method was satisfactory when compared with other methods (99.71 % was obtained by Frag et al, (2012), and it lies in the accepted range by BP, 2011 (95-102%). The estimated drug content with low values of standard deviation established the precision of the proposed method.

Table 4.5: Results of application of spectrophotometric method to the determination of cefixime from pharmaceutical dosage form (capsules)

Label claim ($\mu g\ mL^{-1}$)	**Amount recovered* ($\mu g\ mL^{-1}$)**	**% Recovery ± SD**
4	3.93	98.3±1.41
12	11.87	98.9±0.89
24	23.65	98.6±0.93

*Mean value of three determinations

4.2 Results of glimepiride analysis

4.2.1 Absorption spectra

The absorption spectrum of glimepiride was recorded against DMF (Fig. 4.15). It was found that glimepiride has no absorption in the visible region and exhibits a maximum absorption peak (λ_{max}) at 268 nm with molar absorptivity (ε) of 0.96 X 10^3 $Lmol^{-1}cm^{-1}$. Because of the highly blue shifted λ_{max} of glimepiride, its determination in the dosage forms based on the direct measurement of its absorption for ultraviolet is susceptible to potential interferences from the co-extracted common excipients and/or degradation products. Therefore, derivatization of the drug to a red-shifted light-absorbing derivative was necessary.

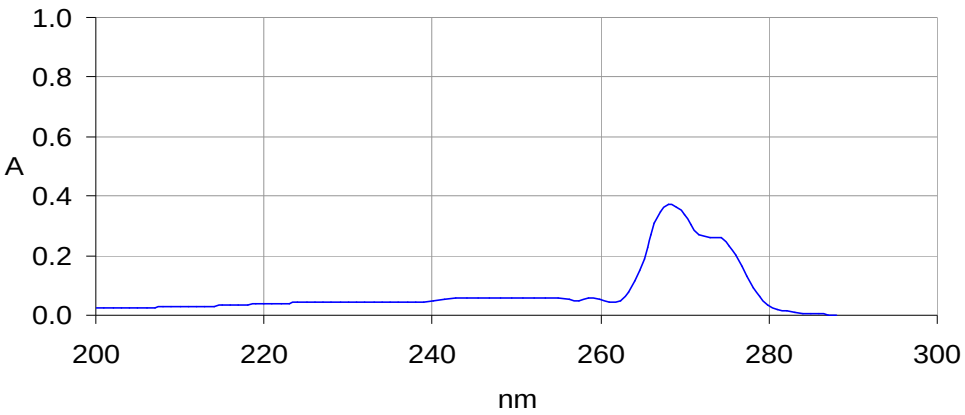

Figure 4.15: Absorption spectrum of glimepiride (200 µg mL^{-1}) in DMF

The absorption spectra of every two components were examined, none of them have shift in λ_{max} to 544 nm. Glimepiride added to eosin didn't cause shift in λ_{max} of eosin alone, (Fig. 4.16). Glimepiride with Copper (II) sulfate have the same λ_{max} of glimepiride alone, and by changing pH values there is no shifting of absorption to visible range. (Fig. 4.17).

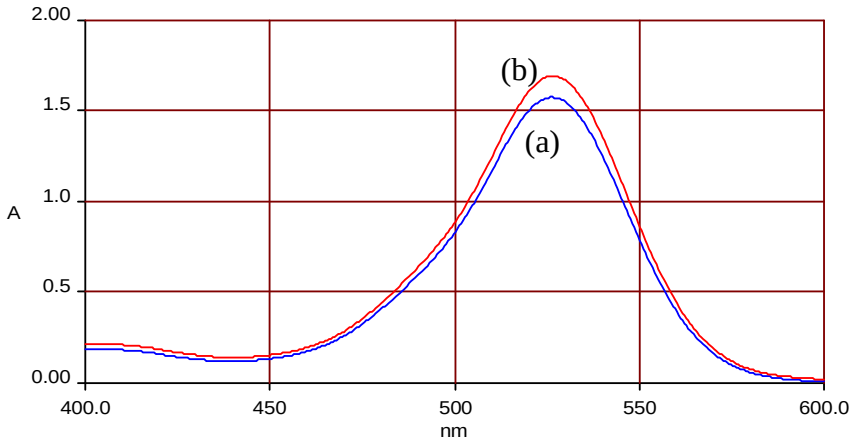

Figure 4.16: Absorption spectrum of eosin (a) versus glimepiride with eosin (b).

