ANALYSIS OF ESCITALOPRAM OXALATE BY VARIOUS ANALYTICAL METHODS

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DOCTORAL THESIS

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This thesis titled "Analysis of Escitalopram Oxalate by Various Analytical Methods" has been prepared and submitted by Wafa Farooq Suleman Badulla in partial fulfillment of the requirements in "Anadolu University Directive on Graduate Education and Examination" for the Degree of PhD in Analytical Chemistry Department has been examined and approved in 24/05/2017.

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ABSTRACT

ANALYSIS OF ESCITALOPRAM OXALATE BY VARIOUS ANALYTICAL METHODS

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Department of Analytical Chemistry
Anadolu University, Graduate School of Health Science, May, 2017
Supervisor: Prof. Dr. Zeki ATKOŞAR
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The main objective of this thesis is development of new analytical methods to determine escitalopram oxalate (ESC-OX) in pharmaceutical formulations, raw material and biological fluids. For this purpose, a novel HPLC method was developed and validated for determination of ESC-OX besides its six impurities. The applicability of the method was initially tested on 12 different columns which were having various surface chemistries and particle characteristics (i.e., fully porous or core-shell particle structure). Among these, six best-performing HPLC columns were selected for further comparison studies. Acceptable separation was obtained in all cases by using acetonitrile: methanol: water: phosphate buffer solution (pH 3.5, 50 mM) (25:5:20:50, *v/v/v/v*) mixture as mobile phase, with a flow rate of 1.2 mL min^{-1}. The analytes were monitored at 210 nm by using an UV-visible detector. The core-shell phenyl-hexyl phase was arisen as a better performing alternative in term of separation efficiency, on the well-known industry standard C_{18} and relatively new PFP phases. On the other hand, quantification of ESC-OX in human urine and pharmaceutical dosage forms using different analytical techniques such as Differential Pulse Voltammetry, Capillary Electrophoresis and Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry was realized. The statistical evaluation of all developed methods showed that there was no significant difference between these methods for the quantification of the ESC-OX.

Keywords: Escitalopram Oxalate, High Performance Liquid Chromatography, Differential Pulse Voltammetry, Capillary Electrophoresis, Liquid Chromatography-Electron Spray Ionization-Tandem Mass Spectrometry.

ÖZET

ÇEŞİTLİ ANALİTİK YÖNTEMLERLE ESSİTALOPRAM OKSALAT ANALİZLERİ

Wafa Farooq Suleman BADULLA
Analitik Kimya Anabilim Dalı
Anadolu Üniversitesi, Sağlık Bilimleri Enstitüsü, Mayıs, 2017
Danışman: Prof. Dr, Zeki ATKOŞAR
İkinci Danışman: Prof. Dr. Göksel ARLI

Bu tezin temel amacı farmasötik formülasyonlar, ham madde ve biyolojik sıvılar içindeki essitalopram oksalatın (ESC-OX) tayini için yeni analitik yöntemler geliştirmektir. Bu amaçla, ESC-OX'ın altı safsızlıkları yanında tayini için yeni bir YPSK yöntemi geliştirilmiş ve yöntem geçerliliği sağlanmıştır. Yöntemin uygulanabilirliği öncelikle çeşitli yüzey kimyaları ve parçacık karakteristiklerine (yani tamamen poröz veya çekirdek-kabuk parçacık yapısına) sahip 12 farklı kolon üzerinde denenmiştir. Bunların arasından, altı en iyi-başarımlı YPSK kolonu sonraki karşılaştırma çalışmaları için seçilmiştir. Kabul edilebilir ayrım her durumda asetonitril: metanol: su: fosfat tampon çözeltisi (pH 3,5, 50 mM) (25:5:20:50, *h/h/h/h*) karışımı kareketli faz olarak kullanılarak, 1.2 mL dk^{-1} akış hızıyla elde edilmiştir. Analitler bir UV-görünür alan detektörüyle 210 nm'de izlenmiştir. Çekirdek-kabuk fenil-hekzil faz, iyi bilinen endüstri standardı C_{18} ve görece yeni PFP fazlarının üzerinde, ayrım verimi yönünden daha iyi başarım gösteren bir alternatif olarak öne çıkmıştır. Ayrıca, ESC-OX'ın insan idrarı ve farmasötik dozaj formlarındaki miktarının Diferansiyel Puls Polarografi, Kapiler Elektroforez ve Sıvı-Kromatografisi-Elektrospray İyonizasyon Tandem Kütle Spektrometrisi gibi farklı teknikler kullanarak tayini gerçekleştirilmiştir. Geliştirilmiş olan tüm yöntemlerin istatistiksel değerlendirilmesi göstermiştir ki ESC-OX'ın miktarının tayininde bu yöntemler arasında anlamlı farklılık yoktur.

Anahtar Sözcükler: Essitalopram Oksalat, Yüksek Performanslı Sıvı Kromatografisi, Diferansiyel Puls Polarografisi, Kapiler Elektroforez, Sıvı-Kromatografisi-Elektrospray İyonizasyon Tandem Kütle Spektrometrisi.

Dedicated to

Almighty Allah

then to

my loving family

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The greatest divine grace of Almighty Allah in my life is my late father, lovely mother, late brother, sisters especially my elder sister Muna and family. Even if I were using sea as ink to write about them it wouldn’t be enough to fulfil their rights.

I am obligated to all those have directly or indirectly helped me and I have missed unintentionally.

Finally, I wish my late father were beside me at this moment because he was always looking for this day. I just pray to Allah to meet him and my brother again in Jannah. Amen.

24/05/2017

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby truthfully declare that this thesis is an original work prepared by me; that I have behaved in accordance with the scientific ethical principles and rules throughout the stages of preparation, data collection, analysis and presentation of my work; that I have cited the sources of all data and information that could be obtained within the scope of this study, and included these sources in the references section; and that this study has been scanned for plagiarism with scientific plagiarism detection program used by Anadolu University, and that "it does not have any plagiarism" whatsoever. I also declare that, if a case contrary to my declaration is detected in my work at any time, I hereby express my consent to all the ethical and legal consequences that are involved.

Wafa Farooq Suleman BADULLA

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LIST OF ABBREVIATIONS

ACN : Acetonitrile
ANOVA : Analysis of Variance
As : Asymmetry factor
BR : Britton Robinson
C mol^{-1} : Coulomb per mole
°C : Degree Celsius
CE : Capillary Electrophoresis
CGME : Controlled Growth Mercury Electrode
CI : Confidence Interval
CIT A : Citalopram related compound A
CIT B : Citalopram related compound B
CIT C : Citalopram related compound C
CIT D : Citalopram related compound D
CIT E : Citalopram related compound E
CIT H : Citalopram related compound H
C.V : Coefficient of variation
CV : Cyclic Voltammetry
CZE : Capillary zone electrophoresis
Da : Dalton
DPV : Differential Pulse Voltammetry
E° : Formal redox potential
***E*pc** : Cathodic peak potential
ESC-OX : Escitalopram Oxalate
F. A : Formic Acid
FDA : Food and Drug Administration
HMDE : Hanging Mercury Drop Electrode
HPLC : High performance liquid chromatography
HPTLC : High Performance Thin Layer Chromatography
i.d. : Internal diameter
ICH : International Conference on Harmonization
***I*pc** : Cathodic peak current

IR : Infrared

IS : Internal Standard

IUPAC : International Union of Pure and Applied Chemistry

K' : Capacity factor

K : Kelvin

k° : Standard heterogeneous rate constant

Kv : Kilo volt

LC- ESI-MS/MS : Chromatography-Electrospray Ionization Tandem Mass Spectrometry

LLE : Liquid–Liquid Extraction

LLOQ : Lower Limit of Quantification

LOD : Limit of Detection

LOQ : Limit of Quantification

m/z : Mass to Charge

mbar : milli bar

MeOH : Methanol

MET : Metoprolol

MRM : Multiple reaction monitoring

$mV\ s^{-1}$: Millivolt per second

mV : Milli volt

N : Theoretical plate number

N/m : Theoretical plate per meter

nA : nanoampere

NMR : Nuclear Magnetic Resonance

ODS : Octadecyl-Bonded Silica

PFP : Pentafluorophenyl

Psi : Pound per square inch

r : Correlation coefficients

r^2 : Square Regression

R_S : Resolution

RSD : Relative Standard Deviation

S/N : Signal to Noise

SD : Standard Deviation

SEM	: Standard Error of Mean
SIM	: Single Ion Monotoring
SPE	: Solid-Phase Extraction
SST	: System Suitability Test
T	: Tailing Factor
T	: Temperature
TEA	: Tetraethylammonium
TOF	: Time of Flight
t_R	: Retention Time
USP	: United State Pharmacopeia
UV/vis	: Ultraviolet and visible spectrophotometer
v	: scan rate
V	: Volt
v/v	: Volume by volume
α	: Selectivity factor
α	: Transfer coefficient
$v^{1/2}$	: square root of the scan rate.

1. INTRODUCTION

In analytical chemistry, one of the most challenging fields is the pharmaceutical analysis field. Pharmaceutical analysts develop and validate suitable methods to achieve the qualitative and quantitative control of Active Pharmaceutical Ingredients (APIs) and drug products. These methods give analytical support for pharmaceutical, biomedical, quality control and another clinical field researches. Instrumental analytical methods are carried out for the most pharmaceutical analysis. The biggest challenge facing the analysts is to develop selective, accurate, precise, cost-effective and fast analytical method which is most accessible and applicable for most users.

Each year, a lot of new drugs are approved by the Food and Drug Administration (FDA). These drugs contribute to improve the quality of human being health. These drugs must be safe and efficient to provide their intention of use. In present time, most drugs are chemically synthesized; in order to be safe, they must be free from unwanted chemicals that may remain from the synthetic steps, or degradants formed during the process or long-period of storage. These impurities must be determined and controlled under suitable limits because; their presence often carries toxicological and pharmacological undesired effects. One of the main issues in drug development is the control of impurities in the pharmaceutical raw material and final products. At the same time, for a drug to be effective it must be able to produce the prospected therapeutic benefits. Toward having drugs that are safe and efficient, analytical methods should be developed to ensure that a drug has its required potency and it is free from potential impurities; in addition, bioanalytical methods must be established for quantitative measurement of an API in biological fluids, which can provide information about the pharmacokinetic profile of the drug.

The development of analytical method for determination of an analyte depends on the selection of suitable technique which consequently based on the consideration of physical and chemical properties of the analyte. In pharmaceutical analysis, it is recommended to develop specific or new analytical methods to enhance selectivity, lower cost per an analysis, increase high throughput, etc. Besides, it is also important to realize that the data obtained from the developed method is correct and valuable from the analytical point of view; however, this is only possible by using reference methods or validating the protocols according to international regulations.

This thesis intentions are to provide integrated analytical data for escitalopram oxalate

ESC-OX; which can be summarized by the following objectives:

1- Development, validation and application of a new high performance liquid chromatography HPLC method for quantification of ESC-OX and six of its impurities named oxalic acid (OX-A), citalopram related impurity A, B, C, D, E (CIT A, CIT B, CIT C, CIT D and CIT E in standard and different pharmaceutical dosage forms.
2- Comparing the separation power of fully porous particle column and the core-shell particle for the newly developed method.
3- Development, validation and employing new Differential Pulse Voltammetry (DPV), Capillary Electrophoresis (CE) and Liquid Chromatography-Electrospray Ionization Tandem mass spectrometry LC-ESI-MS/MS methods for estimation of ESC-OX both in pharmaceutical formulations and spiked human urine.
4- Statistical evaluation of the obtained results from the newly developed methods.

To fulfil these objectives, an isocratic HPLC method was established, validated and utilized to quantify ESC-OX and its impurities in expired and valid pharmaceutical dosage forms. In the same time, evaluation of separation characterizations for core-shell and conventional stationary phase was carried out by using 12 different columns with divers properties. The core-shell columns generally offer better performance and better separation in short time.

On the other hand, voltammetric DPV, CE and LC-ESI-MS/MS methods were developed and applied for determination of ESC-OX in pharmaceutical dosage forms and spiked human urine samples.

The harmonization and comparison of the all developed methods for the determination of ESC-OX in raw material, pharmaceutical dosage forms and spiked urine samples were evaluated statistically by analysis of variance ANOVA test.

2. LITERATURE REVIEW

2.1. Depression and Its Treatment

Depression becomes one of the universal mental disorders that is characterized by gloomy mood, low ability, lack of attention and enjoyment, decrease in self-regard, confusion and irritable sleep and desire for eating. Furthermore, depressant can be manifested as anxiety. This mental disease may become chronic and lead to many social problems and in its worst situation to suicide. According to World Health Organization, depression is approximately influence 350 million people and it is the main cause for increasing incidence of disease and death (Marcus et al., 2012, p. 1-8).

Depression can be classified according to its severity into the bipolar disorder (manic-depression), dysthymia, and major depression (unipolar depression). There are several causes for depressant such as; disturbances in the central nervous system neurotransmitters, hereditary factors and psychosocial factors that may combine and lead to depressive disorders. The decision which kind of treatment that must be chosen is depend on the clinical symptoms, seriousness of illness and the patient's individual and family history. The pathophysiology is related to the imbalance in the level and function of brain neurotransmitters like, serotonin (5-hydroxytryptamine, 5-HT), norepinephrine and dopamine, as well as an increase in the level of adrenocorticotropic hormone (ACTH) (Baldwin and Birtwistle, 2002, p. 17-21). During the last decades, there is an increase in the consumption of antidepressant drugs for the treatment of depression.

2.2. Treatment of Depression

Antidepressant drugs can be classified according to the neurotransmitter/ receptor that participated in the pharmacological action into the following categories:

- **Monoamine oxidase inhibitors (MAOI):**

  Moclobemide, Clorgyline.

- **Tricyclic antidepressants**

A. (Noradrenaline) NA + 5-HT reuptake inhibitors.

Imipramine, Amitriptyline, Trimipramine, Doxepin, Dothiepin, Clomipramine.

B. Predominantly NA reuptake inhibitors.

Desipramine, Nortriptyline, Amoxapine, Reboxetine.

- **Selective serotonin reuptake inhibitors (SSRls)**

  Fluoxetine, Fluvoxamine, Paroxetine, Sertraline, Citalopram, Escitalopram.

- **Atypical antidepressants**

  Trazodone, Mianserin, Mirtazapine, Venlafaxine, Duloxetine, Tianeptine, Amineptine, Bupropion (Tripathi, 2007, p. 439- 449).

In the past, depression was treated mainly with the tricyclic antidepressants (TCAs) and MAOI. However, SSRIs became popular due to adverse effects, toxicity, drug interactions of TCAs and MAOI (Pacher et al., 2001, p. 89-100).

2.3. ESC-OX

ESC-OX can be used for the treatment of depression in children (Rey, 2015, p. 137). It is the drug of choice for treatment of major depressive and anxiety disorder (Waugh and Goa, 2003, p. 343-362).

- **Dosage form and administration**

  It is present as film coated tablets (5, 10, 15 and 20 mg) and an oral solution (10 mg mL^{-1}). It is administered once daily (Cipralex®, 2012, p. 3).

- **Side effect**

  Gastric upset such as nausea, constipation, dry mouth, difficulty in sleeping, dizziness, increase in sweeting and sexual disability (http 1).

- **Cautions**

  It should be used with cautions by pregnant women because complication associated with its usage, especially in the third trimester period. Also, it should not be used with MAOI and to the patient with hypersensitivity to ESC-OX or citalopram (AHFS drug information, 2010, p. 2343).

2.3.1. ESC-OX physical and chemical properties

ESC-OX with the IUPAC chemical name is S-(+)-1-[3-(dimethyl-amino) propyl]-1-(p-fluorophenyl)-5-phthalancarbonitrile oxalate. It is a pure s- enantiomer of the racemic, bicyclic phthalates derivatives of citalopram with chemical structure as shown in Figure 2.1. (Cormier, Das and Ojima, 2009, p. 542).

Some chemical and physical properties of ECS-OX is given below:

- ***Molecular Formula:*** $C_{20}H_{21}FN_2O \cdot C_2H_2O_4$
- ***Molecular Weight:*** 414.43 g mol^{-1}
- ***Physical form:*** White to off-white, crystalline material having no more than a slight odor.
- ***Melting Point:*** 147-152 ℃ (Degree Celsius).

- ***pKa:*** 9.5 (microtitration)
- ***Solubility:*** Water (sparingly soluble), Ethanol (sparingly soluble), Ethyl acetate (slightly soluble), Methanol (MeOH) (freely soluble), Dimethyl sulfoxide (freely soluble), Isotonic saline (soluble) (Lexapro™, 2004, p. 1).
- ***Partition Coefficient:*** Log P (octanol/phosphate buffer pH 7.4) = 3.5 (http 2).

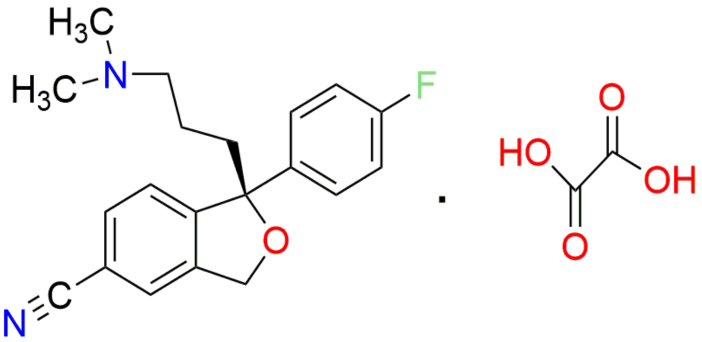


Figure 2.1. *Chemical Structure of ESC-OX.*

2.3.2. Pharmacodynamic

ESC-OX is approved by FDA on August 14, 2002. It is the latest, selective, dose-dependent serotonin reuptake inhibitor and among most currently used drug for the treatment of the depression (Brown and Khan, 1994, p. 341-347; Flint, 1998. p. 269-280; Small, 1998, p. 32-42; Whittington et al., 2004, p. 1341-1345). ESC-OX considered to be more potent than racemic citalopram. It increases the level and action of serotonin by inhibition of its reuptake into the serotonergic nerve ending as shown in Figure 2.2. Serotonin plays essential roles in the maintenance of mood balance and disturbance in its level and function lead to the depression. ESC-OX possess higher efficacy than citalopram because the R-Citalopram antagonize the ESC-OX action by inhibition of ESC-OX binding to the allosteric site on the 5-HT transporter protein (Sidney et al., 2006, p. 123). Also, ESC-OX shows greater effect with fast alleviation of symptom (Sanchez et al., 2004, p. 165).

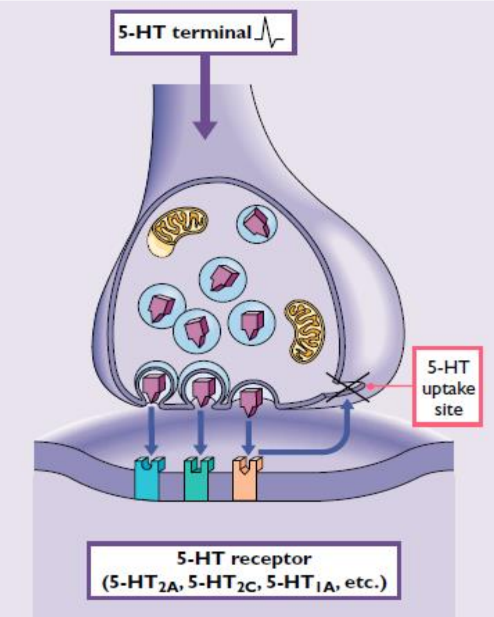


Figure 2.2. *Mechanism of Action of SSRIs by Inhibition of Serotonin Reuptake into Presynaptic Nerve Ending.*
Reference: *Baldwin and Birtwistle, 2002, p. 96.*

2.3.3. Pharmacokinetics

ESC-OX is metabolized mainly in the liver with the formation of main metabolite S-demethylcitalopram which is future biotransformed to the S-didemethylcitalopram. The metabolites have no inhibitory action on serotonin. ESC-OX lose half of its pharmacological action after 27-32 hr (Von Moltke et al., 2001, p. 1102–1109). About 80% of the dose taken by mouth is achieved unvaried to the blood with about 56% binding to the plasma proteins. After administration of 10 mg day^{-1} the plasma steady state concentration 19-37 ng mL^{-1} (Rao, 2007, p. 281-290). Only 8% ESC-OX excreted unvaried in the urine and 10% as its primary metabolites (Søgaard et al., 2005, p.1400-1406).

2.4. Impurity

An impurity as defined by International Conference on Harmonization (ICH) guidelines as “Any component of the medicinal product which is not the chemical entity defined as the active substance or an excipient in the product”, although impurity profiling is recognized to be the analytical activities with the purpose of detecting, identifying or elucidating the structure and quantitatively determining impurities in bulk drugs and pharmaceutical formulations (ICH Topic Q3A, 1995, p. 8).