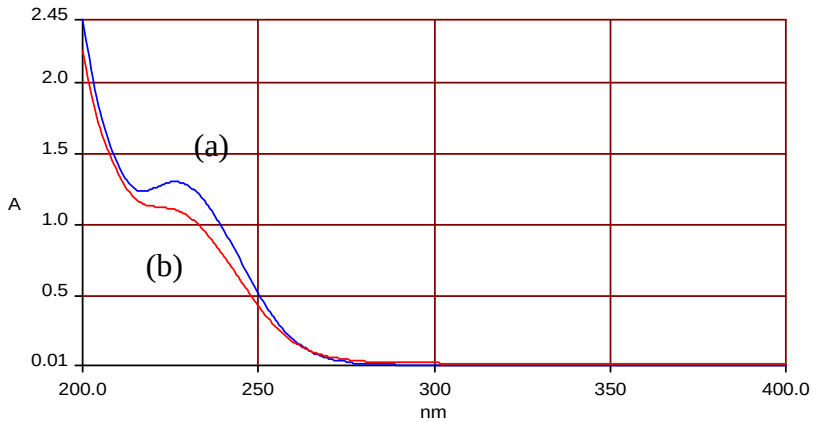

Figure 4.17: Absorption spectrum of glimepiride (a) versus glimepiride with Cu (II) (b).

The reaction between glimepiride and eosin and copper was carried out, and the absorption spectrum of the product was scanned in the range of 400-600 nm against a reagent blank. It was found that the product is exhibiting λ_{max} at 544 nm (Fig. 4.18 and 4.19). Obviously, the λ_{max} of the ternary complex (glimepiride-Cu-Eosin) was red-shifted from the λ_{max} of glimepiride alone (268 nm), eliminating any potential interferences. Therefore, the measurements were carried out at 544 nm. Furthermore, there is a substantial increase in the molar absorptivity of the drug (ε = 1.657 X 10^4 L $mol^{-1}cm^{-1}$) which leads to improvement in the sensitivity of the method of analysis.

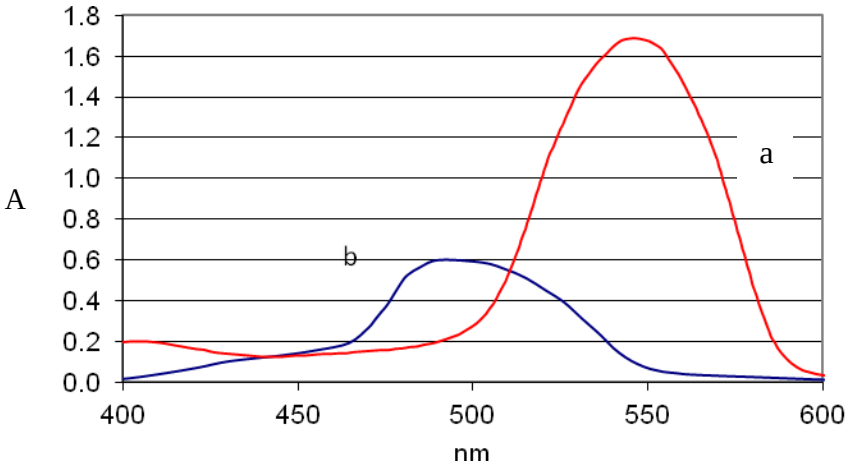

Figure 4.18: Absorption spectrum of ternary complex of glimepiride (a) versus binary mixture of Cu(II) plus eosin (b) in acidic medium (pH 5).