Nowadays, most regulatory authorities such as United States Food and Drug Authority (USFDA, 2010, p.1-10), European Directorate of Quality Medicine (EDQM, 1994, p. 14), Therapeutic Goods Administration (TGA, 2013, p.5-9), and official monographs publications such as United State Pharmacopeia (USP, 2016, p.1176), and others, are emphasized on the purity of the pharmaceutical ingredient.

Presence of impurities, even in small amount may affect drug safety and efficiency. Their presence may lead to the variation in the dosage of active substance (Görög, Lauko and Herenyi, 1988, p. 699). Purity of the drugs depends on various aspects such as crude substances, method of synthesis and the kind of refinement procedure.

2.4.1. Impurity profiling

Impurity profile is the characterization of the known and unknown impurities found in a normal batch of active pharmaceutical ingredients produced by a particular controlled production process (Ayre et al., 2011, p. 76-90). Qualification, quantification and characterization of impurities structure become an essential aspect in the modern pharmaceutical analysis because drug should be free from all hazardous substances that may affect the human being health. There are several analytical methods that can be used for the impurities profiling such as HPLC, Gas Chromatography (GC), Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), CE etc., and their hyphenated techniques. The control of impurities is becoming an essential part of the quality control of API.

2.4.2. Kinds of impurities

As indicated by ICH rules, impurities in the synthesized pharmaceutical substance can be classified into the following groups (ICH Topic Q3A, 1995, p. 2-4).

2.4.2.1. Organic impurities

These kinds of impurities appear in the course of the manufacturing procedure and/or at the time of storage. These involved; raw materials, intermediate, enantiomeric impurities, by-products and degradation products.

2.4.2.2. Inorganic impurities

Likewise, these types of impurities are arising during the synthesis process. They are usually identified and recognized such as; reagent, ligands and catalysts, heavy metals, filter aids, charcoal, etc.

2.4.2.3. Residual solvents

Solvents (organic or inorganic) that are used during the manufacturing process can be remained at the end product. The removal of these solvents form the end product is not an easy issue and they may be harmful.

2.4.3. Formulation related impurities

During the process of formulation, pharmaceutical active substance subject to the different process conditions that may result in its degradation or unwanted reactions. Also, side reaction between the active substances and /or the inactive additives may result in formation of by products which are undesirable, as well as environmental conditions like; humidity, temperature, light, etc. may lead to drug instability (Solanki, 2012, p. 231-238).

The control of impurities has become an essential part of the quality control of API to get critical data about the drug toxicity, safety and determine the lowest quantity of impurities in drug substance or their formulations. The limits for reporting, identification and qualification of impurities in drug substance and drug products according to ICH is summarized in Table 2.1.

Table 2.1. *ICH Reporting, Identification and Qualification Limits of Impurities.*

Maximum daily dose	**Thresholds**		
	Reporting	**Identification**	**Qualification**
Drug substance			
≤ 2 g	0.05%	0.10% or 1 mg day^{-1}	
› 2 gm	0.03%	0.05%	
Drug product			
≤ 1 g	0.1%		
› 1 g	0.05%		
‹ 1 mg		1% or 5 mg day^{-1}	1% or 50 mg day^{-1}
1-10 mg		0.5% or 20 mg day^{-1}	1% or 50 mg day^{-1}
10-100 mg		0.2% or 2 mg day^{-1}	0.5% or 200 mg day^{-1}
100 mg-2 g		0.2% or 2 mg day^{-1}	0.2% or 2 mg day^{-1}
› 2 g		0.1%	0.1%

Reference: *Ahuja and Scypinski, 2011, p. 83-84.*

2.4.4. Impurities Sources

The origin of the impurities classified into three main classes:

- ✓ Process impurities originated during synthesis of the compound.
- ✓ Degradation impurities emerge at the time of storage.
- ✓ Contaminant impurities insert during storage or during drug synthesis but not related to the drug (Ng, Lunn, and Faustino, 2007a, p. 1-4).

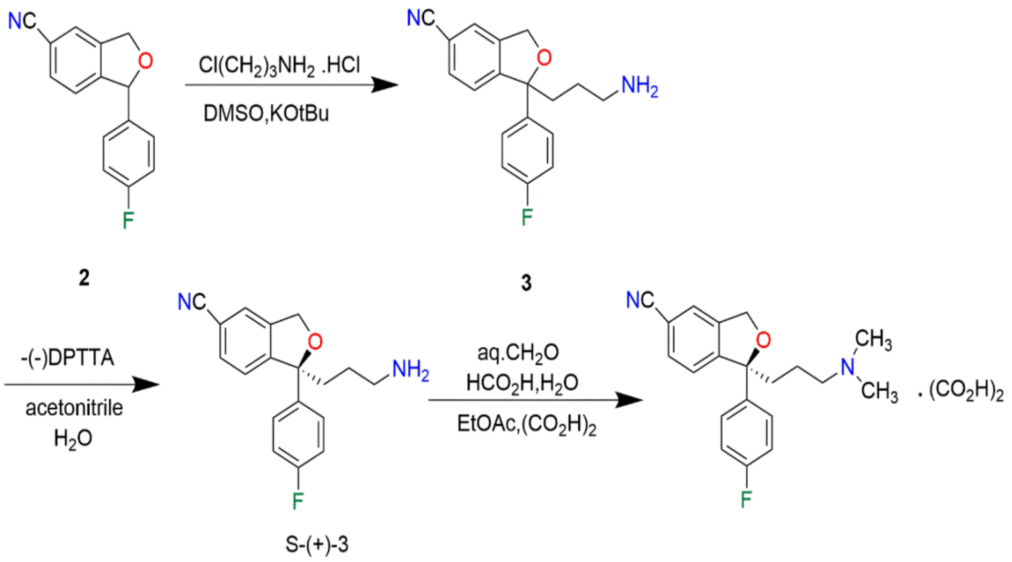
2.4.5. Analytical Methods for Impurities Profiling

There are several analytical methods that can be used for the impurities profiling such as; HPLC, GC, TLC, CE and HPTLC etc., and their hyphenated techniques.

HPLC is the most widespread technique used for determination of impurities due to its versatility. The presence of columns with different type of stationary phase, particle size, length and diameters makes this technique adaptable with different type of impurities. Most organic and inorganic compounds have UV absorbance so they can be detected by UV-visible spectrophotometer.

2.4.6. ESC-OX Impurities

ESC-OX was synthesized in the laboratory by a patent process (Sundaram et al., 2005; Elati et al., 2007, p. 289-290). The starting compound was 1-(4-fluorophenyl)-1, 3-dihydroisobenzofuran-5-carbonitrile **2**, which converted to the intermediate **3** by C-alkylation under the condition which is illustrated in the Scheme 2.1. The intermediate **3** undergo resolution with L- (-)-DPTTA to produce (S)-1-(3-aminopropyl)-1-(fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile (S-(+)-3). The compound S-(+)-3 under Clarke-Eschweiler (Icke, Wisegarvu and Alles, 1945, p. 723) reaction conditions produce ESC which is purified by oxalic acid to give ESC-OX.



Scheme 2.1. *Synthesis of ESC-OX.*

ESC-OX could be contaminated with impurities that may be rise from the starting material, intermediate or degradation with oxidation or alkylation (Vedantham et al, 2012, pp. A-G; Dhaneshwar and Mahadik, 2009, p. 139). The impurities that can be identified in the ESC-OX raw material and pharmaceutical dosage forms according to USP-2016 are mentioned in the following Table 2.2. In this thesis six most common impurity (OX-A, CIT A, B, C, D and E) was selected to be determined. The selection based on the presence of these impurities in both standard and dosage forms. The characterization of the six impurities listed in Table 2.3.

Table 2.2. *The List of Impurities that may be Presented in the Raw Material and Pharmaceutical Dosage Forms According to USP 2016.*

Name of impurity	Presence	Acceptance threshold NMT (%) (respectively)
Oxalic acid	Raw material, Oral solution	Identification
Escitalopram amide	Raw material	0.15%
Escitalopram related compound A	Tablet, Oral solution	0.3%, 0.2%
(CIT A)	Raw material, Tablet, Oral solution	0.1%, 0.5%, 0.2%.
(CIT B)	Raw material, Tablet, Oral solution	0.1% 0.5%, 0.2%
(CIT C)	Raw material, Tablet, Oral solution	0.1%, 0.5%, 0.3%
(CIT D)	Raw material, Oral solution	0.1% and in oral solution as process impurity
(CIT E)	Raw material, Tablet, Oral solution	0.1%, 0.2%, 0.2%
Citalopram related compound (CIT H)	Oral solution	Process impurity
Escitalopram 3-oxo	Raw material	0.15%
Escitalopram fluorophenyl ketone	Raw material	0.10%
Desfluorocitalopram	Oral solution	Process impurity
Citalopram chloromethyl quartenary ammonium salt	Oral solution	Process impurity
Citalopram alkene dimer	Oral solution	Process impurity
5-Dimethylaminobutyryl citalopram	Raw material, Oral solution	0.2% and in oral solution as process impurity.

Table 2.3. *Structural Information About ESC-OX Impurities.*

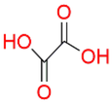
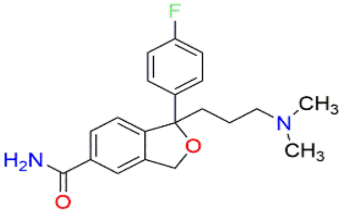
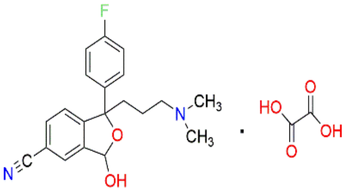
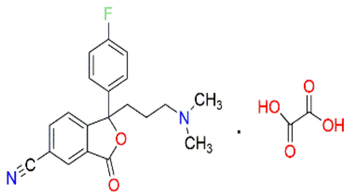
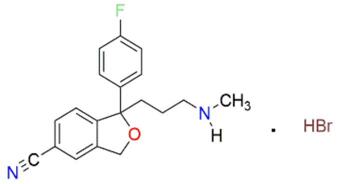
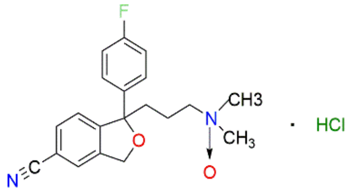
Impurity name **IUPAC name**	**Chemical structure**	**Physical and chemical properties**
OX-A		Molecular formula: $C_2 H_2O_4$ Molecular weight: 90.034 g mol^{-1} Physical form: solid. Melting point: 189.5 °C. (http 2) pKa: 1.46; pKa: 4.40 (http 2) Partition coefficient: Log P (octanol/phosphate) -0.81 (http 2)
CIT A IUPAC name: (1-(3-Dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carboxamide).		*Molecular formula:* $C_{20}H_{23}FN_2O_2$ *Molecular weight:* 442.41 g mol^{-1} *Physical form:* solid. *Melting point:* 125-135 °C (http 3). 123.6-135.3 °C (as measured in laboratory). *pKa:* 16.06 ± 0.40 (predicted) (http3). *Partition coefficient:* Log P (octanol/phosphate) – 2.4 (http 1).
CIT B IUPAC name: (1-(3-Dimethylaminopropyl)-1-(4-fluorophenyl)-3-hydroxy-1,3-dihydroisobenzofuran-5-carbonitrile oxalate.		*Molecular formula:* $C_{22}H_{23}FN_2O_6$ *Molecular weight:* 430.43 g mol^{-1} *Physical form*: solid. *Melting point:* 136 – 140 °C (http 3), 144.3- 166.8 °C (as measured in laboratory). *pKa:* 11.90 ± 0.40 (predicted) (http 4). *Partition coefficient:* Log P (octanol/phosphate) – 2.7 (http 2).

Table 2.3. *(Continued) Structural Information About Impurities.*

CIT C IUPAC name: (3-(3-Dimethylaminopropyl)-3-(4-fluorophenyl)-6-cyano-1(3H)-isobenzofuranone oxalate).		*Molecular formula:* $C_{20}H_{19}FN_2O_2 \cdot C_2H_2O_4$ *Molecular weight:* 428.42 g mol^{-1} *Physical form:* Solid. *Melting point:* 115 - 120 °C (http 3). 177.8- 182.0 °C (as measured in laboratory). *pKa:* 9.48 ± 0.28 (predicted) (http 4). *Partition coefficient:* Log P (octanol/phosphate) – 3.4 (http 2).
CIT D IUPAC name: (1- (4-Fluorophenyl) -1- (3-methylaminopropyl) -1,3-dihydroisobenzofuran-5-carbonitrile hydro bromide)		*Molecular formula:* $C_{19}H_{19}FN_2O \cdot HBr$ *Molecular weight:* 391.28 g mol^{-1} *Physical form:* solid. *Melting point:* 187.2-198.5 °C (as measured in laboratory). *pKa:*10.50 ± 0.1 (predicted) (http 4). *Partition coefficient:* Log P (octanol/phosphate) 2.93 (http 5)
CIT E IUPAC name: (1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuron-5-carbonitrile-N-oxide hydrochloride)		*Molecular formula*: $C_{20}H_{21}FN_2O_2 \cdot HCl$ *Molecular weight:* 376.85 g mol^{-1} *Physical form:* solid. *Melting point:* 173.1-183.4 °C (as measured in laboratory). *pKa:* Not available. *Partition coefficient: Log P (octanol/phosphate) 3.17 (http 5)*

2.5. Analytical Methods

In this thesis four different analytical instruments were used. HPLC was utilized for determination of the ESC-OX impurities in both standard and pharmaceutical dosage forms (tablets and oral solution). CE, Voltammetry (DPV) and LC- ESI-MS/MS were applied for determination of the ESC-OX in pharmaceutical dosage forms and spiked human urine.

2.5.1. HPLC

Since the development of this technique by the Russian botanist M.S. Tswett in 1903 (Ettre and Sakodynskii, 1993, p. 223-231), there has been tremendous progress in this technique which lead to make it one of the most essential techniques for the analysis of organic compounds, pharmaceuticals, drug purity (Jansson, 1986, p. 615-624), and biological samples (Watson, 1987, p. 117-189). The concept of separation of the analytes depends on partition of the substances between the stationary and the mobile phase. The substances, then eluted at different times from the column depending to their partition behavior. HPLC can be used for identification by taking the retention time of the compound, in the case of quantitative analysis, the concentration of the compound calculated from the area under the peak.

There are several types, size and shape characteristic of stationary phase binding to the silica surface. The cutting-edge period of column technique was started in the late 2000s by using core-shell and sub-2µm particle. The concept of core-shell particles and superficially porous silica particles was introduced by Horváth and co-workers (Horvath, B Preiss and Lipsky, 1967, p. 1422; C. Horvath andLipsky,1969, p. 109) after that, Kirkland et al (Kirkland, 2000, p. 3-13) continued in the development and improvement of the concept until it gets a wide application in the HPLC technique. This type of column provides analysis in a short time with high flow rate, maintaining column efficiency and relatively low back pressure (Unger, Skudas and Schulte, 2008, p. 393–415; Cunliffe and Maloney, 2007, p. 3104–3109). The type of column provides analysis with the advantages of ultra-performance liquid chromatography UHPLC by providing a large surface area for separation with low back pressure in comparison UHPLC (Brice, Zhang and Colón, 2009, p. 2723). In order to know the phenomenon of peak boarding, one must know the van Deemter equation. This equation explains all factors that involve in band boarding, as follows:

$$HETP = A + \left(\frac{B}{u}\right) + Cu \tag{1.1}$$

where:

$HETP$ = Height Equivalent to a Theoretical Plate.
A = Eddy diffusion
B = Longitudinal diffusion
C = Resistance to mass transfer
u = Average mobile phase velocity

The most important advantage of core-shell particles is the small diffusion path due to smaller pore volume, which result in a reduction in the longitudinal diffusion B thus provide narrow peaks. Besides that, the short diffusion path due to thin, porous shell result in fast mass transfer (Wu and Clausen, 2007, p. 1167; Van Deemter, Zuiderweg and Kinkenberg, 1956. p. 271). All this, lead to improve the column efficiency and fast analysis (Blue and Jorgenson, 2011 p. 7989; Omamogho et al., 2011, p. 1942) as shown in Figure 2.3.

In this thesis, the performance and separation power of the core-shell column with different types of stationary face such as; Kinetex C_{18}, Kinetex Phenyl-Hexyl and Kinetex Pentafluorophenyl compared with the fully porous Inertsil C_{18}, Luna Phenyl-Hexyl, Luna Pentafluorophenyl.

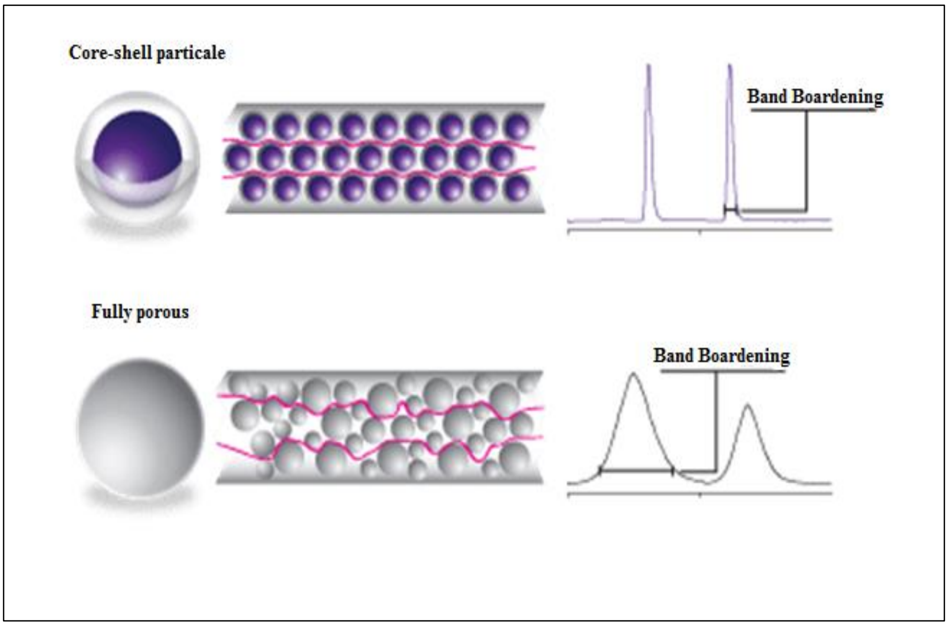


Figure 2.3. *Represent Band Boarding by Using Fully Porous and Core-Shell Particle.*

Reference: *http 5.*

There are two types of elution in HPLC; isocratic and gradient elution. In isocratic elution, the composition of the mobile phase remains constant throughout the analysis time. While in case of gradient elution the composition of the mobile phase is changed during the analysis- usually by increasing the percentage of the organic solvent and sometimes combine by alteration in the flow rate. It is better to use isocratic elution if the analysis is not required to use gradient because of several reasons which can be briefed in the following:

- ✓ Isocratic elution is simple in comparison to gradient elution.
- ✓ Isocratic elution doesn't require an advanced instrument.
- ✓ Isocratic elution can be used with all types of detectors, but gradient elution cannot be used with the refractive index detector.
- ✓ Problems associated with gradient elution such as; base line noise, possibility of precipitation of salt by changing the organic phase percentage, column equilibration between runs and reproducibility are not present in isocratic elution (Snyder et al, 1997, p.351).

In spite of gradient elution disadvantages, it must be used in some analysis to get effective separation with good resolution and reasonable analysis time.

In the current study, isocratic elution was used for the effective separation of ESC-OX and its six impurities with good resolution and short analysis time.

2.5.2. CE

This method was introduced to the analytical separation field in the 1930's by Swedish chemist Arne Tiselius. After that Jorgenson and co-workers (Jorgenson and Lukacs, 1983, p. 266-272) improved it and applied to the analysis of biopolymers. However, this method becomes a popular for the pharmaceutical analysis after its introduction in the European Pharmacopoeia (EP)- Supplement only in 2001(European Pharmacopoeia-Supplement, 2001, p. 35-39). After that this method has been applied for the analysis of several compounds including; uncharged or charged and structurally similar or isomers. In electrophoresis, the concept of separation depends on the difference in charge-to-size ratio of the sample ions in back ground electrolyte (BGE) which consequently result in different migration rate under the force of the electrical field. The analyte ions move through the capillary under the effect of their own electrophoretic mobility and electro-osmotic flow (EOF), which moves from anode to cathode. The system of CE is represented in Figure 2.4.

There are several modes of CE separation such as:

- ✓ Capillary zone electrophoresis (CZE).
- ✓ Micellar electrokinetic capillary chromatography (MEKC or MECC).
- ✓ Capillary gel electrophoresis (CGE).
- ✓ Capillary isoelectric Focusing(CIEF).
- ✓ Capillary Isotachophoresis(CITP). (Baker, 1995 a, p. 53-91).

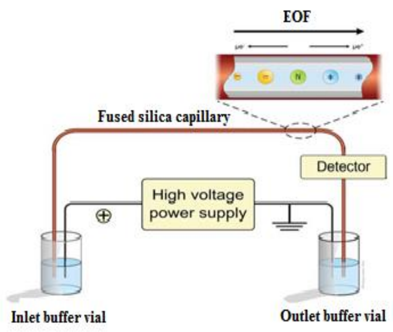


Figure 2.4. *Structural Representation of Capillary Electrophoresis.*
Reference: *Johannesson, 2006, p. 8.*

In this study CZE was used to determine the quantity of ESC-OX in the pharmaceutical preparations and urine. In recent times, there is a wide application of CE methods in the industry and quality control laboratories for the routine drug analysis. CE has many advantages over the traditional HPLC technique in term of using lower solvent, high throughput analysis and simple operation. At the same time HPLC is reproducible, sensitive and robust. So, CE and HPLC are correlative techniques for the pharmaceutical analysis. There are some limitations of the CE technique for analysis of pharmaceuticals in biological samples like; injection reproducibility and low sensitivity (Altria and Rogan,1993, p. 50-51). In this thesis in order to overcome the injection precision limitation, internal standard (IS) Metoprolol (MET) and the replenishment system which allows fresh BGE in every injection were both was used and to improve the technique sensitivity, pretreatment of the analyte was done by using liquid-liquid extraction (LLE).

2.5.3. LC-MS/MS

Liquid chromatography is considered as an essential separation technique in most scientific fields. Combining the separation ability of liquid chromatography with the detection selectivity of the mass spectroscopy result in technique with extraordinary sensitivity and selectivity. The high selectivity of the LC-MS hyphenation makes it the most used analytical technique used for detection of compound in complex matrix at low quantity.

To overcome the difficulty associated with the interface between the LC and MS, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) was introduced by John Bennett Fenn and he won the Nobel prize in chemistry in 2002 (Herschbach and Kolb, 2014, p. 9-11). Also, other types of ionization source are present such as; matrix-assisted laser desorption, chemical ionization, etc. (Hoffmann and Stroobant, 2007, p. 15).

The mass spectrometer separates ions according to mass to charge ratio (m/z). The most important part of the LC-MS instrument is the analyzer. There are mainly four types of analyzer; quadrupole, time of flight, ion trap and Fourier transform-ion cyclotron resonance. Quadrupole analyzer is considered the prominently utilized analyzer because of its simplicity, coverage of wide mass range, satisfactory linearity for quantitative determination, resolution and reasonable cost. To increase the selectivity and sensitivity of the analyzer two quadrupoles conduct with a collision cell construct together in series. This type of system is called tandem or triple quadrupole mass spectrometry (MS/MS). However triple quadrupole does not define the system, realistically. Because collision cell structure consists of hexapole or octupole. Using MS/MS results in improving signal to noise ratio. So, it is useful for the quantitative analysis in complex samples such as; serum, hair, brain and urine with less sample purification. The first quadrupole is used to select the precursor ion or molecular ion, the fragmentation of the ion occurs in the collision cell by the influence of high energy and collision by gas (N_2, Ar or He). In second quadrupole, a fragment is chosen, known as product or daughter ion, and is transferred to the detector. This kind of monitoring is known selected reaction monitoring or multiple reaction monitoring (MRM) (Yost and Boyd, 1990, p. 154) as represented in Figure 2.5.

In this work, LC-MS/MS technique was used because only 8% of the unchanged ESC-OX is secreted in urine. So, there is a demand to use more sophisticated and sensitive technique for detection of the lower amount of drug in urine sample.

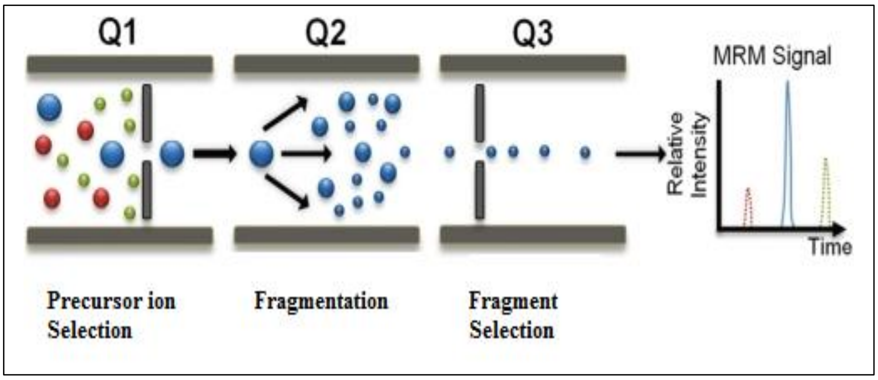


Figure 2.5. *An Illustrative Diagram of a Triple Quadrupole Mass Spectrometer Using MRM.*
Reference: *Boja and Rodriguez, 2011, p. 64.*

2.5.4. Electrochemical methods

Electroanalytical methods are a wide field of chemical analysis. The electrochemical techniques discovered by Heyrovsky in 1922 is called polarography. He reported electrochemical reaction on dropping mercury electrode (DME) and he was awarded Nobel Prize in chemistry in 1959 (Heyrovsky, 1922, p. 303). The main subdivision of electrochemical methods is the polarographic and voltammetric procedure. The most widely used technique nowadays is voltammetry. The voltammetric analysis depends on measurement of current developed due to redox reaction on the electrode surface after the implementation of potential difference to an electrochemical cell. Several techniques of voltammetry are present such as; Cyclic Voltammetry (CV), DPV, Osteryoung Square Wave Voltammetry (OSWV), Adsorptive Stripping Voltammetry (AdSV) and others. These methods get attention for application in the analysis of pharmaceuticals and drugs in dosage forms and biological fluids. This advanced application of these techniques for drug analysis comes from the methods less sensitivity to the excipients and matrix in biological samples, effective cost, low solvent consumption and brief analysis time in comparison to other analytical methods.

In this thesis, the electrochemical behavior of ESC-OX was studied by using CV and DPV on Hanging Mercury Drop Electrode (HMDE) as well as its determination in pharmaceutical dosage forms and urine samples.

2.5.4.1. CV

This technique provides the basic information about the electrochemical behavior of the electroactive compound, its kinetic specification, and electrochemical reversibility.

The redox characterization of the drug can give an idea about the pharmacological activity and pharmacokinetics information. This technique is based on measuring the current while the potential on the electrode varied in both forward and backward direction (Özkan, Bengi and Aboul-Enein, 2003, p. 157).

2.5.4.2. DPV

The pulse voltammetric methods was introduced in order to reduce the limit of detection of the electrochemical measurement. The DPV technique is based on application of a series of potential as steps with fixed amplitude (10-100 mV) on gradually altering base potential. The current measuring takes place at two points, immediately at the beginning and end of the applied of pulse potential. The plot of potential against the current difference measurement at these points is used for analytical evaluation. This results in a reduction in measuring of charging current against faradaic current (Skoog et al., 2014, p. 624).

2.5.4.3. Electrodes in electrochemical cell

The electrochemical reaction takes place in a cell that contain three electrodes. The working electrode, is the main electrode in the electrochemical cell on which redox reaction takes place. There are several types of electrodes such as; mercury electrode, carbon electrode, gold electrode and another modified electrode. The reference electrode, must be inert to the content of the electrochemical cell and the potential of the working electrode is measured against it. The usually used reference electrodes are standard hydrogen electrode, silver-silver, calomel electrode etc. The auxiliary electrode, is used to prevent the change in the potential of the reference electrode as a result of current passage. Platinum electrode is the most widely used (Özkan, Kauffmann and Zuman, 2015 a, p. 48) as shown in Figure 2.6.

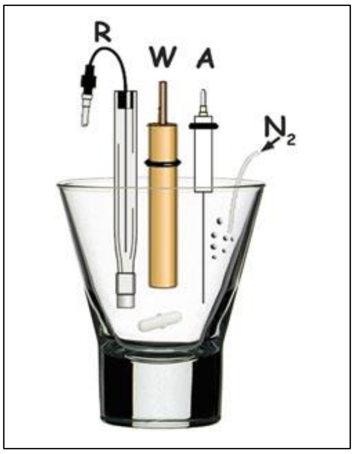


Figure 2.6. *Electrochemical Cell with Three Electrodes:*
(W) working, (A) auxiliary and (R) reference; and (N_2) a nitrogen gas line.
Reference: *Özkan, Kauffmann and Zuman, 2015, p. 48.*

2.5.4.4. Mercury electrode

It is an electrode in a liquid form that can be used in dropping and hanging forms. The mercury electrode has many advantages like, a new electrode surface is formed for each analysis so contamination from the previous analysis does not occur, has a wide potential range (from +0.4 to –2.5 V corresponding to supporting electrolyte), has smooth surface so does not require polishing, has high hydrogen overpotential which makes it the best electrode for the cathodic reaction but it is rarely used for oxidation reaction due to rapid oxidation of mercury. Mercury electrode application has been limited in the current time for the analysis of pharmaceuticals under the argument of its toxicity. However, mercury toxicity can occur through inhalation, ingestion or skin contact but the exposure to mercury during analysis is short and low. It can be used without any harm, by taking special care during storage and discard (Barek et al., 2001, p. 295).

2.6. Bioanalysis of Pharmaceuticals

Bioanalysis is a branch of analytical chemistry that deals with the measurement of drug quantity and their metabolites in the biological samples. The obtained data from bioanalysis can be used for the pharmacokinetics, toxicological, clinical and bioequivalence purpose. The critical step in the bioanalysis is the sample preparation (Wells, 2002, p. 41). The biological samples represent a complex matrix that contains several components; these components may interfere with the precise biological estimation. In order to get sensitive and selective bioanalysis, it is recommended to choose a proper extraction method with reasonable time. The most used biological samples are plasma and urine for the investigation of drugs in human. The two essential steps in bioanalysis are; the sample preparation and the compound detection. There are a different types of extraction techniques like LLE, solid-phase extraction (SPE) and solid-phase micro extraction (SPME), etc. (Xu, 2007, p. 323-329). The SPE is more efficient and faster than LLE, however, it is more expensive, can be clogged with the matrix component and require an expert technician (Mislanova and Hutta, 2003, p. 99).

2.6.1. LLE

The aim of LLE is to transfer specific analyte from the biological sample to an organic phase. LLE is considered the most common and broadly used technique for sample pretreatment in the laboratories. The concept of extraction is based on partition of the analyte between two immiscible solvent. The degree of effective extraction is based on knowing the chemical properties of the analyte, in order to choose the proper solvent and the adjustment of the medium pH if the drug is ionizable (most drugs present as the salt of weak acid or base). In most case, the urine pH is recommended to be ±2 units below or above the pKa of the analyte if the drug is acid or base respectively (Hansen, Pedersen-Bjergaard and Rasmussen, 2012 a, p. 109).

The unionized molecules pass to the organic layer which is then evaporated to get drug free from the endogenous material of biological matrix. The free drug is dissolved in the suitable solvent and became ready for analysis. The step of reconstitution of extracts from biological sample to the low volume solvent is powerful for the preconcentrating of the drugs and obtaining adequate LLE. The percent of recovery is an indicator for the extraction technique selectivity (Jones, 1976, p. 107-113).

It is recommended to use solvent of low polarity to prevent from co-extraction of the other material in the biological sample. The more efficient the extraction or cleaning step is, the more sensitive the detection is.

For extraction of basic or acidic drugs by the LLE, pH adjustment must be carried out. For basic drugs addition of base makes the drug in its unionized form which facilitates its passage to the organic layer. Ammonium hydroxide is a popular option for increasing the extraction power (Woodcock, 2013, p. 23).

The LLE of ESC-OX from urine sample was done by using water-hexane because hexane has polarity index of 0.1 among non-polar solvents (Hansen, Pedersen-Bjergaard and Rasmussen, 2012 b, p. 282) and has low carcinogenic risk potential in comparison with the other organic solvents such as chloroform.

Even of the drawbacks of LLE in recent time by the development of SPE however, it is still broadly used due to low cost, efficient extraction and rapid standardization. By taking care of all parameters that may affect the LLE, it is possible to get a clean sample that is intended for analysis.

2.6.2. Analysis of drugs in urine

Urine is considered as liquid biological sample of relatively uncomplex matrix and easy for analysis in comparison with other biological samples. Urine contains about 95% water, various components such as inorganic salts (NaCl), urea, small amount of protein, lipids, creatinine and other constituents that depend on intake food, drink and body state. The pH of the urine range from 4.5-8.0, taking pH 6 as a mean (Ji, 2013, p. 307).

Urine should be stored in a correct manner because urea can be decomposed by the action of bacteria to the ammonia, which may result in changing the urine pH (Cook et al., 2007, p. 486). Increasing the urine pH can lead to drug degradation. For this reason, urine freezing is recommended immediately after collection.

Analysis of drugs in urine is desirable for its collection is non-invasive procedure, does not require trained person for collection, and large quantity could be obtained and more stable than the blood which make it suitable for both forensic toxicological and urinary excretion and bioavailability studies.

2.7. Review of Analytical Methods Used for ESC-OX Analysis

Based on getting information from approved scientific source (listed in science citation index or science citation expand), the literature survey showed that there have been a few number of analytical studies related to the determination of ESC-OX impurities in the bulk and different pharmaceutical dosage forms. However, most of these methods related to isolation and detection of the R- enantiomer of ESC-OX and/or identification of other impurities that are not listed in the official USA monograph of ESC-OX. In USP 2016 there are different methods for determination of impurities in both standard and dosage forms. The mentioned methods are time consuming up to 45 to 68 minutes per analysis. In addition, these methods used gradient elution by changing both concentrations of organic solvent and flow rate. Those methods are cumbersome to perform in comparison with the developed method in this thesis. One of this thesis objectives was to develop simple, cost effective and time saving analytical method that can be used easily in the quality control and industrial laboratories.

The analytical studies relating to the determination of impurities are listed in Table 2.4. According to the previous studies there were no analytical method have been developed yet for the determination of ESC-OX impurities named CIT A, B, C, D and E, at the same time for their detection in both raw material and different pharmaceutical formulations.

For estimation of ESC-OX in biological fluids, plasma, serum and hair were used in most of the previously developed methods. Blood and hair are representing one of the complex biological samples, for this reason the former methods either used special electrode for its detection or utilized hyphenated methods such as fused silica capillary connected to the LC-MS/MS for its extraction and evaluation. These methods are listed in Table 2.5. There have been only a few analytical methods used for its determination in the urine. The second aim of this thesis was to develop analytical methods for its determination in spiked human urine by using different analytical instruments and to compare the data obtained from these methods. By achieving these two objectives, this work covers part of the limitation in the evaluation of ESC-OX in urine and its impurities detection.

Table 2.4. *Different Analytical Methods for the Determination of ESC-OX and Its Impurities.*

Compound	Column, Plate or Capillary	Mobile Phase, F.R, t_R or R_f or Volt	Detector, IS, Inj. Vol.	LOD, LOQ, Range ($\mu g\ mL^{-1}$)	Reference
ESC-OX CIT	Chiralcel OD-H (250 mm × 4.6 mm) 5 µm	n-hexane:2-propanol: TEA (95:05:0.1 *v/v/v*) 1.0 mL min^{-1} ESC-OX, 15 min CIT, 12.5 min	UV 240 nm and polarimetry 20 µL	0.5, 1.3 50 - 600	Rao, Raju and Nagaraju, 2006, p. 280–285.
(R, S) CIT, (R, S) Citadiol	Fused-silica capillaries (50 µm × 360 µm) 47/40 cm	Phosphate buffer pH 2.5+ β-CD and sulfated -β-CD, ESC-OX, 5.1 min CIT, 4.49 min, (S)-Citadiol, 6.67 min, (R)- Citadiol, 7.81 min, -20 kV	UV 205 nm Salicylic acid 0.5 psi /6 s^{-1}	CIT, 1.0, 2.5- 150 Citadiol 2.5, 2.5- 50	Sungthong, Jac and Scriba 2008, p. 959–965.
ESC-OX and degradation products	PerfectSil-100 ODS-3 -C_{18} (25 cm × 4.6 mm) 5 µm	MeOH: Acetate buffer pH 3.8 (45:55 *v/v*), 1 mL min^{-1}, ESC, 12.8 min, Degradation products, 5.5, 8.6 min	LC-MS, IR, -, 20 µL	0.25 0.60 20-100	Dhaneshwar, 2009, p. 138-147.

F.R: flow rate, R_f: Retention Factor, Inj. Vol.: injection volume, CIT: citalopram, β-CD: Beta Cyclodextrin.

Table 2.4. *(Continued) Different Analytical Methods for the Determination of ESC-OX and Its Impurities.*

ESC-OX, ESC-I ESC-II ESC-III	C_{18} column (150 mm × 4.6 mm) 3 µm	Solution A: 0.15% NH_4HCO_2 Solution B: ACN, 1.0 mL min, ESC 10.2 min, ESC-I, 4.3 min ESCII, 4.9 min, ESCIII, 11 min	Q-TOF, LC/ESI-MS, NMR, -, -	-	Raman et al., 2010, p. 895–901.
Di-p-Toluoyl-d-Tartaric acid	C_8 (150 mm × 4.6 mm) 5 µm	ACN: Phosphate buffer pH 3 (50:50 *v/v*) ,1.0 mL min^{-1}, 4.1 min	UV-240 nm, -, 25 µL	0.040, 0.120 0.05-2.0	Kaleemullah et al, 2011, p. 197-205.
ESC-OX, ESC-cyanodiol, R-ESC- N-oxide	silica gel 60 F254 plates (10 cm × 10 cm) with β-CD	ACN: A. A: MeOH: water (5:0.5:3:1 *v/v/v*), R_f, ESC-OX, 0.75, ESC-cyanodiol 0.40, R-enantiomer 0.31 and ESC- N-oxide 0.23	Densitometer 254 nm, -, 25 µL	ESC–OX 13.3 44.0 25-1000	Soliman and Youssef, 2011, p. 475–481.
ESC-OX, CIT	Chiralcel OD (25 cm × 4.6 mm) 10 µm	n-heptane: isopropanol: diethylamine (94.5:5:0.5 *v/v/v*), 1 mL min^{-1}, CIT 14.6 min, ESC-OX 17.3 min	UV-240 nm, -, 10 µL	ESC-OX and CIT 0.16 0.50, 0.05- 7.5	Dighe et al, 2012, p. 4804-4809.
ESC-OX and R-enantiomer	fused-silica capillary (50.2 cm ×75 µm)	25 mM phosphate buffer at pH 7.0, containing 1.6% (*w/v*) sulfated-β-CD, 2 min, -20 kV	UV-205 nm, -, 0.5 psi for 5 s	ESC-OX 0.08 and 0.25	Deng et al, 2012, p. 1648–1651.

ESC-I, ESC-II, ESC-III: Type of impurity. A. A. Acetic acid

Table 2.4. *(Continued) Different Analytical Methods for the Determination of ESC-OX and Its Impurities.*

ESC-OX, Degradation impurities	BEH C_8 (10 cm × 2.1 mm) 1.7 µm	Phosphate Buffer pH 7: ACN: MeOH (60:28:12 *v/v/v*), 0.4 mL min^{-1}, 3.5 min	UV-239 nm, -	-, -, 6- 18	Vaghela and Rao, 2013, p. 1018-1030.
(Tablet) ESC, CIT A CIT B, CIT C CIT E	C_8 (10 cm × 4.6 mm) 3 µm	Acetate buffer pH 5.2: ACN: MeOH (60:7:33 *v/v/v*), 1 mL min^{-1}, -.	UV 239 nm, -, 10 µL	-, -, -	USP 39, 2016 p. 3768.
(Raw material) ESC CIT A, CIT B CIT C, CIT D CIT E	C_8 (25 cm × 4.6 mm) 5 µm	Gradient over 68 min, Solution A: ACN: Phosphate buffer pH3(10:90 *v/v*) and Solution B: ACN: Phosphate buffer pH3 (65:35 *v/v*) ,1-2 mL min^{-1}, -.	UV 237 nm, -, 20 µL	-, -, -	USP 39, 2016 p. 3770.
(Oral solution) ESC CIT A, CIT B CIT C, CIT D CIT E, CIT H	C_8 (15 cm × 4.6 mm) 3 µm	Gradient over 20 min, Solution A: Phosphate buffer pH 3 and Solution B: ACN: MeOH 1.5 mL min^{-1}	UV 220 nm, -, 20 µL	-, -, -	USP 39, 2016 p. 3765.