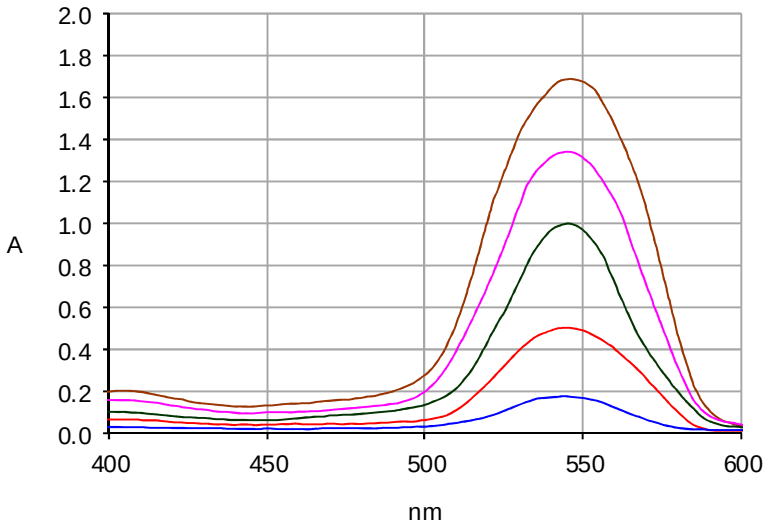

Figure 4.19: Absorption spectrum of ternary complex of glimepiride (concentrations from lower to higher curve: 5, 15, 30, 40 and 50 µg mL^{-1}) against reagent blank

4.2.2 Optimization of reaction conditions

To optimize the assay conditions, the effects of surfactant, pH, reaction time, eosin and copper (II) concentrations and temperature on the absorbance of the ternary complex formed were studied through control experiments. These factors were optimized with respect to maximum sensitivity, adherence to Beer's law and stability

The optimum conditions were established by varying one parameter and observing its effect on the absorbance of the colored products.

4.2.2.1 Effect of surfactant

The effect of surfactant on the absorbance of the glimepiride ternary complex was studied using methylcellulose (MC) and sodium lauryl sulfate (SLS). The addition of surfactants to solubilize and stabilize the ternary complex had been previously reported (Fujita, 1987). Cationic surfactants such as cetylpyridinium chloride were found to depress the colored complex formation probably due to the formation of an ion-pair complex between eosin and the cationic surfactant. MC, which is a non ionic water-soluble polymeric surfactant, was reported to be the best dispersing agent with respect to sensitivity (Fujita, 1987). Accordingly, MC was used. In this experiment, the use of surfactants excluded prior extraction steps. However, the addition of MC was found to improve the complex stability and to produce the highest sensitivity when compared to SLS and methanol. The best results (regarding maximum and constant absorbance) were obtained with 1 mL of 0.3% w/v MC solution (Fig.4.20).

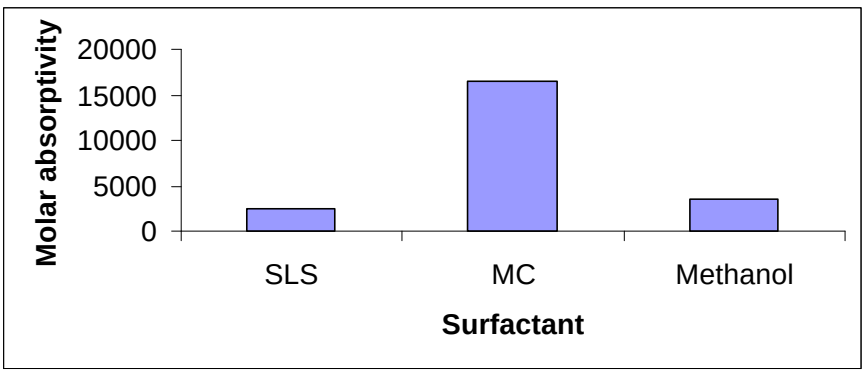

Figure 4.20: Effect of surfactant on absorptivity of glimepiride ternary complex.

4.2.2.2 Effect of pH

The effect of pH on the absorbance of ternary complex was studied using acetate buffer of pH values; 4, 5 and 5.6 and also without buffer (pH = 7). Maximum absorbance was achieved at pH 5.0 as shown in figure 4.21.

The effect of pH values on ternary complex formation and its stability might be attributed to the effect of ionization of eosin and/or drug. It was reported that in the

presence of MC and at pH 4.3 about 80% of eosin was found to be in the form HR^- (El-Enany, 2004).