Table 2.5. *Different Analytical Methods for the Determination of (ESC-OX) In Pharmaceutical Dosage Form and Biological Fluids.*

Compound/ Sample	**Extraction Method**	**Column, Plate, Electrode**	**Mobile Phase, Flow Rate, T_R or Volt,**	**Detector, IS, Inj. Vol.**	**LOD, LOQ, Range (μg mL^{-1})**	**Reference**
ESC-OX (Spiked human plasma)	LLE	ODS (15 cm × 4.6mm) 5.0 μm	Ammonium acetate pH 5: ACN (54:46 *v/v*) 1.0 mL min^{-1}, 7.0 min	LC–ESI-MS, Paroxetine, 10.0 μL	1.0 ng mL^{-1} 1.0- 200 ng mL^{-1}	Singh et al, 2004, p. 209–215.
ESC-OX (Urine)	On line extraction by a sol–gel monolith fused-silica capillary	C_{18} modified sol–gel (75 μm × 360 μm)	MeOH: water with 5mM acetic acid (50:50 *v/v*), 2μL. min^{-1}, 20.0 min	ESI-TOF-MS, -, 2.25 μL	10 pg mL^{-1} , 34 pg mL-^{1}, -	Johannesson and Bergquist, 2007, p. 1045–1048.
ESC-OX (Hair)	Soxhlet extraction	Luna C_{18} (15 cm × 4.6 mm) 5 μm	Gradient for 25min Solution A: MeOH: water + 0.1% F. A (20:80 *v/v*) Solvent B: MeOH: water (90:10 *v/v*) + 0.1% F. A 0.5 mL min^{-1}, 14.98 min	Ion trap mass spectrometry, -, 20 μL	0.03 ng mL^{-1}, -, -	Doherty et al, 2007, p. 755–761.
ESC-OX, CIT (Plasma)	Centrifuge and filtration	-	Tris buffer pH 7	Spectrofluorimeter-285 nm, -, -	-, -, 17-175 ng mL^{-1}	Serebruany et al, 2007 p. 513–520.

Table 2.5. *(Continued) Different Analytical Methods for Determination of (ESC-OX) In Pharmaceutical Dosage Form and Biological Fluids.*

ESC-OX (Bulk and Tablet)	-	-	Complex of ESC with Bromocresol green in chloroform	UV/vis 417 nm, -, -	0.00345 µg mL^{-1}, 0.01045 µg mL^{-1}, 2-10 µg mL^{-1}	Vetrichelvan et al, 2010, p. 269-271.
ESC Desmethyle CIT (Hair)	LLE	Fused-silica capillaries (75 mm ×100 cm)	NaOH: isopropanol (1:1 *v/v*), and 0.2% of F.A, 15 kV, Desmethyle CIT, 10.8, 11.5 min, ESC, 11.3, 14.1, 12 min	TOF-MS, Folcodine, 30 s at 7 kV.	-, -, -	Gottardo, 2012, p. 101- 107.
ESC-OX (Human plasma)	precipitation extraction with ACN	C_{18} (ACE) (125 mm × 4.6 mm) 3 µm	ACN: water (60:50 *v/v*) + 0.2% F.A, -, 3.0 min, -.	LC-MS/MS, Paroxetine, -	0.5 ng mL^{-1}, -, 0.5-50 ng mL^{-1}	Sousa et al, 2012, p. 310-314.
ESC-OX (Bulk Tablet)	-	-	*Method I*: cerium(IV) and tris (2,2-bipyridyl) ruthenium(II) (Ru (bipy) 32+) *Method II:* 2- (2,2-bipyridyl) ruthenium(II) (Ru(bipy)32+) and H_2SO_4	photomultiplier tube, - , 150 µL	*Method I:* 1 x 10^{-5} µg mL^{-1}, 0.001 µg mL^{-1}, 0.001-50 µg mL^{-1} *Method II:* 1 x 10-7 µg mL^{-1}, 1 x 10^{-5} µg mL^{-1}, 1 x 10^{-5} – 70 µg mL^{-1}	Alarfaj, A. Aly and Al-Qahtany 2012, p. 84–92.

Table 2.5. *(Continued) Different Analytical Methods for Determination of ESC-OX in Pharmaceutical Dosage Form and Biological Fluids.*

ESC-OX and metabolites, (Human urine)	chiral imprinted polymer (CIP)-coated stir bar	A Zorbax Eclipse XDB-CN (150 mm × 4.6 mm) 5 µm from	Ammonium format pH 3 (30 mM): ACN (30:70 *v/v*), 0.6 mL min^{-1}, (+) -(S)-didesmethylcitalopram, 7.1 min, (+) -(S)-desmethylcitalopram, 7.8 min, ESC-OX and ESC-d6 8.6 min	Ion trap mass spectrometry, Deuterated ESC-d6, 20 µL	2 ng mL^{-1}, 5 ng mL^{-1}, 5-2000 ng mL^{-1}	Unceta et al., 2013 p. 448- 453.
ESC-OX (Bulk, Tablet Spiked urine, serum)	-	*plastic membrane* (ESC with phosphomolybdic acid) *coated Al wire* (ESC with ammonium reineckate) *coated graphite rod* (ESC with phosphotungstic acid)	pH 3-7	Potentiometric, -, -	*plastic membrane* 5.0×10^{-7} mol L^{-1}, 1.0×10^{-2} -1.0×10^{-6} mol L^{-1} *coated Al wire* 4.8×10^{-7} mol L^{-1} 1.0×10^{-2} -1.0×10^{-6} mol L^{-1} *coated graphite rod* 5.0×10^{-8} mol L^{-1} 1.0×10^{-2} -1.0×10^{-7} mol L^{-1}	Al-Amri, Alarfaj and A.Aly 2013, p. 10044 – 10058.
ESC-OX (Bulk and Tablet)	-	Bismuth (III) oxide (Bi_2O_3) nano-particlesmodified-glassy carbon electrode	pH 2, 0.4% sodium lauryl sulphate	-, - ,-	53.67 µg mL^{-1}, -, 150–400 µg mL^{-1}	Jain, Dhanjai and Sharma 2013, p. 178–184.

Table 2.5. *(Continue) Different Analytical Methods for Determination of (ESC-OX) In Pharmaceutical Dosage Form and Biological Fluids.*

ESC-OX (Oral Solution)	-	C_8 (25 cm × 4.6 mm) 5 µm	ACN: Phosphate buffer pH 2.5 (32:68 *v/v*), 1 mL min^{-1}, -.	UV 240 nm, -, 20 µL	-, -,-	USP 39, 2016, p. 3765.
ESC-OX (STD)	-	C_8 (25 cm × 4.6 mm) 5 µm	Solution A: ACN: Phosphate buffer pH 3 (10: 90 *v/v*), Solution B: ACN: Phosphate buffer pH 3 (65: 35 *v/v*), gradient mode, 1-2 mL min^{-1}, -.	UV 237 nm, -, 20 µL	-, -,-	USP 39, 2016, p. 3769.
ESC-OX (Tablet)	-	C_8 (10 cm × 4.6 mm) 3 µm	MeOH: ACN: Acetate buffer pH 5.2 (33:7:60 *v/v/v*), 2 mL. min^{-1}, -.	UV 240 nm, -, 10 µL	-, -,-	USP 39, 2016, p. 3767.

3. MATERIALS AND METHODS

3.1. Chemicals

- Acetic acid (49-51%) (Sigma-Aldrich, Switzerland).
- Acetonitrile, methanol and water (HPLC grade) (Sigma-Aldrich, Switzerland).
- Ammonium format (≥ 99.0%) (Fluka, India).
- Ammonium hydroxide (29.2%) (Sigma-Aldrich, USA).
- Boric acid (≥ 99.5%) (Sigma Life Science, Japan).
- Dipotassium phosphate hydrogen (≥ 98.5%) (Fluka, Switzerland).
- ESC-OX, CIT A, CIT B, CIT C, CIT D and CIT E reference standards (United States Pharmacopeial Convention, USA).
- Formic acid (98-100%) (Riedel-de Haen, Germany).
- Hexane (95%) (Sigma-Aldrich, USA).
- Phosphoric acid (~86%) (Fluka Analytical, Switzerland).
- Potassium chloride (≥ 99.99%), Sodium hydroxide (≥ 99.99%) (Fluka Analytical, Germany).
- Potassium dihydrogen phosphate (≥ 99.5%) (Fluka, Switzerland).
- Sodium acetate (≥ 99.0%) (Fluka, Switzerland).
- Ultrapure water (produced by using Milli-Q A10 Synthesis Water Purification System, France).

3.2. Pharmaceutical Formulations

- Oral Solution (Citoles® 10 mg mL^{-1}, Cipralex ®10 mg mL^{-1}), were obtained from a local (Turkey) pharmacy.
- Tablet Samples (Esram® 20 mg, Citoles® 10 mg, Eslorex ®10 mg).

3.3. Devices and Instruments

- Analytical balance (Mettler Toledo, Switzerland Brand Excellence Plus XP-205 model).
- Centrifuge (Eppendorf, centrifuge 5810 R, Germany).
- Filter paper (Cronus filter PTFE (Poly Tetra Fluoro Ethylene) 4 mm 0.22 µm, DK blue, UK).
- Filter paper (Sartorius AG.37070 Goetting, Germany, pore size 0.45µm).

- Filter paper (Syringe filter Nylon 25 mm, 0.45 μm).
- pH meter (Mettler Toledo, Switzerland, brand Seven multi-model pH meter).
- Pipettes (Eppendorf, Germany, 100-1000 μL and 20-100 μL).
- Ultrasonic Bath (Bandelin, Germany, brand, RK 100H Sonorex model).
- **Electrochemical System**

Voltammogram were recorded on a fully automated BAS-100 voltammetric analyzer (made in India) connected to a PC computer with proper BAS 100B version 2.3 software for overall monitoring of the examination and data achievement and analysis. A CGME polarographic stand with a cell (10 mL) consisted of controlled growth mercury electrode as the main electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl electrode as the reference electrode was used. All measurements were done at laboratory temperature (25 ± 2°C). Ultra-pure nitrogen gas (99.999%) was used for deoxygenation.

- **HPLC Method**

  HPLC applications were carried out by using two separate instruments. One of them (System A) was used for method development and validation, and the other one (System B) was used for method transfer, ruggedness and specificity studies.

- **HPLC System A**

The method development was carried out in HPLC system A, which contain gradient pump with LC-20ATmodel LPGE unit, SIL-20AC automatic sampler, CBM-20A model communication unit, CTO-10-ASvp model column furnace, SPD 20A model UV/VIS detector and DGU-20ASR model degassing, An Intel Pentium 4 processor computer and Shimadzu LabSolutions LC solution version 1.25 data analysis program were used in addition to the HPLC product of Shimadzu (Japan). 12 columns were used as it is represented in the Table 4.1.

- **HPLC System B**

This system was used for the ruggedness study. It contains of the following parts; the gradient pump, SIL-20A automatic sampler, CBM-20A model communication unit, CTO-10-ASvp model column oven, SPD-M20A model photo diode array detector (DAD) and DGU-20A5 model degassing equipped with LP- The Intel Pentium 4 processor computer and the Shimadzu Lab Solution LC solution v1.11 SP1 data analysis program were used in addition to the HPLC product of Shimadzu (Japan). Phenomenex Synergi Hydro® RP (4 μm, 150 × 4.6 mm).

- **CE System**

An Agilent 7100 model CE (Agilent Technologies, U.S.A.) with DAD (200 nm) was used for CE experiments. Separation was achieved by a fused silica capillary with 40 cm effective (48.5 cm total, 75 µm i. d.) length.

- **LC-MS/MS System**

Agilent 1290 LC System was used. The system contains the following parts: Agilent Technologies 6460 Triple Quad LC-MS/MS, Agilent Mass Hunter Data Acquisition software, Electrospray Ionization Source (positive mode) and Ion multiplier detector. The chromatographic separation was performed by using Zorbax Eclipse RP C_{18}, Particle size 1.8µm (50 mm × 2.1mm).

4. METHODS

4.1. UV- Visible Spectrophotometric Method

4.1.1. Preparation of standard solutions

Standard solutions of ESC-OX (15.05 µg mL^{-1}), CIT A (12.05 µg mL^{-1}), CIT B (10.40 µg mL^{-1}), CIT C (10.10 µg mL^{-1}), CIT D (10.40 µg mL^{-1}) and CIT E (10.30 µg mL^{-1}) were prepared in ACN: water (30:70, *v/v*) and used for UV-visible spectrophotometric method.

4.1.2. Absorbance maxima detection of analytes

A diluted standard solution of a selected target compound (either ESC-OX, CIT A, CIT B, CIT C, CIT D or CIT E herein) were prepared as described under Section 4.1.1. and injected into a liquid chromatograph coupled with a DAD. After performing an acceptable elution, UV-visible spectrum of the principal peak for each of the analytes was recorded between 190 nm to 400 nm, and its absorptivity characteristics were examined for detection of possible absorption maxima.

4.1.3. Analysis parameters

Method parameters for the analysis are given in Table 4.1.

Table 4.1. *Method parameters applied in the detection of absorbance maxima.*

Parameter	**Value**
Scan range	190 – 400 nm
Measurement mode	Absorbance
Scan mode	Spectrum
Sampling frequency	0.2 nm
Slight width	1.2 nm
Buffer and pH	Phosphate buffer pH 3.5 (50 mM)
Mobile phase composition	Buffer: ACN: MeOH: water (50:25:5:20 *v/v/v/v*)
Column temperature	40°C
Flow rate	1.2 mL min^{-1}

4.2. HPLC Method

4.2.1. Preparation of mobile phase

The mobile phase consisted of ACN: MeOH: water: phosphate buffer (50 mM, pH 3.5) (25: 5: 20: 50, *v/v/v/v*). For preparation of phosphate buffer solution (50 mM, pH 3.5), 6.8 g of $KHPO_4.2H_2O$ and 115.6 µL of H_3PO_4 (d= 1.695 g mL^{-1} and 86 %) was added to a volumetric flask (V=1000 mL), and after appropriate dissolution the volume was made up to volume with ultrapure water. The pH of the buffer was checked by using a calibrated pH-meter. The prepared buffer solution was filtered through an 0.45 µm pore size cellulose membrane filter under vacuum to remove any insoluble particles. For removal of the dissolved gases in the solution, it was left in an ultrasonic bath for about 15 minutes. Mixing of the specified solvents at the desired ratios was carried out using the low-pressure gradient mixing unit of the HPLC device.

4.2.2. Preparation of standard solutions

To prepare standard stock solutions of the analytes, appropriate amount of USP grade reference standard was weighed and dissolved in 30 mL of ACN: water (30:70, *v/v*) solution. For this purpose, 3.01 mg of ESC-OX, 2.41 mg of CIT A, 2.08 mg of CIT B, 2.02 mg of CIT C, 2.08 mg of CIT D and 2.06 mg of CIT E were used. Consequently, the stock solutions were mixed and diluted using the same diluent to obtain the test solution used in the method development studies. The final concentration of the analytes was as follows: ESC-OX 15.05µg mL^{-1}, CIT A 12.05 µg mL^{-1}, CIT B 10.40 µg mL^{-1}, CIT C 10.10 µg mL^{-1}, CIT D 10.40 µg mL^{-1} and CIT E 10.30 µg mL^{-1}.

4.2.3. Analysis parameters

The test solution mentioned in the previous section was used for preliminary studies and the content of the mobile phase were changed applying different ratios of ACN, MeOH, water and buffer. Main chromatographic parameters that was examined for evaluations were system suitability parameters such as asymmetry factor, resolution, theoretical plate, etc.; the conditions that resulted in the best chromatographic performance were selected.

In this work, 12 HPLC columns from different brands and models were utilized in method development studies; the columns and manufacturers at this step were as follows: Fluophase® RP (Thermo Scientific) (5 µm, 10 cm × 4.6 mm), Synergi® Hydro-RP (Phenomenex) (4 µm, 15 cm × 4.6 mm), Nucleosil® C_{18} (Teknokroma) (3 µm, 10 cm

× 4.6 mm), Ascentis® Express F5 (Supelco Analytical) (2.7 µm, 10 cm × 4.6 mm), Ascentis® Express C_{18} (Supelco Analytical) (2.7 µm, 10 cm × 4.6 mm), Ascentis® Express Phenyl-Hexyl (Supelco Analytical) (2.7 µm, 10 cm × 4.6 mm), Kinetex® C_{18} (Phenomenex), Kinetex ® PFP (Phenomenex), Kinetex® Phenyl-Hexyl (Phenomenex) (2.6 µm, 150 × 4.6 mm), Luna® PFP (Phenomenex) (3 µm, 150 × 4.6 mm), Inertsil® C_{18} (GL Sciences) (3 µm, 150 × 4.6 mm) and Luna® Phenyl-Hexyl (Phenomenex) (3 µm, 150 × 4.6 mm). After examination of system suitability results and the chromatograms recorded, the last six columns in the list, which showed best performance were utilized for further studies. The specific properties of the columns are given in Table 4.1.

4.2.4. Evaluation of results

The area under curve for each chromatographic peak was used as the analytical response throughout the study. The calibration curve was constructed by using the peak area versus corresponding concentration; the equation calculated from linearity studies were used for quantitation.

4.2.5. Method validation

The validation of the proposed method was carried out according to ICH Q2(R1) and USP guidelines (ICH-Topic Q2(R1), 2005, p. 1-15; USP 37, 2014), including accuracy, precision, LOD, LOQ, etc. In addition, system suitability was tested to verify analytical performance of the instrument and the columns.

4.2.5.1. System suitability test (SST)

One of the integral parts of the method development is to verify the performance of the analytical instrument. Parameters such as resolution (R_S), tailing (T) or symmetry factor (As), theoretical plate number (N), capacity factor (K') and selectivity factor (α). Each of these parameters was calculated using the Shimadzu Lab Solutions version 1.25 data analysis software according to the USP 30. The formulas used in the calculations are given in Table 4.2.

Table 4.2. *Equations Related to the System Suitability Parameters*.

Parameter	Equation	
Theoretical plate	$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$	t_R: Retention time of the peak. $W_{1/2}$: Width of the peak at half height.
Tailing factor	$T = \frac{W_{0.05}}{2 \times \alpha_{0.05}}$	T: Tailing factor. $W_{0.05}$: Width of the peak at 5% height. $\alpha_{0.05}$: Front half-width at 5% height.
Resolution	$R_S = 2 \times \frac{t_{R2} - t_{R1}}{W_1 + W_2}$	R_S: Resolution. t_{R2} and t_{R1}: Retention times of the two compounds. W_2 and W_1: the corresponding widths.
Capacity factor	$K' = \frac{t_R - t_0}{t_0}$	K': Capacity factor. t_R: Retention time of the peak. t_0: Dead time.
Selectivity Factor	$\alpha = \frac{K'_2}{K'_1} = \frac{t_2 - t_0}{t_1 - t_0}$	α: Selectivity factor K'_1 and K'_2: Capacity factor of first and second peak. $t_1$and t_2: Retention time of first and second peak. t_0: Dead time.

4.2.5.2. Linearity

The linearity of an analytical method can be defined by linear correlation between the analyte concentration versus the instrumental response (USP 37, 2014). The linearity of the developed method was studied for ESC-OX in the range of 0.0753 to 117.80 µg mL^{-1}, for CIT A 0.0603 to 120.5 µg mL^{-1}, for CIT B 0.0520 to 104.0 µg mL^{-1}, for CIT C 0.0505 to 101.0 µg mL^{-1}, for CIT D 0.052 to 104.0 µg mL^{-1} and for CIT E 0.0515 to 103.0 µg mL^{-1}. Then the calibration curve was constructed in the following range for ESC-OX 0.236 to 70.69 µg mL^{-1}, CIT A 0.241 to 72.30 µg mL^{-1}, CIT B 0.208 to 62.40 µg mL^{-1}, CIT C 0.202 to 60.60 µg mL^{-1}, CIT D 0.208 to 62.40 µg mL^{-1} and CIT E 0.206 to 61.40 µg mL^{-1}. Three sets of the compound mixture were prepared and analyzed for three days. Linear regression analysis was performed for calculation of slope and intercept, and the equation was used in the calculation of pharmaceutical dosage form potency. In addition, intergroup variations were examined by one-way ANOVA. Statistical calculations were done with Graph Pad Prism v 6.0 b program.

The calibration applications were carried out using three best performing columns, namely Kinetex® C_{18} (Phenomenex), Kinetex ® PFP (Phenomenex), Kinetex® Phenyl-Hexyl (Phenomenex).