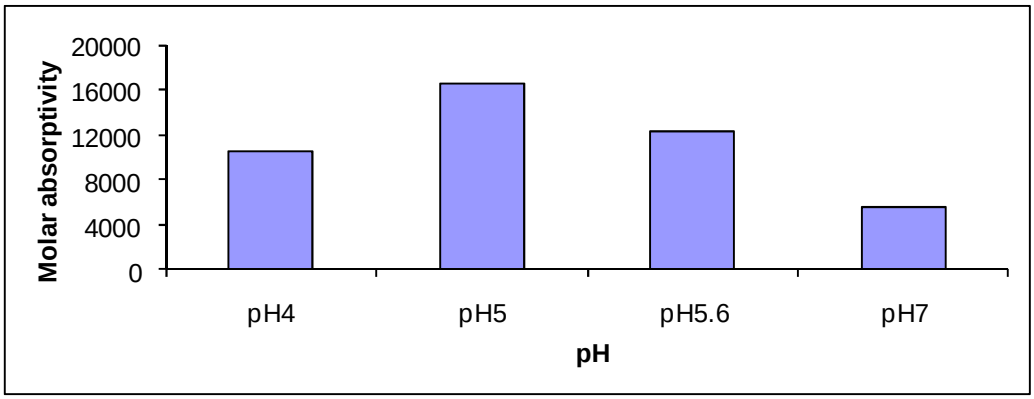

Figure 4.21: Effect of pH on the absorptivity of glimepiride ternary complex.

4.2.2.3 Effect of temperature and reaction time

In order to examine the effect of temperature and reaction time on the absorbance of the ternary complex, the above mentioned procedure was carried out at room temperature and at 40, 50, 60 and 70 °C using thermostatic water bath. It was found that the absorbance of ternary complex was not affected by increasing the temperature (Fig. 4.22) and the reaction at room temperature (25 ± 5 ºC) went to completion in 20 minutes after dilution (reached maximum and constant absorbance, Fig. 4.23). The color formed under the above mentioned optimum conditions was stable for at least 2 h. Therefore, further experiments were carried out at room temperature (25 ± 5 ºC) for 20 minutes.

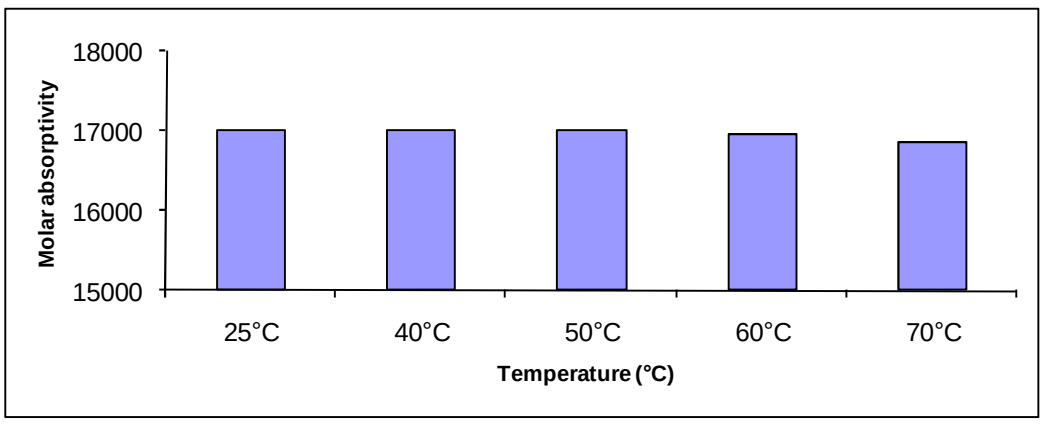

Figure 4.22: Effect of temperature on absorptivity of glimepiride ternary complex.