4.2.5.3. Accuracy

One of the important parameters in analytical method validation is accuracy; it is the estimation of the degree of closeness of the determined value to the real value (ICH-Topic Q2(R1), 2005, p. 9). The percentage of recovery was used for this purpose, and performed by standard addition method for both tablet and oral solution matrices. Three sets of high, medium and low concentration levels (50,100 and 150%) were examined three times for three days. The concentrations used are for ESC-OX 2.72, 5.45 and 8.17 µg mL^{-1}, for CIT A 2.41, 4.82 and 7.23 µg mL^{-1}, for CIT B 2.08, 4.16 and 6.24 µg mL^{-1}, for CIT C 2.02, 4.04 and 6.06 µg mL^{-1}, for CIT D 2.08, 4.16 and 6.24 µg mL^{-1} and for CIT E 2.06, 4.12 and 6.18 µg mL^{-1}.

4.2.5.4. Precision

The precision is essential to obtain reliable results; it is the degree of closeness of the repeated measurements among a series of experiments. Precision was tested at three different schemes: Repeatability (intra-day precision), intermediate precision (inter-days' precision) and reproducibility (between laboratories precision). The precision is expressed as the coefficient of variation (C.V), relative standard deviation (RSD) and standard deviation (SD).

In this work, lower concentration from the calibration curve was selected as the test solution for precision studies and analyzed for six times for three days. For ESC-OX 0.236 µg mL^{-1}), for CIT A 0.241 µg mL^{-1}, for CIT B 0.208 µg mL^{-1}, for CIT C 0.202 µg mL^{-1}, for CIT D 0.208 µg mL^{-1} and for CIT E 0.206 µg mL^{-1} were used for precision study.

4.2.5.5. Limit of detection and quantification (LOD) and (LOQ)

LOD can be defined as the lowest quantity of an analyte that can be detected but not necessary to be quantified. LOQ can be defined by the lowest quantity that can be determined with high degree of precision and accuracy. In the HPLC part, signal-to-noise was applied for calculation of LOD and LOQ. The LOD and LOQ were determined from the chromatograms by taking (Signal to Noise) S/N ratio of 3 for LOD and 10 for LOQ.

4.2.5.6. Specificity and selectivity

The specificity and selectivity can be defined as the ability of the developed method to differentiate the analyte (s) response from all other responses (e.g., impurities, degradation products and matrix components, etc.). Specificity is verified by means of possible interference from the pharmaceutical dosage forms matrix. The examination was done in all the examined columns and there was no interference exhibited in the chromatograms.

4.2.5.7. Robustness and ruggedness

Robustness can be defined by the capability of the developed analytical method to be stable against small variations in the operational parameters. To study the effect of flow rate on the resolution, the flow rate was changed within ± 0.1 units (1.1 and 1.3 mL min^{-1}) The effect of pH on the resolution of impurities was studied by varying the pH of the buffer within ± 0.2 pH units (at 3.3 and 3.7 buffer pH). The effect of column temperature on the resolution was studied at two different temperatures (35°C and 45°C). The effect of mobile phase composition was studied by changing components' ratios by ± 2% (i.e., 48% and 52% for buffer, 3% and 7% for MeOH, and 23% and 27% for ACN). The influence of wavelength was also evaluated at two different wavelengths of UV-detector (205 and 215 nm) (Heyden et al., 2001, p. 743). In all cases, the test solutions were re-analyzed and proper analytical interpretation was realized.

According to USP, ruggedness is defined as the ability of the developed method to produce reproducible results also when different instruments, laboratories, reagents, environmental status, etc. were used. The ruggedness of the developed method was examined by using an additional HPLC system (Instrument B) and Synergi® Hydro-RP column (4 µm, 150 × 4.6 mm).

4.2.5.8. Stability

The stability of the standard solutions under different conditions was also determined during method validation. The conditions used in stability tests reflected the circumstances that was probably to be experienced during real samples processing and analysis (Shah et al., 2001, p. 6-7). The following are the stability conditions that are conducted in stability tests.

- ***Short-Term Temperature Stability***

  The stability of the standard solutions that were kept at room temperature for 24 h was examined. It was also called as the “benchtop stability”.

- ***Freeze and Thaw Stability***

  The stability of the analytes after three thaw-and-freeze (-24°C) cycles was determined.

- ***Long-Term Stability***

  The stability of the frozen stock solutions for one month was examined.

4.2.6. Preparation of pharmaceutical dosage form samples

ESC-OX tablets (10 mg and 20 mg) and oral solution (10 mg mL^{-1}) were used for application of the developed method. First of all, each tablet was weighed, and then the mean weight of ten tables was calculated. The tablets were completely crushed in a mortar to get a fine powder. An amount of the powder equal to the mean weight of one tablet was dissolved in 10 mL ACN: water (30:70, *v/v*) solution and sonicated for 10 min to initiate dissolution; the resulting mixture was filtered through nylon syringe filters (0.45 µm), then further diluted to 10 µg mL^{-1} and, injected. For oral solution, 1 mL of the sample was diluted in ACN: water (30:70, *v/v*) filtered through nylon syringe filters (0.45 µm), then diluted to 10µg mL^{-1}, and injected into the system.

4.3. Electrochemical Methods

4.3.1. Preparation of standard solutions

The stock solution of ESC-OX at 414.4 µg mL^{-1} concentration was prepared by dissolving in ultrapure water, then sonicated for 10 min; all of the further dilutions were realized using the stock solution. Solutions for the calibration curve were prepared from the stock solution by adequate dilution with water.

4.3.2. Preparation of supporting electrolyte solution

Supporting electrolyte solution was consisting of: Britton-Robinson (BR) buffer, MeOH and KCl.

BR buffer was prepared in ultrapure water by mixing equal amounts of 0.04 M H_3BO_3, 0.04 M H_3PO_4 and 0.04 M CH_3COOH; resulting solutions were then adjusted to the desired pH (between 2.0 to 12.0) with 0.2 M NaOH solution (Britton and Robinson, 1931). The solution was agitated after blending and put for one night to achieve equilibrium.

Solutions of KCl, which was used as supporting electrolyte, was also prepared in ultrapure water between 1.0 M to 3.0 M concentrations. All solutions were kept in a refrigerator at 4.0°C.

Methanol 50% was used without dilution. The final supporting electrolyte contains: 1 mL of BR of 6.5 pH, 1 mL of KCl 0.3 M and 1 mL of 50% MeOH.

4.3.3. Analysis parameters

Prior to starting every measurement, the electrodes were cleaned with ultrapure water and smoothly wiped with a piece of tissue. In this work, DPV, OSWV and CV were used as the analytical voltammetric techniques. The experiments in DPV and OSWV mode was carried out in the range between - 0 V to + 1.50 V, while in CV mode the potential was scanned over the range of −0.2 V to +1.50 V, at various scan rates (ν) from 10 mV s^{-1} to 1000 mV s^{-1}. The blank DP voltammograms was obtained by employing the following procedure: 10 mL of supporting electrolyte was put into the cell and air is eliminated by purging with purified nitrogen gas for 30 sec. To get DP voltammograms of ESC-OX, the stock solution of 41.44 µg mL^{-1} was diluted to achieve desired concentration with the supporting electrolyte. A voltammogram was recorded after 5 sec of equilibration time, under the static state of the cell.

4.3.4. Evaluation of results

The area under curve for each voltammetric peak was used as the analytical response throughout the study. The calibration curve was constructed by using the peak area versus corresponding concentration; the equation calculated from linearity studies were used for quantitation.

4.3.5. Method validation

All validation parameters were performed for both standard solution and urine.

4.3.5.1. Linearity

Linearity of the calibration curve was examined by plotting the cathodic peak current (*I*pc) versus the concentration of ESC-OX in triplicate (n=3). The linearity was examined in range of 4.14 to 29 µg mL^{-1}. Linearity was checked by using the least square regression equation. Calibration plots were chosen in this range and concentrations were analyzed for three consecutive days.

In case of urine, matrix matched calibration was carried out by spiking 100 µL of ESC-OX into raw urine samples. The calibration curve was ranged between 4.14 to 29 µg mL^{-1}. LLE was also applied consequently as mentioned in section 4.3.7.

4.3.5.2. Accuracy

Regarding the study of the accuracy of the developed method, recovery studies were applied in three subsections: At the first section, the accuracy of the proposed method was determined by using reference standard solutions of 4.14 µg mL^{-1}, 16.5 µg mL^{-1}, and 29 µg mL^{-1}.

At the second section, the accuracy of urine analysis was tested by addition of ESC-OX in the following concentrations 4.14 µg mL^{-1}, 16.5 µg mL^{-1}, and 29 µg mL^{-1} to the blank urine samples (n=6) for three consecutive days.

At the third section, the accuracy of the pharmaceutical samples analysis methods was evaluated by comparing the results of the samples analysis results with those of obtained in the HPLC method, as recommended in the ICH Q2 (R1) guideline.

4.3.5.3. Precision

The measurement of repeatability (intra-day) and intermediate precision (inter-days) was done to evaluate the precision of the developed method. Standard solutions of ESC-OX at 4.14, 16.5 and 29 µg mL^{-1} concentrations were examined in three consecutive days, including six independent determinations per day. The precision is expressed as (C.V), RSD and SD.

4.3.5.4. Limit of detection and quantification (LOD) and (LOQ)

The calculation of LOD and LOQ were done by the following equations respectively (3.1, 3.2):

$$\mathrm{LOD} = \frac{3.3\ x\ \sigma}{S} \tag{3.1}$$

$$\mathrm{LOQ} = \frac{10X\ \sigma}{S} \tag{3.2}$$

where σ is the SD of the response, and S is the slope of the calibration curve.

4.3.5.5. Specificity and Selectivity

The specificity and selectivity of the method was evaluated by the examination of blank human urine and different pharmaceutical dosage forms matrix.

4.3.5.6. Robustness

Development of a new method for the analysis of the pharmaceuticals or in the biological fluids requires the evaluation of its reliability in routine use. This can be achieved by studying the experimental and analytical variables that affect the robustness of the developed method (Ozkan, 2012, p.323-325). The changed instrumental conditions are as following: drop size 2-16 au (arbitrary unit), scan rate 15-50 mV s^{-1}, sample width 25 ms, pulse width 50-100 ms, pulse amplitude 25-100 mV, pulse period 100-350 ms.

4.3.5.7. Stability

The stability of standard solutions was studied by analyzing standard solution of ESC-OX. The freshly prepared solutions were initially analyzed and then kept at +4 °C in the dark; after a one month waiting period the solutions were re-analyzed and the results were compared with the initial ones. The stability of the spiked urine samples was evaluated by analyzing the urine samples three times over the period of one day.

4.3.6. Preparation of pharmaceutical dosage form samples

The developed method was applied to three different dosage forms. The same procedure was followed as mentioned in section 4.2.6. except that the final dilution was done with water. Then further dilution was carried out with supporting electrolyte to get the final concentration of 24.86 μg mL^{-1} and they were analyzed to measure the amount of the analyte. For oral solution 500 µL was taken and diluted to 100 mL, then further dilution with supporting electrolytes was done to achieve 24.86 µg mL^{-1} final concentration in the measuring cell. The content amount of the ESC-OX was calculated from the related regression equation.

4.3.7. Preparation of urine samples

The ESC-OX-free urine samples were collected from healthy volunteers and kept frozen till examination time. After a gentle thawing, a portion of the urine samples was spiked with the 100 µL of analyte and the procedure for LLE was carried out as follows: 0.5 mL of urine and 1.5 mL saturated ammonium hydroxide was vortexed. After vortex for 1 min, ESC-OX was extracted from the spiked mixture with 1 mL hexane by mixing for 10 min with vortex. After centrifuging for 10 min at 4000 rpm, the organic layer at the top was transferred to another Eppendorf tube. The hexane phase was evaporated under vacuum. The extract was reconstituted with ultrapure water, filtered through 0.45 nm nylon filter, and then diluted to 10 mL with supporting electrolyte in the measuring cell of the electrochemical system. The steps of LLE are illustrated in Figure 4.1.

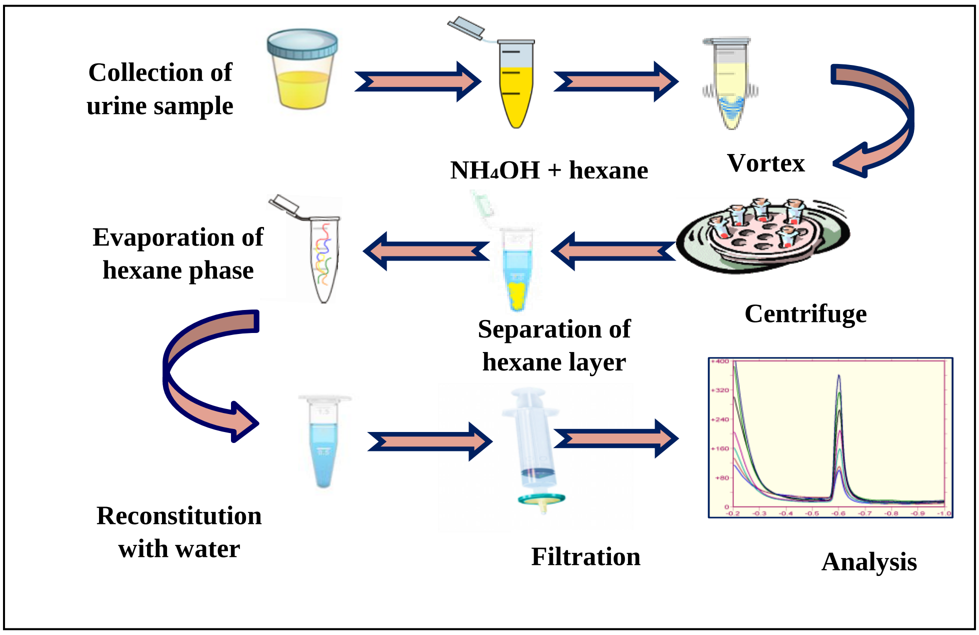


Figure 4.1. *LLE of Urine Samples.*

4.4. Capillary Electrophoresis

4.4.1. Preparation of standard solutions

Standard stock solutions of ESC-OX (430 µg mL^{-1}) and MET, which was used as IS (1063.66 µg mL^{-1}) were prepared in distilled water. They were both diluted with 1/10 run buffer solution to the desired concentration. All solutions were stored in dark at + 4℃ and working solutions were prepared freshly by appropriate dilution just before use.

4.4.2. BGE

15 mM phosphate buffer at pH 2.5 was obtained by diluting the stock solution of 100 mM to 15 mM and adjusting 15 mM NaH_2PO_4 solution accurately to the desired pH with diluted phosphoric acid. All solutions were filtered through 0.45 µm nylon filters before use.

4.4.3. Analysis parameters

At the very first use of the fused-silica capillary, it was conditioned by flushing with the following solutions consequently: 1.0 M NaOH for 30 min, then 0.1 M NaOH for 10 min, ultrapure water for 10 min, and BGE for 10 min. In the following days, the capillary was washed and re-conditioned again by rinsing for 10 min for each with 0.1 M NaOH, ultrapure water, and BGE every day. The samples were then injected by vacuum injection into the fused-silica capillary filled with BGE at 50 mbar pressure for 10 sec. Between each run the capillary was rinsed with 0.1 M NaOH (2 min), distilled water (2 min), and BGE (2 min). At the end of each working day, it was washed with 0.1 M NaOH and ultrapure water for 10 min and left air-aspirated. During analysis, the applied potential was +22.5 kV, under voltage-controlled conditions. All CE runs were conducted at 25°C. Detection was performed at 200 nm using DAD.

4.4.4. Evaluation of results

Quantification was accomplished using ESC-OX to MET correct peak area ratios [i.e., (peak area of ESC/migration time of ESC) / (peak area of MET/migration time of MET)] (Yang, Bose and Hage, 1996, p. 209-220; http 10) as the analytical response.

4.4.5. Method validation

4.4.5.1. Linearity

The linearity of the method was investigated in the range of 0.016 to 32.25 µg mL^{-1}, evaluating by linear regression analysis using the least square regression method.

Calibration plots were constructed within this range, and seven reference solutions at different concentrations (0.0264 to 2.75 μg mL^{-1}) were injected into the system for three consecutive days for this purpose.

The calibration curve was carried out in the urine samples matrix by spiking 0.5 mL of urine samples with the 10 μL of the ESC-OX in the range of 0.026-2.75 μg mL^{-1} then LLE was carried out as mentioned in section 4.4.7.

4.4.5.2. Accuracy

The accuracy of intended method was evaluated by calculating the percentage of recovery of three concentrations 0.0264, 0.3302, and 2.75 μg mL^{-1} for three days, six times in a day for the reference standard solution. The accuracy of the spiked urine samples was verified for three days, six times in a day by spiking of ESC-OX in the following range; 0.0269, 0.3343 and 4.3515 μg mL^{-1} to the urine samples. Then the percentage of recovery was calculated. The accuracy for the pharmaceutical products was performed as mentioned in section 4.3.5.2.

4.4.5.3. Precision

The precision of the method was determined by the measurement of repeatability (intra-day) and intermediate precision (inter-days). Standard solution of ESC-OX at 0.0264, 0.3302, and 2.75 μg mL^{-1} concentration were injected for three consecutive days six times per day. The precision of the spiked urine samples was verified for three days, six times in a day by spiking of ESC-OX in the following range; 0.0269, 0.3343 and 4.3515 μg mL^{-1} to the urine samples. Then the RSD % was calculated.

4.4.5.4. Stability

The stability of standard solutions was studied as mentioned in section 4.3.5.2.

4.4.6. Preparation of pharmaceutical dosage form samples

The preparation of the pharmaceutical dosage form was performed as mentioned in section 4.2.6. Then the amount equivalent to 0.3302 μg mL^{-1} of all resulting solutions were diluted 1 to 10 BGE with solution to the desired concentrations, and consequently they were analyzed to measure the amount of ESC-OX. For oral solutions, 500 μL was taken from the pharmaceutical product into the 10-mL volumetric flask, further dilution was carried out to get an amount equivalent to the 0.3302 μg mL^{-1}, filtered as mentioned above and injected into the system.

4.4.7. Preparation of urine samples

For this purpose, 500 μL of urine sample was spiked with ESC-OX, then 200 μL saturated ammonium hydroxide solution was added. The sample constituents are extracted from aqueous samples to a water-immiscible organic phase by adding 1 mL of hexane Eppendorf tubes. Then the same procedure as mentioned in section 4.3.7 was carried out. The extracts were reconstituted with distilled water, filtered with 0.45 μL nylon filter, 0.266 μg mL^{-1} MET was added to sample and transferred to CE auto sampler vials for analysis.

4.5. LC-ESI-MS/MS

4.5.1. Preparation of mobile phase

The mobile phase consists of (A) 0.1% F. A in ACN and (B) 0.1% F. A in HPLC grade water.

4.5.2. Preparation of standard solutions

The stock solution of ESC-OX (50 μg mL^{-1}) was prepared by dissolving ESC-OX standard in MeOH 50% (*v/v*), and then sonication for 10 min to enhance dissolution. All further dilutions were prepared from the stock solution. Solutions for calibration curve were prepared from the stock solution by adequate dilution with MeOH 50% (*v/v*).

4.5.3. Analysis parameters

The chromatographic separation was performed by using Zorbax Eclipse RP C_{18} (50 mm x 2.1 mm) column with a particle size of 1.8 μm. Several gradient programs were tried and the final gradient elution is as shown in the Table 4.3. Re-equilibration time was 3 min. The instrumental parameters are summarized in Table 4.4.

Table 4.3. *Gradient elution used in LC-ESI-MS/MS.*

Time	0.1% F. A in ACN	0.1% F. A in water
0	20	80
4	65	35
5	30	70
6	85	15

Table 4.4. *The instrumental parameters for the developed LC-ESI-MS/MS.*

Analyte	Ionization source	Injection volume	Column Temp. ℃	Flow rate mL min^{-1}	Precursor ion (m/z)	Product ion (s)
ESC-OX	ESI (+ mode)	2 µL	40	0.35	325	109. 262

4.5.4. Evaluation of results

Analyte qualification and measurement were based on their retention times and product ion full scan spectra. The calibration curve was constructed by using the peak area versus related concentration. Weighted-Least Squares equation (1/x) was used for the calculation method validation parameters.

4.5.5. Method validation

4.5.5.1. Linearity

The linearity of the method was investigated in the range of 0.0078 to 391.370 ng mL^{-1}. Calibration plots were constructed within this range 0.079 to 196.450 ng mL^{-1} and injected into the system for three consecutive days for this purpose.