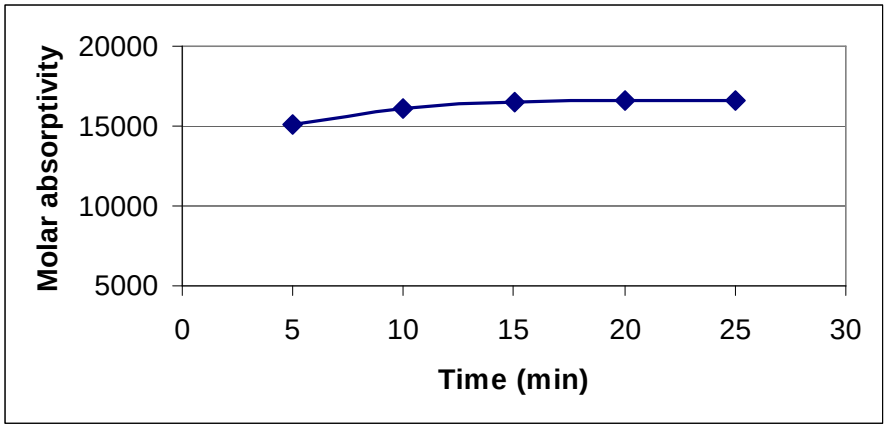

Figure 4.23: Effect of reaction time on the absorptivity of glimepiride (25 µg mL^{-1}) ternary complex at room temperature.

4.2.2.4 Effect of concentration of Cu (II) and eosin solutions

Concerning the effect of eosin concentration on the absorbance of the complex formed, the optimum results i.e., maximum and constant absorbance were obtained using 1 mL of 0.1% w/v eosin. Also the effect of Cu(II) concentration was studied by keeping the concentration of eosin constant and varying the metal concentration. Optimum results were obtained by using 1 mL of 0.1% w/v copper sulfate. Higher concentration of reagents did not affect the color intensity, therefore, further experiments were carried out using 1 mL of 1% solution of each of Cu (II) and eosin.

4.2.3 Validation of the proposed method

4.2.3.1 Linearity

Under the above optimized experimental conditions, the calibration curve was constructed by plotting concentration versus absorbance (Fig. 4.24). A linear calibration graph was obtained between absorbance and glimepiride concentration in the range given in Table 4.6. The correlation coefficient, intercept and slope for the calibration data for glimepiride are calculated using the least squares method.

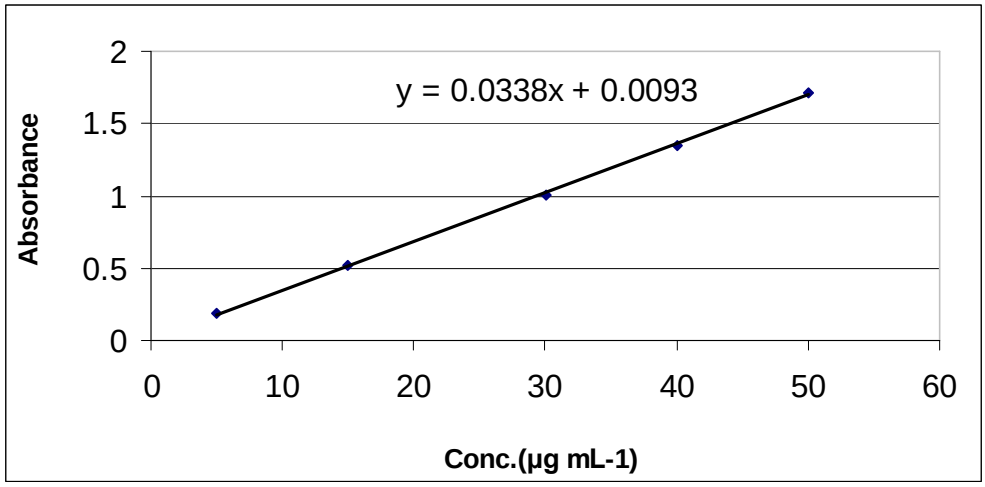

Figure 4.24: Calibration curve for glimepiride determination

Table 4.6: Optical characteristics and statistical data of the regression equation for the ternary complex formation with glimepiride.