The calibration curve was carried out in the urine sample matrix by spiking 0.5 mL of urine samples with the 10 µL of the ESC-OX in the range of 0.079 to 196.450 ng mL^{-1} then LLE was carried out as mentioned in section 3.5.7. The lower limit of quantification (LLOQ), is the lowest concentration that can be measured with suitable accuracy and precision usually ± 20%, was calculated by using C.V%. LOD was calculated by using signals to noise ratio. Same validation parameters were calculated for the urine matrix.

4.5.5.2. Accuracy

The accuracy of intended method was evaluated by calculating the percentage of recovery of three concentrations 0.079, 7.800 and 196.450 ng mL^{-1} for three days, six times in a day for the reference standard solution. The accuracy of the spiked urine samples was verified for three days, six times in a day by spiking of ESC-OX in the following range; 0.079, 7.800 and 196.450 ng mL^{-1} to the urine sample. Then the percentage of recovery was calculated. The accuracy for the pharmaceutical products was performed as mentioned in section 4.3.5.2.

4.5.5.3. Precision

The precision of the method was determined by the measurement of repeatability (intra-day) and intermediate precision (inter-days). Standard solutions of ESC-OX at 0.079, 7.800 and 196.450 ng mL^{-1} concentrations were analyzed for three consecutive days and six times per day.

The precision of the spiked urine samples was verified for three days, six times in a day by spiking of ESC-OX in the following range; 0.079, 7.800 and 196.450 ng mL^{-1} to the urine sample. Then the RSD% was calculated.

4.5.5.4. Stability

The stability of standard solutions was carried out as mentioned in section 4.3.5.7.

4.5.6. Preparation of pharmaceutical dosage form samples

The developed method was applied for tablet dosage forms (10 mg or/and 20 mg) and oral solution (10 mg mL^{-1}). For the preparation of tablets, the same manner was performed as mentioned before for other methods. The final injected concentration for all dosage forms was 10 ng mL^{-1}.

4.5.7. Preparation of urine samples

LLE was accomplished by using 0.5 mL from urine sample spiked with 10 μL of ESC-OX, then 100 μL saturated ammonium hydroxide was added. The remaining steps were finalized in the similar procedure for LLE. The extracts were reconstituted with 50% MeOH, filtered through a PTFE filter.

5. RESULTS AND DISCUSSION

5.1. Investigation of Spectral Properties of ESC-OX and its Impurity in UV Region

One of the objectives of this thesis was to develop a single HPLC method for determination of ESC-OX with its impurities (n=6). Standard solutions of the target compounds were analyzed using the instrument B for preliminary studies: All of the standard solution of ESC-OX (15.05 µg mL^{-1}), OX-A (12.85 µg mL^{-1}), CIT A (12.05 µg mL^{-1}), CIT B (10.40 µg mL^{-1}), CIT C (10.10 µg mL^{-1}), CIT D (10.40 µg mL^{-1}) and CIT E (10.30 µg mL^{-1}) were prepared in ACN: water (30:70, *v/v*) and used for determination of the absorbance maxima in the range of 190 to 400 nm. The wavelength selection for HPLC-UV studies was based on two major points at this step: 1. Utilization in simultaneous analysis of the compounds without interfering and overlapping each other, 2. Determination of maximum absorbing wavelengths to obtain highest signal intensity.

Nearly all of the target compounds were absorbed in relatively short wavelengths. Besides, significant absorbance was occurred in 196-210 nm region, and in accordance, it was decided that signal detection could be set at 210 nm wavelength in HPLC with UV-detection studies. Related spectrums are given in Figure 5.1 to Figure 5.7.

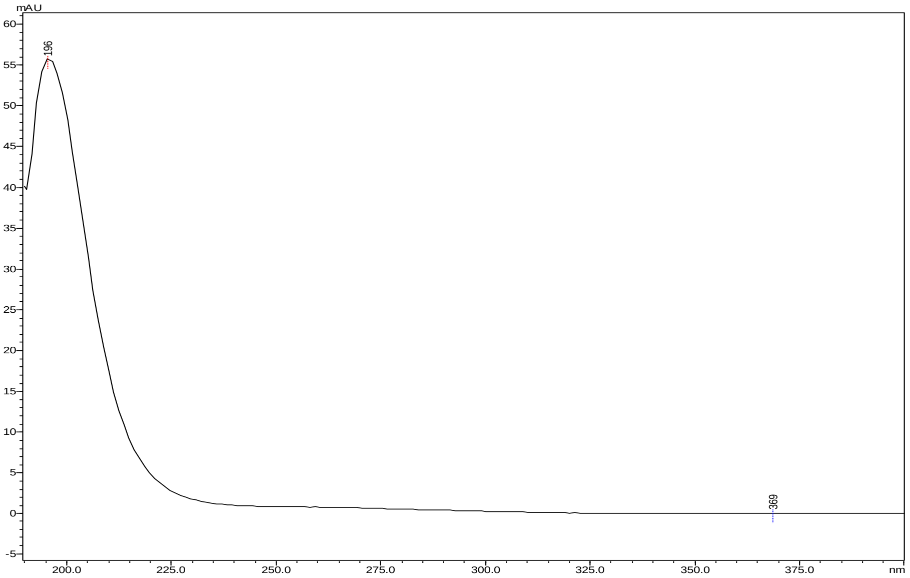


Figure 5.1. *Spectrum of the OX-A in the UV-Visible Region (12.85 µg mL^{-1}).*

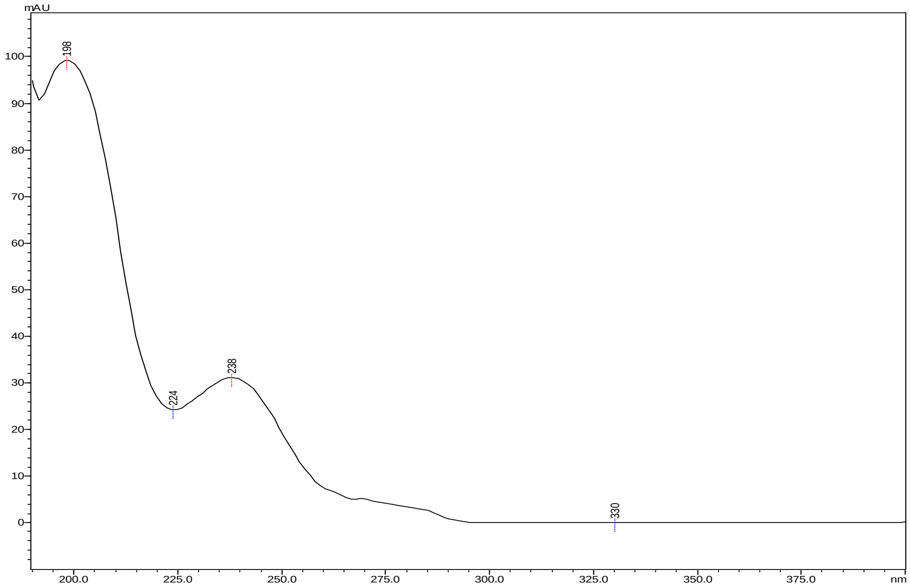


Figure 5.2. *Spectrum of the CIT A in the UV-Visible Region (12.05 μg mL^{-1}).*

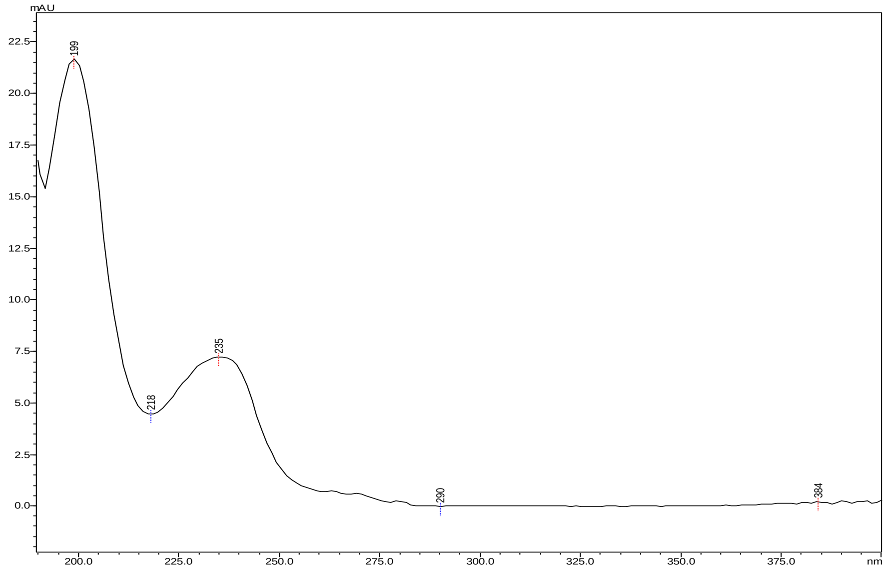


Figure 5.3. *Spectrum of the CIT B in the UV-Visible Region (10.40 μg mL^{-1}).*

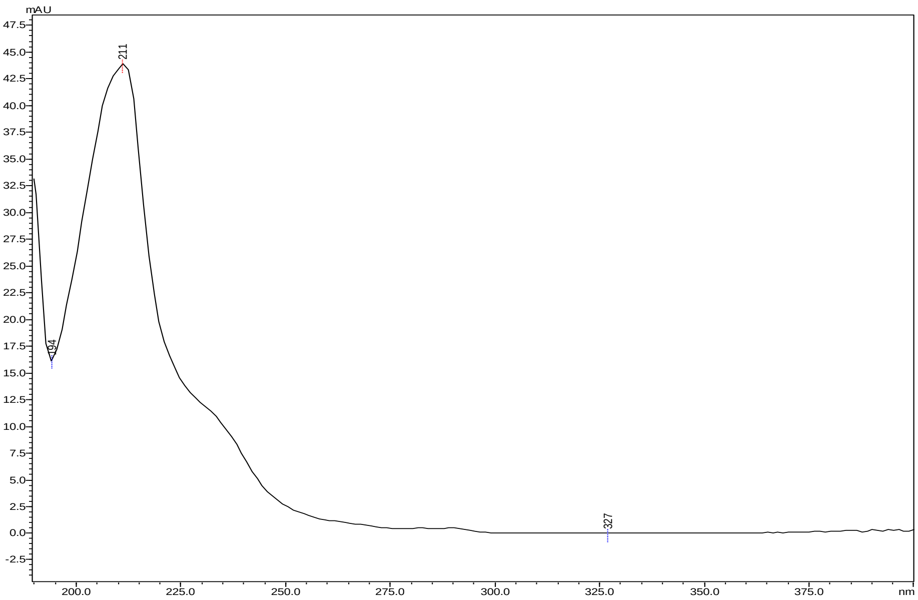


Figure 5.4. *Spectrum of the CIT C in the UV-Visible Region (10.10 µg mL^{-1}).*

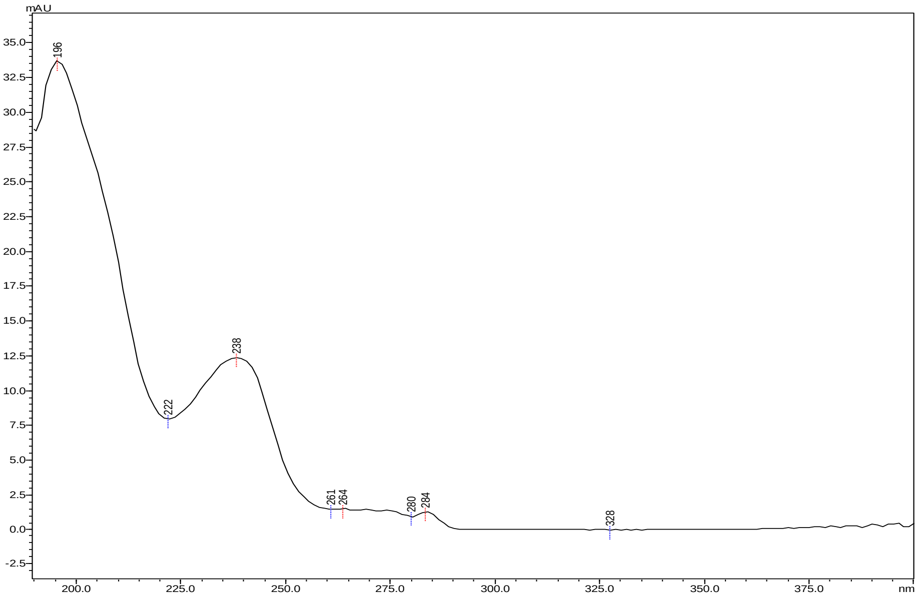


Figure 5.5. *Spectrum of the CIT D in the UV-visible region (10.40 µg mL^{-1}).*

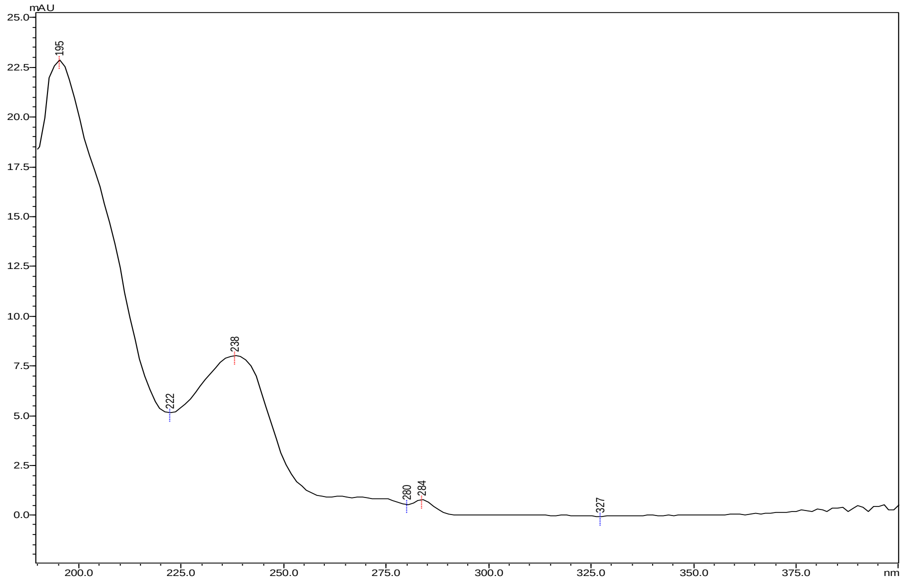


Figure 5.6. *Spectrum of the CIT E in the UV-visible region (10.30 µg mL^{-1}).*

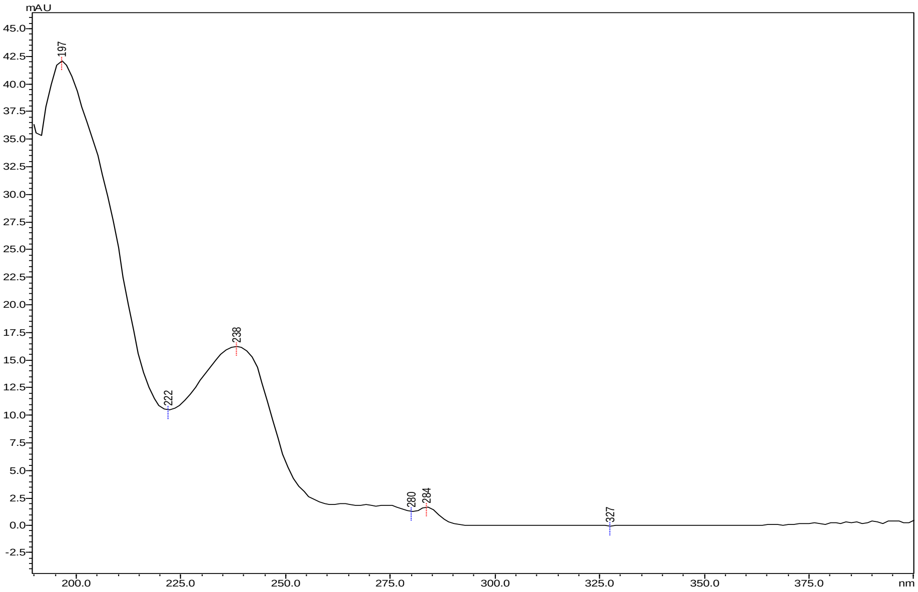


Figure 5.7. *Spectrum of the ESC-OX in the UV-Visible Region (15.05 µg mL^{-1}).*

5.2. Development and Optimization of the HPLC Method

The literature survey showed that there is no analytical method has been developed till current time for determination of previously mentioned ESC-OX impurities simultaneously in one analysis run except the method presented in USP 36. However, this method required long analysis time, as well as, each dosage forms and raw material has a separated analytical method with different analytical and instrumental conditions. This study intended to develop simple and short HPLC method for determination of all mentioned ESC-OX for all dosage forms and raw material at the same time.

In order to develop an analytical method by using HPLC instrument, it is important to take into consideration a number of factors and parameters that affect the analysis, such as chemical properties of the compounds to be determined, selection of the mobile phase content, the column where separation occur and other analytical and instrumental parameters.

5.2.1. Selection of mobile phase

The selection of mobile phase composition was performed by using Phenomenex Kinetex ® PFP core-shell column (3 μm, 150 × 4.6 mm). The choice of mobile phase depends on the chemical properties of the solutes such as pKa, partition coefficient, solubility and polarity. The most frequently used solvents in HPLC are water and water-miscible organic solvents (e.g., ACN, MeOH, 2-propanol, etc). The buffer is used to control retention and provide the reproducible results if the compounds are ionizable. The most frequently used buffers are the phosphate, acetate, format and citrate buffers; these can be used covering different ranges between pH 2-12, and especially phosphate buffer can be used at wavelength below 200 nm. The examination of pH effect was conducted by using mobile phase that contain, buffer: ACN: MeOH: H_2O (50:25:5:20, *v/v/v/v*).

ESC-OX and its impurities are basic compounds; the pKa values of ESC-OX and its impurities are ranging from 9.5- 16.06. Using a buffer within a pH range of 2 to 6 results in ionization of these basic compounds and getting relatively more hydrophilic, so reduce in their retention time. The effect of buffer pH on the retention time of ESC-OX and its related impurities is summarized in Figure 5.8. When phosphate buffer at pH 2 was used, CIT B peak was odd-shaped as shown in Figure 5.9 (a), this phenomenon may be explained by interconversion between chiral isomers of CIT B (Kern and Kirkland, 1997 b, p.562). At pH 3, the peak shape of CIT B was broad (Figure 5.9 (b)). By using acetate buffer at pH 4, the ESC-OX and CIT E eluted together and the peak shape of CIT B was sharp and symmetric (The detection was carried at 239 nm in this part of the study, because UV-cut off of acetic acid is at 210 nm (McPolin, 2009, p. 57) (Figure 5.10 (a)). At pH 2 to 4 the ESC-OX and its impurities retention time are depended mainly on their polarities (log P). The co-elution of ESC-OX with CIT E at pH 4 can be explained by variation in the ionic strength by increasing and changing the type of buffer. At pH 5 and pH 6, CIT C and CIT E eluted together as represented in Figure 5.10 (b, c). The elution order was changed at pH 5 and 6, the CIT E eluted between CIT C and CIT D, ESC-OX was the latest eluting compound in contrast to the pH 2 to 3.5 where CIT E was the latest compound. Changing the order of elution can be explained by alteration in ionization degree of the analytes and electrostatic interaction between the F atom in PFP stationary phase and amine lone pair (Marin and Barbas, 2006, p. 262–270). By using mobile phase of higher pH resulted in delay of retention time that can be explained by decrease in the compounds ionization degree, some π-π interaction between the aromatic rings of analytes and stationary phase (Przybyciel and Santangelo ,2003, p. 210-215) and some electrostatic interaction or by strong adsorption of compounds with the stationary phase (Sun and Liu, 2011, p.279). Nevertheless, the exact mechanism for the complex interaction of these compounds with stationary phase by changing pH is complicated and still unexplained (Przybyciel, 2003, p.2-5)

According to above mentioned results, it was concluded that ESC-OX and their impurities are extremely influenced by the mobile phase pH, so it must be well-controlled to develop a robust method. By trying phosphate buffer at pH 3.5 and at concentration of 50 mM, there was a good resolution between the peaks, and the peak shape of CIT B was slightly sharper than that at pH 3; for this reason, pH 3.5 was selected for further analysis. Potassium phosphate was used instead of sodium phosphate due to better solubility of potassium in the organic mobile phase (Snyder, Kirkland, and Dolan, 2010 a, p. 314).

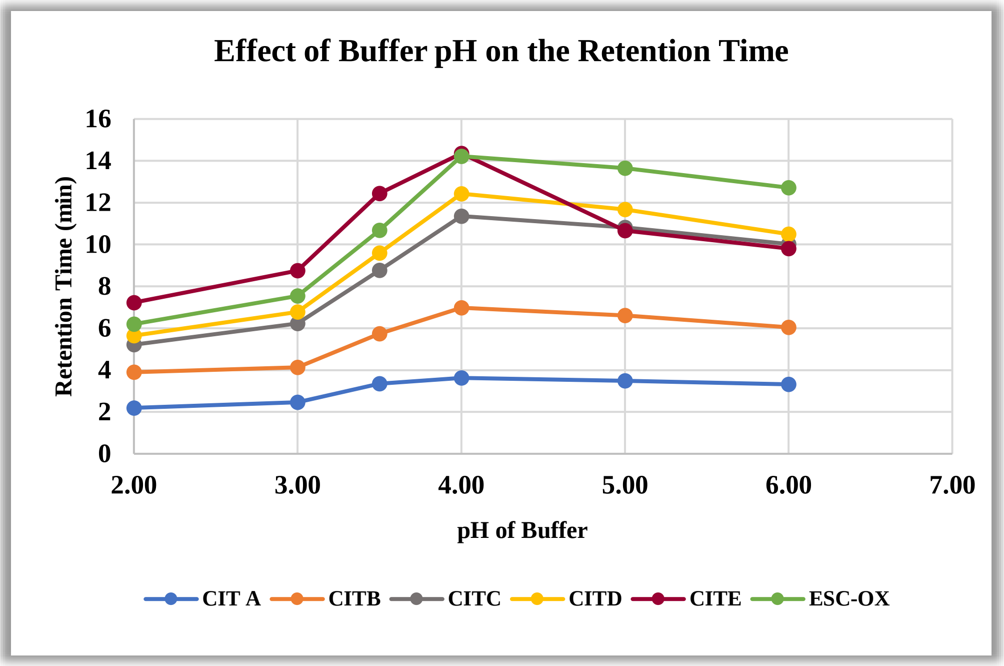


Figure 5.8. Effect of Buffer pH on the Retention Time of ESC-OX and It’s Impurities.

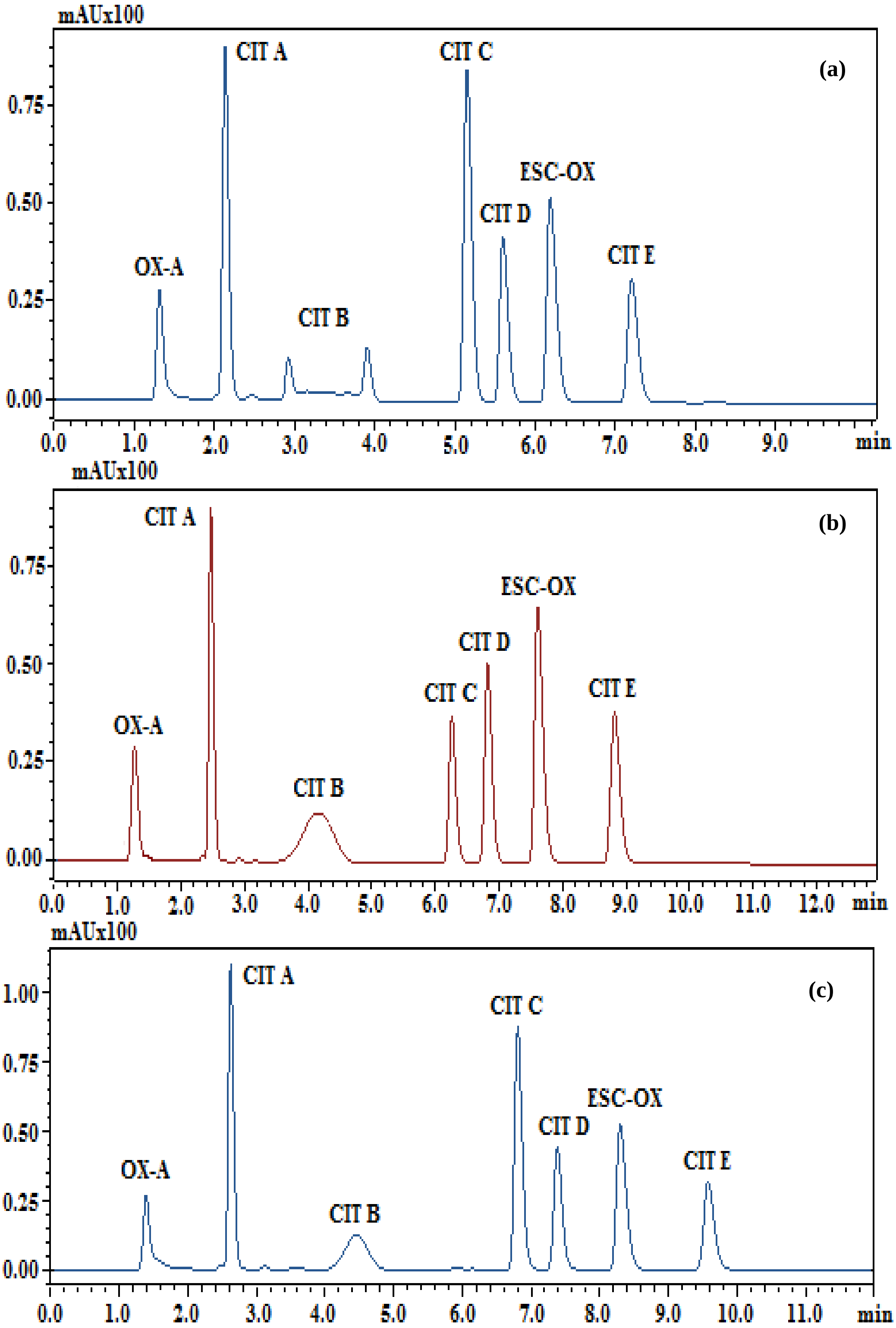


Figure 5.9. *The Effect of Mobile Phase pH on The Retention Of ESC-OX and Its Impurities by using (Column: Kinetex ® PFP Core-Shell Column (3 µm, 150 × 4.6 mm), Mobile phase; Buffer: ACN: MeOH: H_2O (50:25:5:20, v/v/v/v). (a) pH=2, (b) pH=3, (c) pH=3.5, All Phosphate Buffer.*

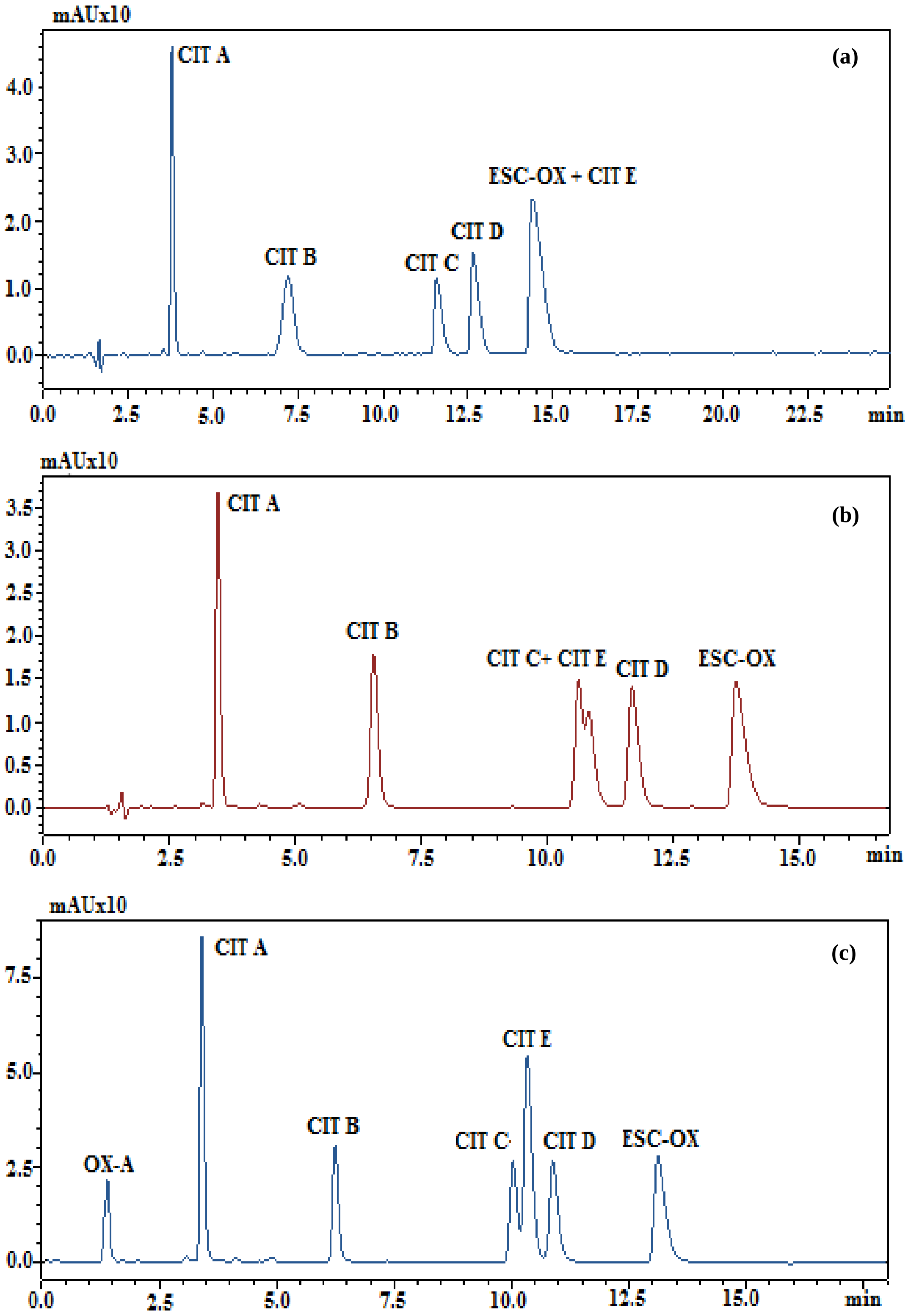


Figure 5.10. *The Effect of Mobile Phase pH on The Retention Of ESC-OX and Its Impurities by using (Column: Kinetex ® PFP Core-Shell Column (3 μm, 150 × 4.6 mm), Mobile phase; Buffer: ACN: MeOH: H_2O (50:25:5:20, v/v/v/v). (a) pH=4(Acetate Buffer), (b) pH=5(Acetate Buffer), (c) pH=6 (Phosphate Buffer).*

ACN is the solvent of choice in RP-HPLC due to several properties such as generation of low back-pressure and low UV cut-off point of 200 (Gill, 1986 a, p. 209). Different percentages of ACN was tested and the best result was obtained at 25% by volume, but the resolution between the CIT C and CIT D was low; thus, to improve the resolution between these compounds and peak symmetry, and reduce tailing of CIT E peak, MeOH was added. MeOH has an UV cut-off of 205 nm (Gill, 1986 b, p. 225). For getting absorbance of MeOH less than 0.05 AU (absorbance unit), the working wavelength must be chosen to be greater than 235 nm or the MeOH percentage must be lower than 15% (Afosah, D. 2010, p. 23; http 12). Various percentages of MeOH with 25% of ACN was tested and the best result was obtained by using 5% of MeOH; there was an improvement in the resolution of adjacent peaks due to change in solvent selectivity. This result is also in accordance with the approaches in the solvent-selectivity triangle (Snyder, Kirkland, and Dolan, 2010 b, p. 287). The result was evaluated by application of SST for each compound with selection of the shortest analysis time. The best separation with good resolution was obtained by using; Buffer: ACN: MeOH: H_2O (50:25:5: 20, *v/v/v/v*). The chromatograms related to the method development are given in Figure 5.11.

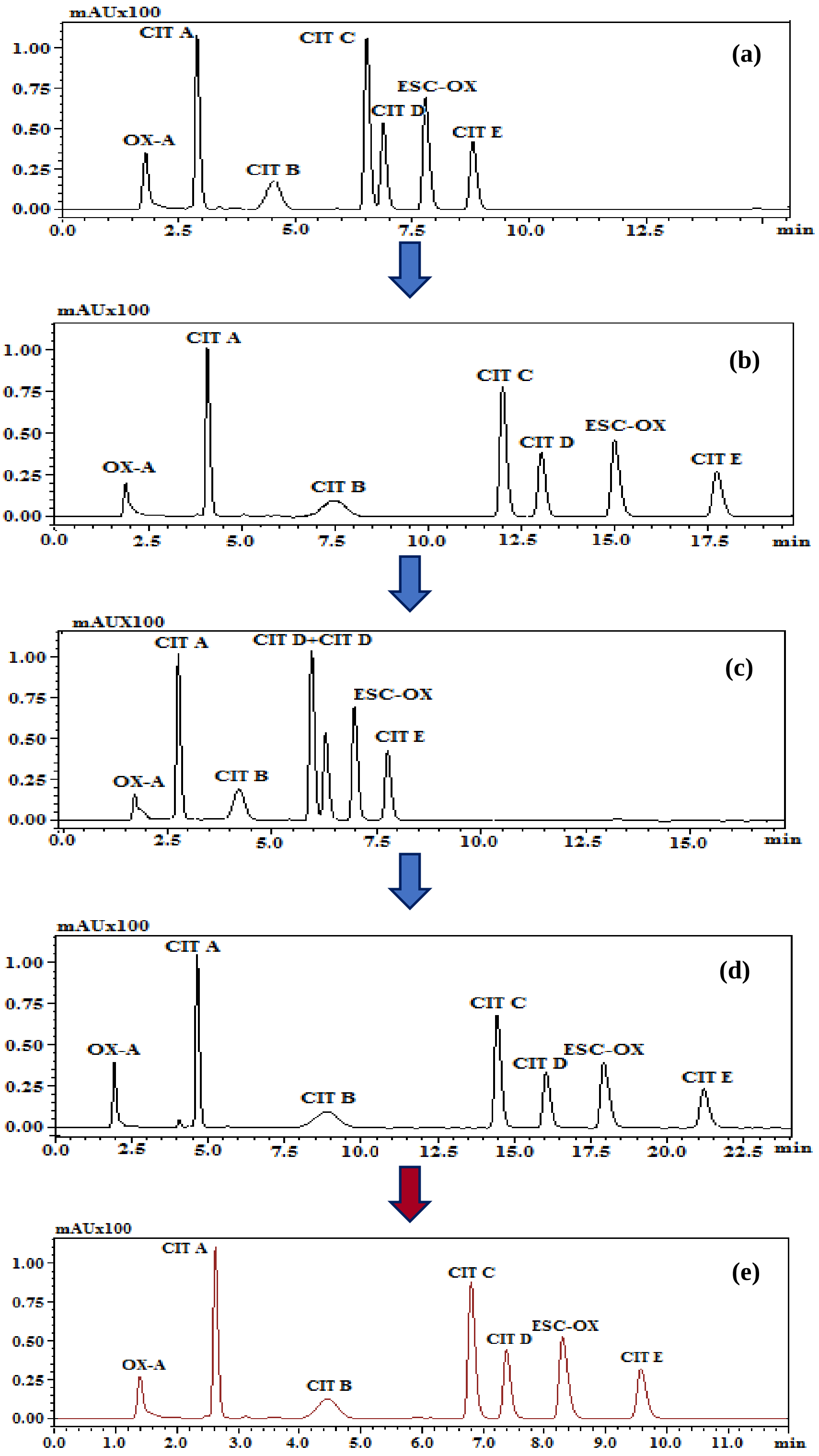


Figure 5.11. *The Chromatograms Related to the Method Development by Using Kinetex® PFP Core-Shell Column (a) Buffer: ACN: H_2O (50:30:20) v/v/v/v, (b) Buffer: ACN: H_2O (50:25:25, v/v/v/v), (c) Buffer: ACN: MeOH: H_2O (50:30:5:15, v/v/v/v), (d) Buffer: ACN: MeOH: H_2O (50: 20:10:20, v/v/v/v), (e) Buffer: ACN: MeOH: H_2O (50:25:5:20, v/v/v/v).*

5.2.2. Selection of the columns

The main part of the HPLC system is the column where the separation takes place. The choice of the column depends on the chemical properties of the target compounds. The physical and chemical properties of the column packing material play important role in providing efficient separation. Frequently used stationary phases are octadecyl-bonded silica (ODS) (known as C_{18}) and octyl bonded silica (known as C_8). In this work, 12 different columns with different particle size and chemical properties were used, as illustrated in Table 5.1. The chromatograms recorded by using different columns having ligand chemistries are presented in Figure 5.12 to Figure5.16. In addition, the chromatogram of each compound by using phenyl-hexyl column is illustrated in Figure 5.17.

The concept of using different stationary phases was to test orthogonality. This technique allows separation of unresolved peaks of two compounds. These compounds may not be separated by using one type of the stationary phase column, but can be detected with suitable resolution by using a diverse stationary phase column (Ng, Lunn, and Faustino, 2007 b, p.12). The effect of stationary phase on the retention of target analytes was examined by using the same instrumental conditions and mobile phase; Buffer: ACN: MeOH: water (50: 25:5:20, *v/v/v/v)*.

Table 5.1. *Physicochemical Properties of the Tested Columns.*

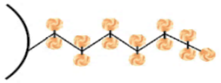
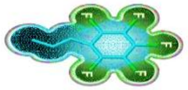
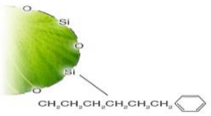
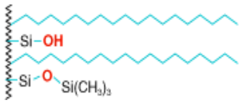
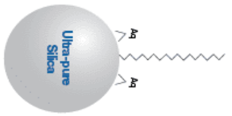
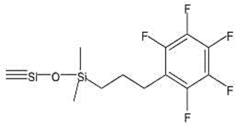
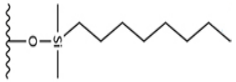
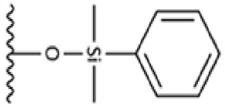
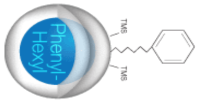
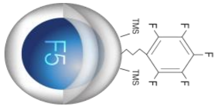
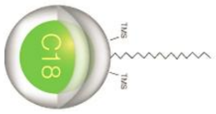
Column (Brand)	L x I. D.	Particle Size	Pore Size	pH Range	Surface Area	Matrix	Carbon Load	Scheme of stationary phase
Inertsil® ODS-3 [a] **(GLSciences)**	15 cm × 4.6 mm	3µm	100 Å	2 - 7.5	450 m^2 g^{-1}	3 Series High Purity Silica Gel	15%	
Fluophase® RP [b] **(Thermo Scientific)**	10 cm × 4.6 mm	5 µm	100 Å	2 - 8	100 m^2 g^{-1}	High Purity, base deactivated	10%	
Luna® PFP [c] **(Phenomenex)**	15 cm × 4.6 mm	3µm	100 Å	1.5 - 9.0	400 m^2 g^{-1}	Ultra-pure, metal-free silica (99.99 % purity)	11.5	
Luna ® Phenyl-Hexyl [c] **(Phenomenex)**	15 cm × 4.6 mm	3µm	100 Å	1.5 - 9.0	400 m^2 g^{-1}	Ultra-pure, metal-free silica (99.99 % purity)	11.5%	
Nucleosil® C_{18} [d] **(Teknokroma)**	10 cm × 4.6 mm	3µm	100 Å	2–8	350 m^2 g^{-1}	Porous Silica	15%	
Synergi® Hydro-RP [c] **(Phenomenex)**	15 cm × 4.6 mm	4 µm	80 Å	1.5 - 7.5	475 m^2 g^{-1}	Fully Porous Silica	19 %	

Table 5.1. *(Continued) Physicochemical Properties of the Tested Columns.*

Ascentis® Express F5 [e] (Supelco Analytical)	10 cm × 4.6 mm	2.7 µm	90 Å	1 - 9	150 $m^2\ g^{-1}$	Fused-Core particle platform	2-8	
Ascentis® Express C_{18} [e] (Supelco Analytical)	10 cm × 4.6 mm	2.7 µm	90 Å	2 - 9	150 $m^2\ g^{-1}$	Fused-Core particle platform	2-9	
Ascentis® Express [e] Phenyl-Hexyl (Supelco Analytical)	10 cm × 4.6 mm	2.7 µm	90 Å	2 - 9	150 $m^2\ g^{-1}$	Fused-Core particle platform	2-9	
Kinetex® Phenyl-Hexyl[c] (phenomenex)	15 cm × 4.6 mm	2.6 µm	100 Å	1.5 - 8.5	200 $m^2\ g^{-1}$	Core-Shell particles	11%	
Kinetex® PFP [c] (phenomenex)	15 cm × 4.6 mm	2.6 µm	100 Å	1.5 - 8.5	200 $m^2\ g^{-1}$	Core-Shell particles	9%	
Kinetex® C_{18} [c] (phenomenex)	15 cm × 4.6mm	2.6 µm	100 Å	1.5 - 8.5	200 $m^2\ g^{-1}$	Core-Shell particles	8%	