Parameters	**Glimepiride Spectral Data**
λ_{max} (nm)	544
Beer's law limits, µg mL^{-1}	5 – 50
Molar absorptivity, L mol^{-1} cm^{-1}	1.657 X 10^4
Sandell's sensitivity, µg/cm^2	0.029
Limit of detection, µgmL^{-1} (LOD)	1.70
Limit of quantitation, µgmL^{-1} (LOQ)	5.10
Regression equation*	**Y = 0.0093 + 0.0338X**
Intercept (a)	0.0093
Slope (b)	0.0338
Correlation coefficient (*r*)	0.9997

*Y= a + bX, where Y is the absorbance, a intercept, b slope and X concentration in µgmL^{-1}.

4.2.3.2 Sensitivity (LOD, LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) for the proposed methods were calculated using the following equations:

LOD = 3.3 SD/S LOQ = 10SD/S

SD is calculated as the standard deviation of the residuals around the regression line, S is the slope of calibration curve. LOQs and LODs for glimepiride are listed in Table 4.6.

4.2.3.3 Precision

The precision and accuracy of the proposed method were tested by means of replicate measurements of the tested drug within Beer's law limits. The precision of the analytical procedure is usually expressed as the standard deviation of a series of measurements. Intraday and interday precision were assessed using triplicate analysis of three different concentrations. The calculated relative standard deviation values were found to be very small below 2% indicating good repeatability and reliability of the proposed methods. The results and their statistical analysis were summarized in Table 4.7.

Table 4.7: Evaluation of precision of the analytical procedure of glimepiride

Statistical Parameters		**10 µgmL^{-1}**	**25 µgmL^{-1}**	**50 µgmL^{-1}**
Intraday				
	1	10.06	24.74	49.51
	2	10.13	24.73	49.57
	3	10.05	24.81	49.62
	Mean recovery	10.08	24.76	49.57
	Mean % recovery	100.8	99.04	99.14
	S.D.	0.0436	0.0436	0.0551
	R.S.D. (%)	0.433	0.176	0.111
	Relative error	0.8%	0.96%	0.86%
Interday				
	1	10.08	24.80	49.60
	2	10.065	24.82	49.53
	3	10.146	25.09	49.85
	Mean recovery	10.097	24.90	49.66
	Mean % recovery	100.97	99.6	99.3
	S.D.	0.043	0.162	0.168
	R.S.D. (%)	0.43	0.651	0.338
	Relative error	0.97%	0.4%	0.68%

S.D. = Standard Deviation, R.S.D. = Relative Standard Deviation

4.2.3.4 Accuracy

Accuracy of the proposed method was further confirmed by performing recovery studies using standard addition method. A fixed amount of glimepiride from dosage form was taken and pure standard drug at three different concentrations within Beer's range was added. The total concentration was determined by the proposed method. The determination for each concentration was repeated three times and the average percent recovery of the added standard was calculated. The results of the recovery study are tabulated in Table 4.8. The results obtained in Tables 4.7 and 4.8 showed excellent mean recovery percent values, close to 100 %, and low standard deviation values (S.D. < 1.0) which indicate high accuracy of the proposed analytical methods.

Table 4.8: Results of recovery study for glimepiride

Base level ($\mu g\ mL^{-1}$)	**Amount spiked ($\mu g\ mL^{-1}$)**	**Amount recovered* ($\mu g\ mL^{-1}$)**	**% Recovery ± SD**
5	5.00	5.047	100.94±0.61
5	20.0	20.068	100.34±0.27
5	40.0	39.88	99.7±0.37

* Mean value of three determinations

4.2.3.5 Specificity

The commonly used additive and excipients in the preparation of tablets (such as starch, lactose, talc, and magnesium stearate) were found not to interfere in the analysis (Table 4.9).

Table 4.9: Results of specificity study for glimepiride

Concentration ($\mu g\ mL^{-1}$)	**Amount recovered* ($\mu g\ mL^{-1}$)**	**% Recovery ± SD**
5.00	5.053	101.06±0.22
20.0	20.074	100.37±0.17
40.0	39.86	99.65±0.37

***Mean value of three determination

4.2.4 Strcture of ternary complex of glimepiride

The nature of the ternary complex (glimepiride–Cu(II)–eosin) was determined using Job's method of continuous variation. The result of applying this method can be summarized as follows: the [glimepiride:copper(II)] ratio in presence of excess eosin was 1:1 (Fig. 4.25a), the [glimepiride:eosin] ratio in presence of excess Cu(II) sulfate was 1:1 (Fig. 4.25b), and the [eosin:copper(II)] ratio in presence of excess glimepiride was 1:1 (Fig. 4.25c). Hence the composition of the ternary complex formed may be expressed as glimepiride–copper(II)–eosin (1:1:1).

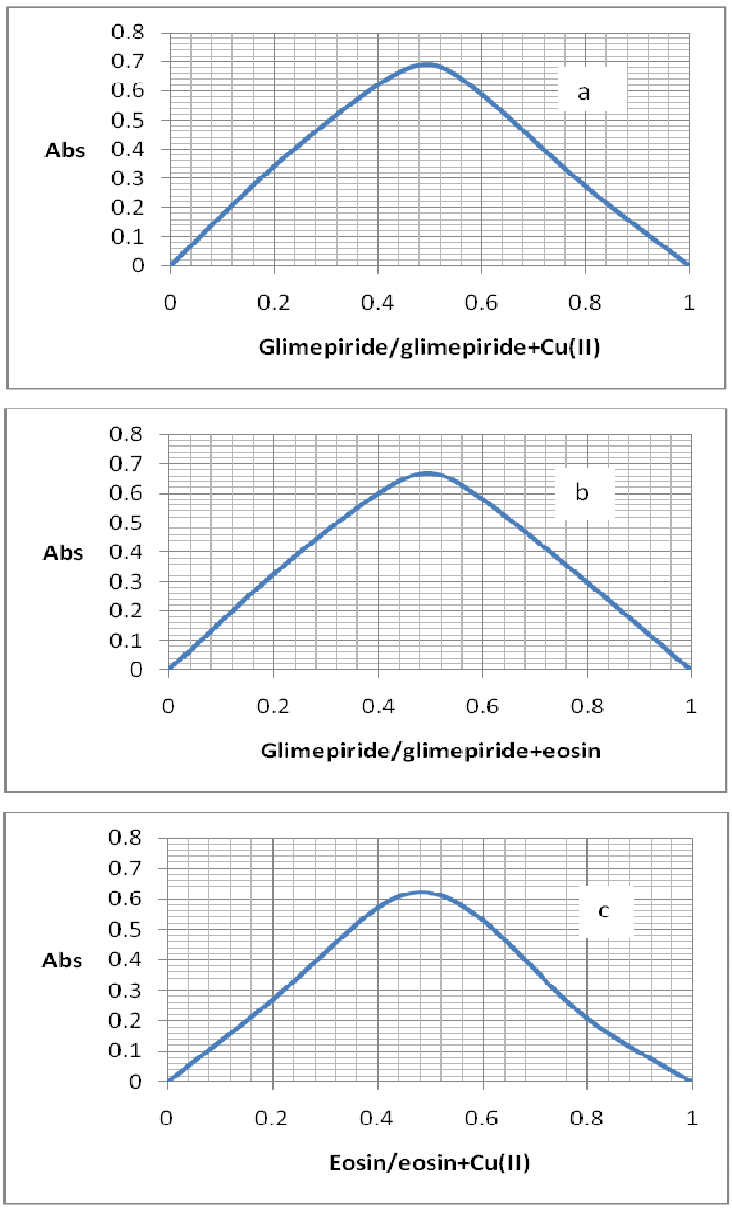

Figure 4.25: (a) Continuous variation plots for glimepiride: Cu(II) in the presence of excess eosin. (b) Continuous variation plots for glimepiride: eosin in the presence of excess Cu(II). (c) Continuous variation plots for eosin:Cu(II) in the presence of excess glimepiride

According to results of Job's method of continuous variation, a proposed structure of glimepiride ternary complex is shown in figure 4.26.

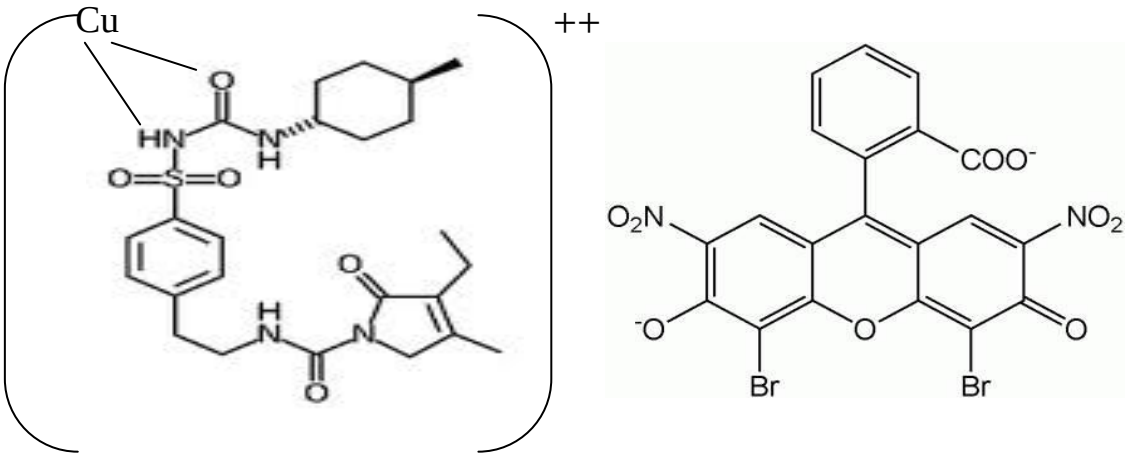

Figure 4.26: proposed structure of glimepiride-Cu-eosin ternary complex.

4.2.5 Application to pharmaceutical dosage form (tablets)

The assay for the marketed tablets of glimepiride was established with present optimized spectrophotometric conditions and it was found to be accurate and reliable. The results are shown in Table 4.10. The assay values of glimepiride for tablet formulation ranged from 98.2 % to 98.6 %, with relative standard deviation of not more than 1.96. The assay values for the formulations were very close to the label claim, indicating that the interference of excipient matrix is insignificant in the estimation of glimepiride by the proposed analytical method. Recovery percentage obtained by the proposed method was satisfactory when compared with other methods (99.73 % obtained by Khan et al, 2005), and it lies in the accepted range by BP, 2011 (97-102%). The estimated drug content with low values of standard deviation established the precision of the proposed method.

Table 4.10: Results of application of spectrophotometric method to the determination of glimepiride from pharmaceutical dosage form (tablets)

Label claim ($\mu g\ mL^{-1}$)	**Amount recovered* ($\mu g\ mL^{-1}$)**	**% Recovery ± SD**
5	4.91	98.2±1.96
15	14.75	98.33±1.3
30	29.58	98.6±0.46

* Mean value of three determinations

Chapter (5)

5. Conclusion

5.1 Conclusion

An accurate spectrophotometric method was developed and validated for the determination of two important drugs, namely cefixime and glimepiride, through ternary complex formation with copper (II) and eosin. The color reaction wouldn't require neither stringent conditions nor many reagents or solvents. The data given before reveal that the proposed analytical method is simple, sensitive, rapid and specific. Obviously, the λ_{max} of the ternary complex (Drug-Cu-Eosin) was red-shifted from the λ_{max} of the drugs alone and this increases the selectivity of the method and could minimize the interferences from other substances. The method was suitable to determine concentrations in the range of 4 - 28 and 5 - 50 mg mL^{-1} for cefixime and glimepiride, respectively, precisely and accurately. The limits of detection and quantitation for the drugs were (0.900;1.70) and (2.70; 5.1) μg mL^{-1}, for cefixime and glimepiride, respectively. Furthermore, the mean relative standard deviation (RSD) and the mean relative analytical error can be considered to be very satisfactory. No interference is noticed from the presence of additives and excipients. The sample recovery from the formulation was in good agreement with its respective label claim. In conclusion, with these developed methods, analysis can be run fast with low cost and without prior extraction or losing accuracy. Finally, the proposed methods can be used as alternative to the reported ones for the routine analysis and the quality control of the two drugs in pure form and in pharmaceutical dosage forms.

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