Reference: [a] *http 7,* [b] *http 8,* [c] *http 6,* [d] *http 9,* [e] *http 1*

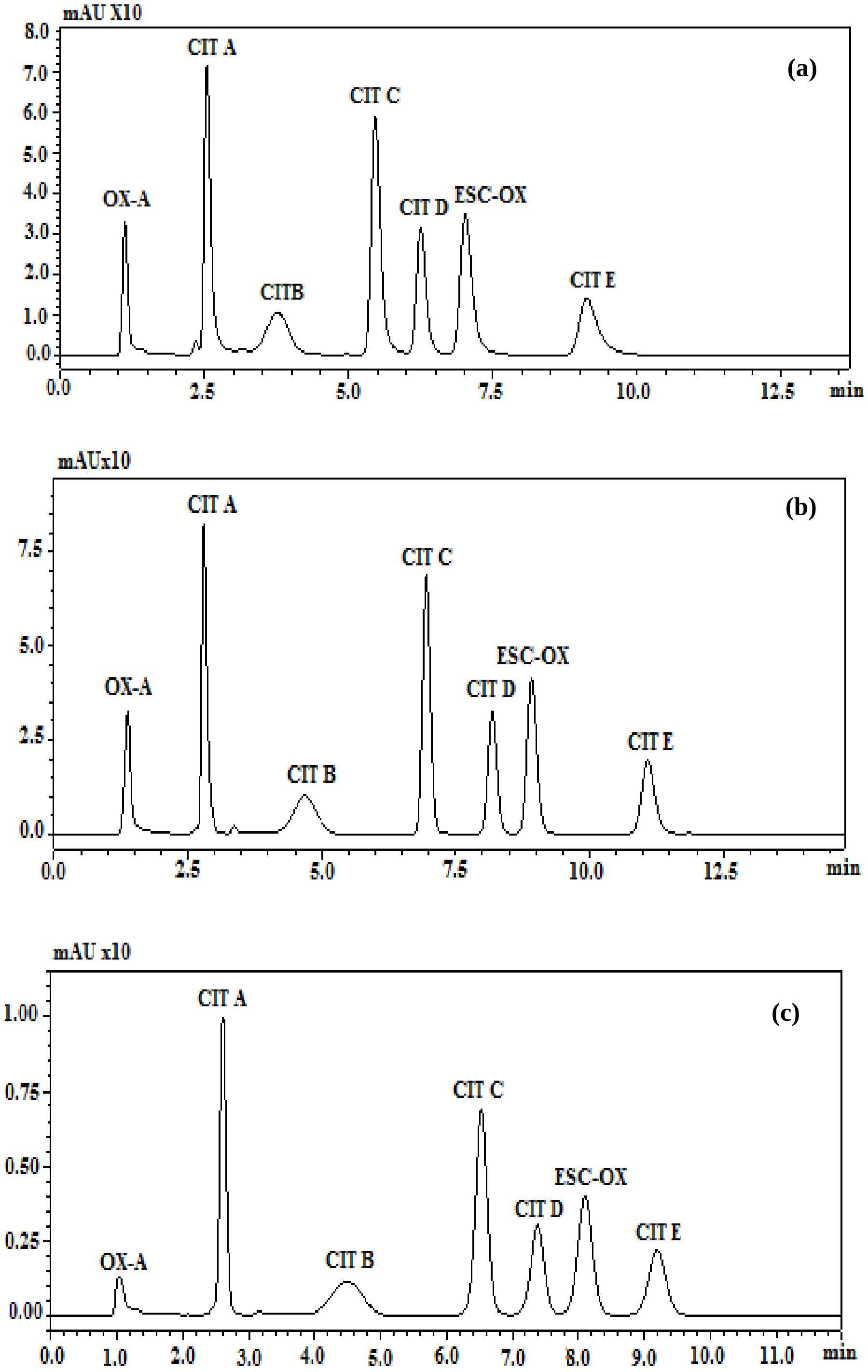


Figure 5.12. *Chromatogram of ESC-OX and its Impurities by Using;(a) Nucleosil ® 100 C_{18} (3 µm, 10 × 0.46 cm), (b) Synergi Hydro-RP ® (4 µm, 15 × 0.46 cm), (c) Fluophase RP ® (3 µm, 10 cm × 4.6mm).*

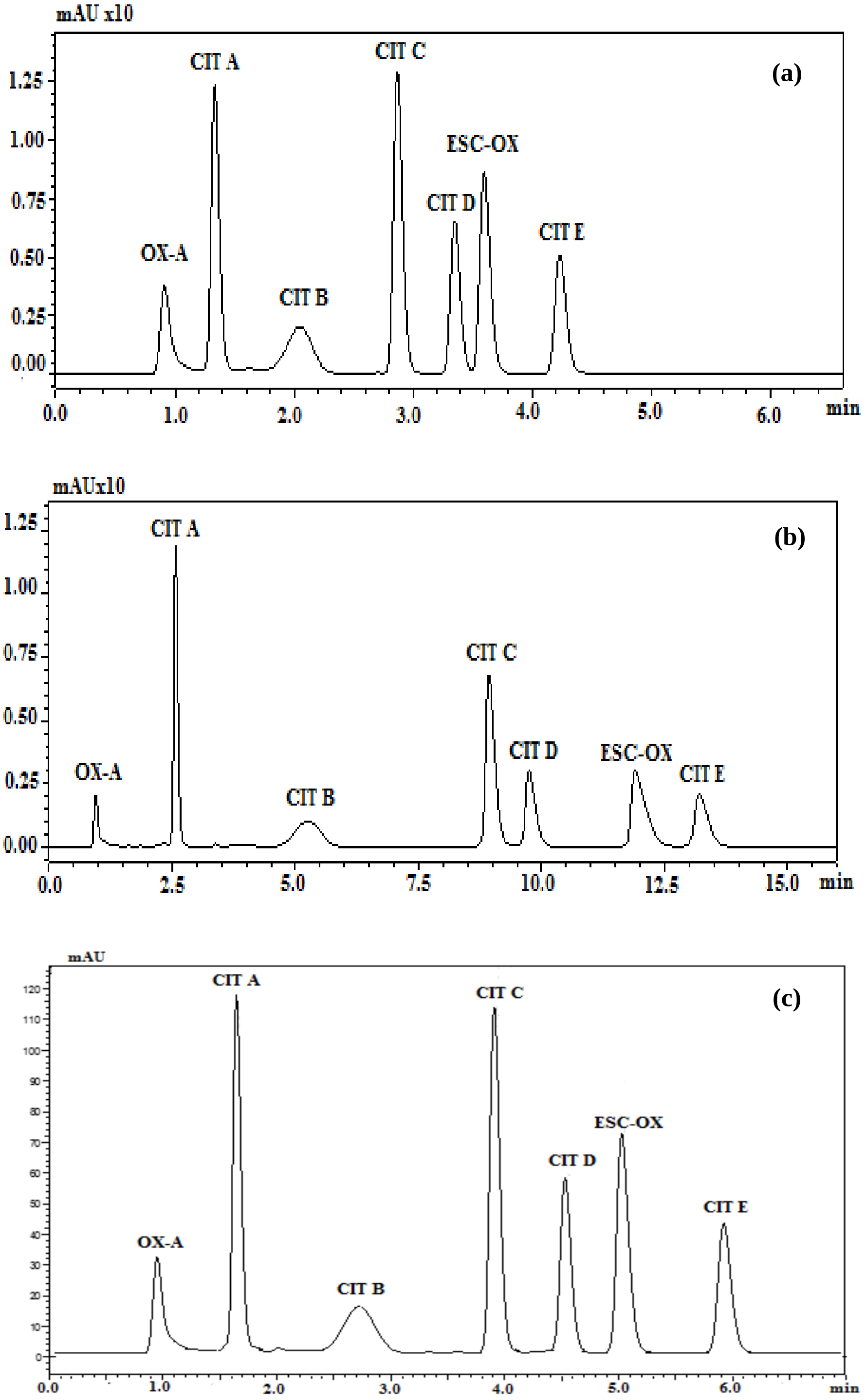


Figure 5.13. *Chromatogram of ESC-OX and its Impurities by Using;(a) Ascentis® Express C_{18} (2.7 μm, 10 cm × 4.6mm), (b) Ascentis® Express F5 (2.7 μm, 10 cm × 4.6mm), (c) Ascentis® Express Phenyl-hexyl (2.7 μm, 10 cm × 4.6mm).*

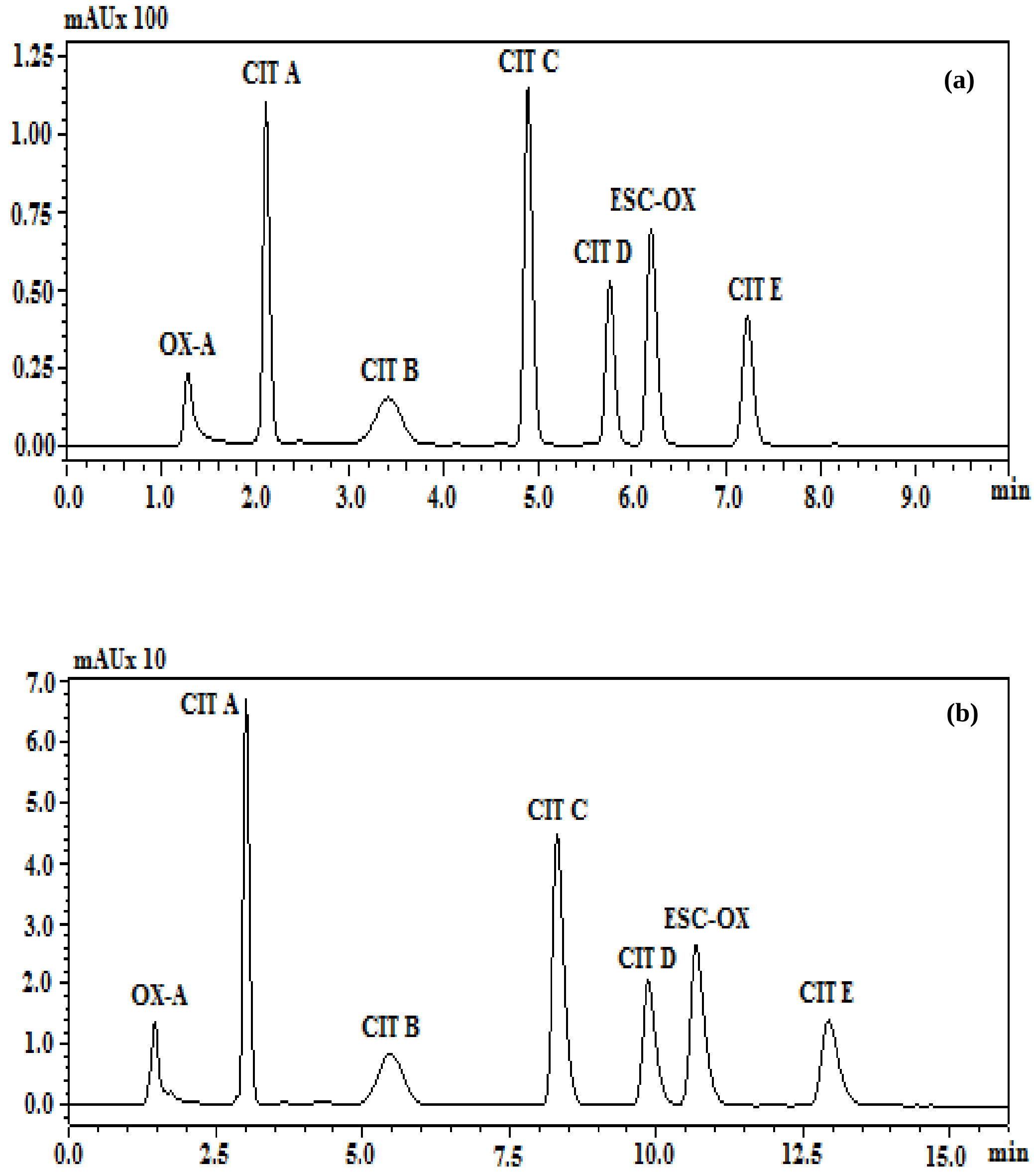


Figure 5.14. *Chromatogram of Standard Solution of ESC-OX and its Impurities by Using(a) Kinetex ® C_{18} (2.6 µm, 150 × 4.6 mm) Core-Shell Column, (b) Inertsil® C_{18} (3 µm, 150× 4.6 mm) Fully Porous Column.*

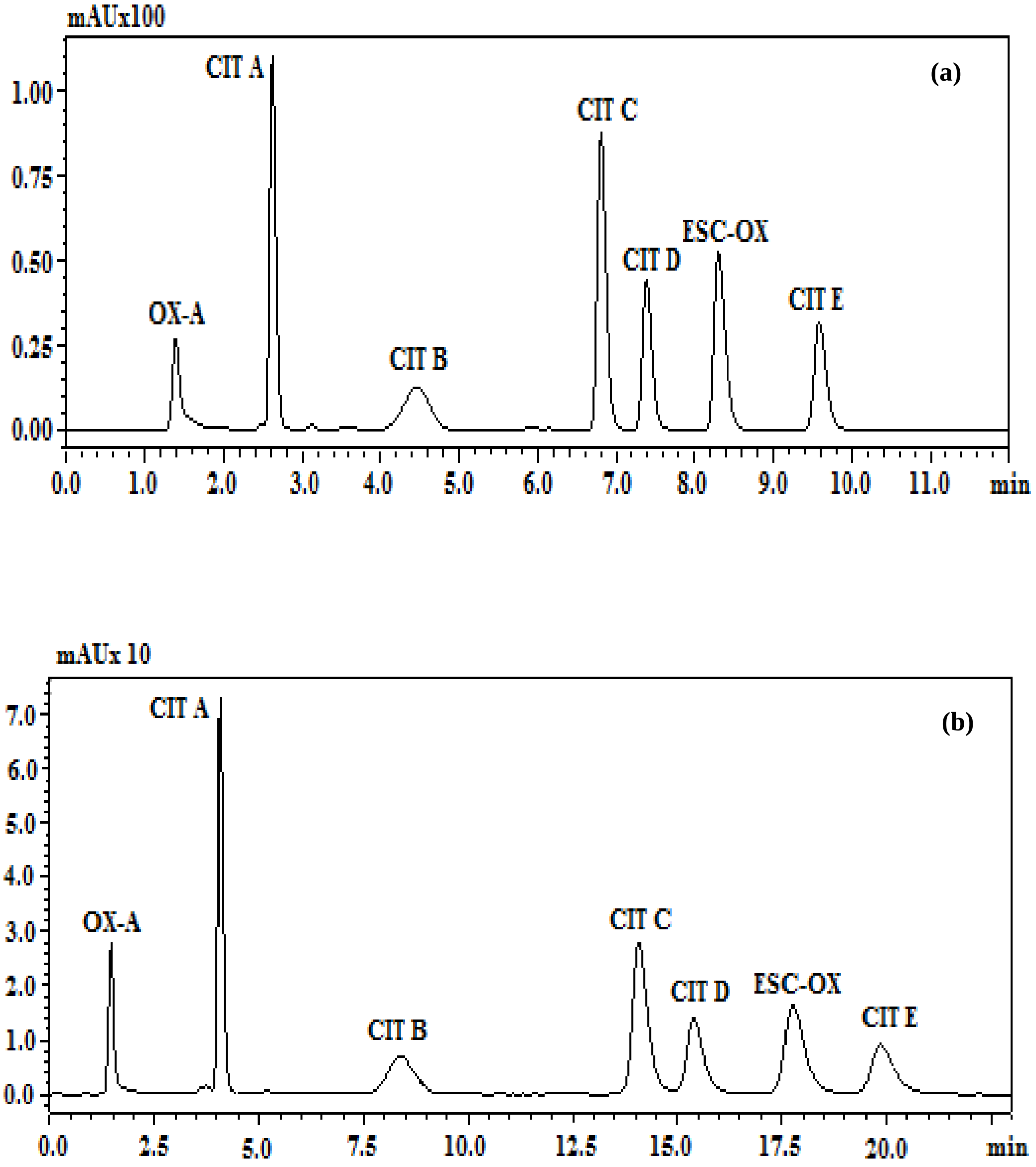


Figure 5.15. *Chromatogram of Standard Solution of ESC-OX and its Impurities by (a) Kinetex ® PFP (2.6 µm, 150 × 4.6 mm) Core-Shell Column, (b) Luna® PFP (2.6 µm, 150 × 4.6 mm) Fully Porous Column.*

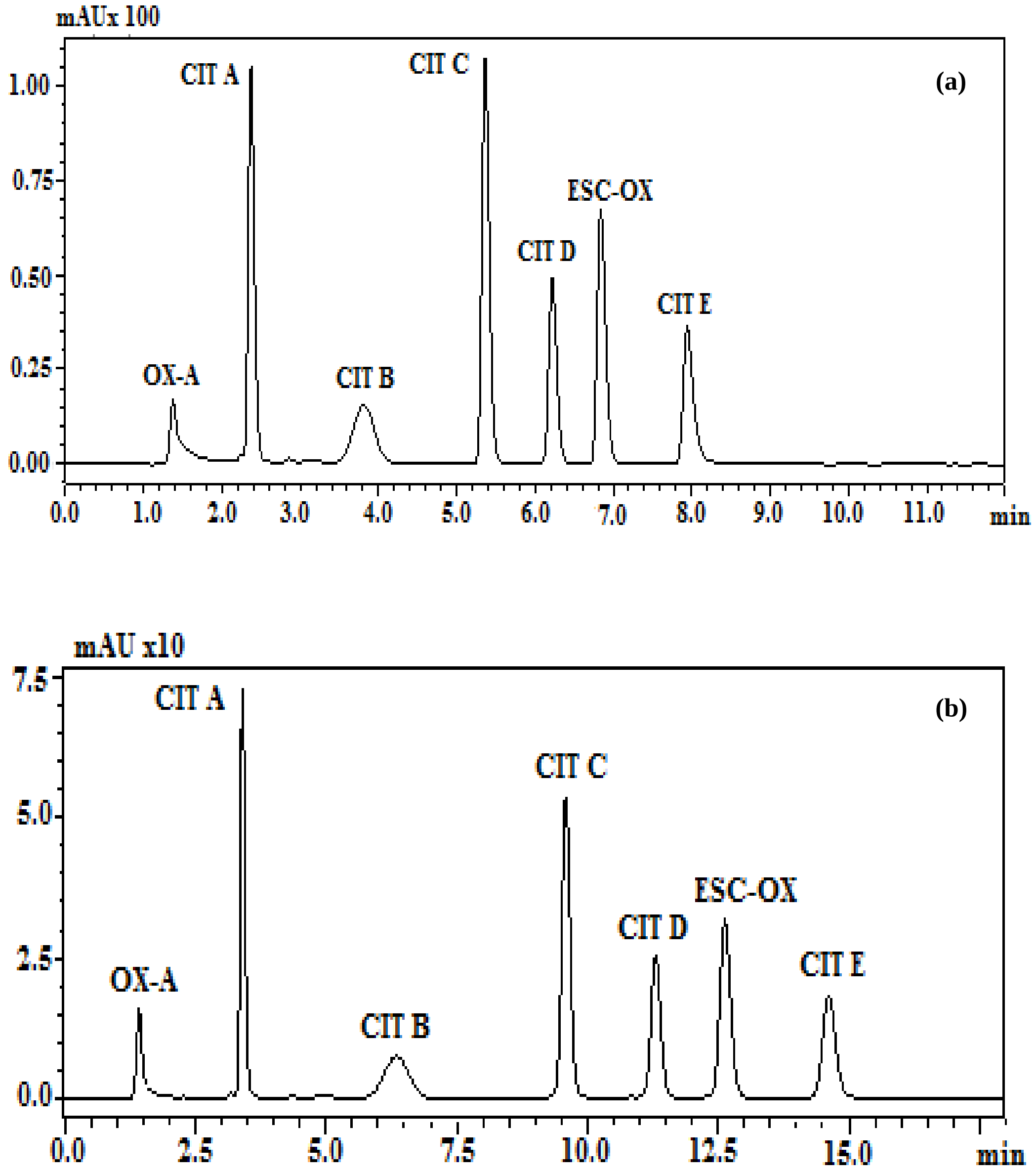


Figure 5.16. *Chromatogram of Standard Solution of ESC-OX and its Impurities by using (a) Kinetex ® Phenyl- Hexyl (2.6 µm, 150 × 4.6 mm) Core-Shell Column. (b) Luna® Phenyl- Hexyl (2.6 µm, 150 × 4.6 mm) Fully Porous Column.*

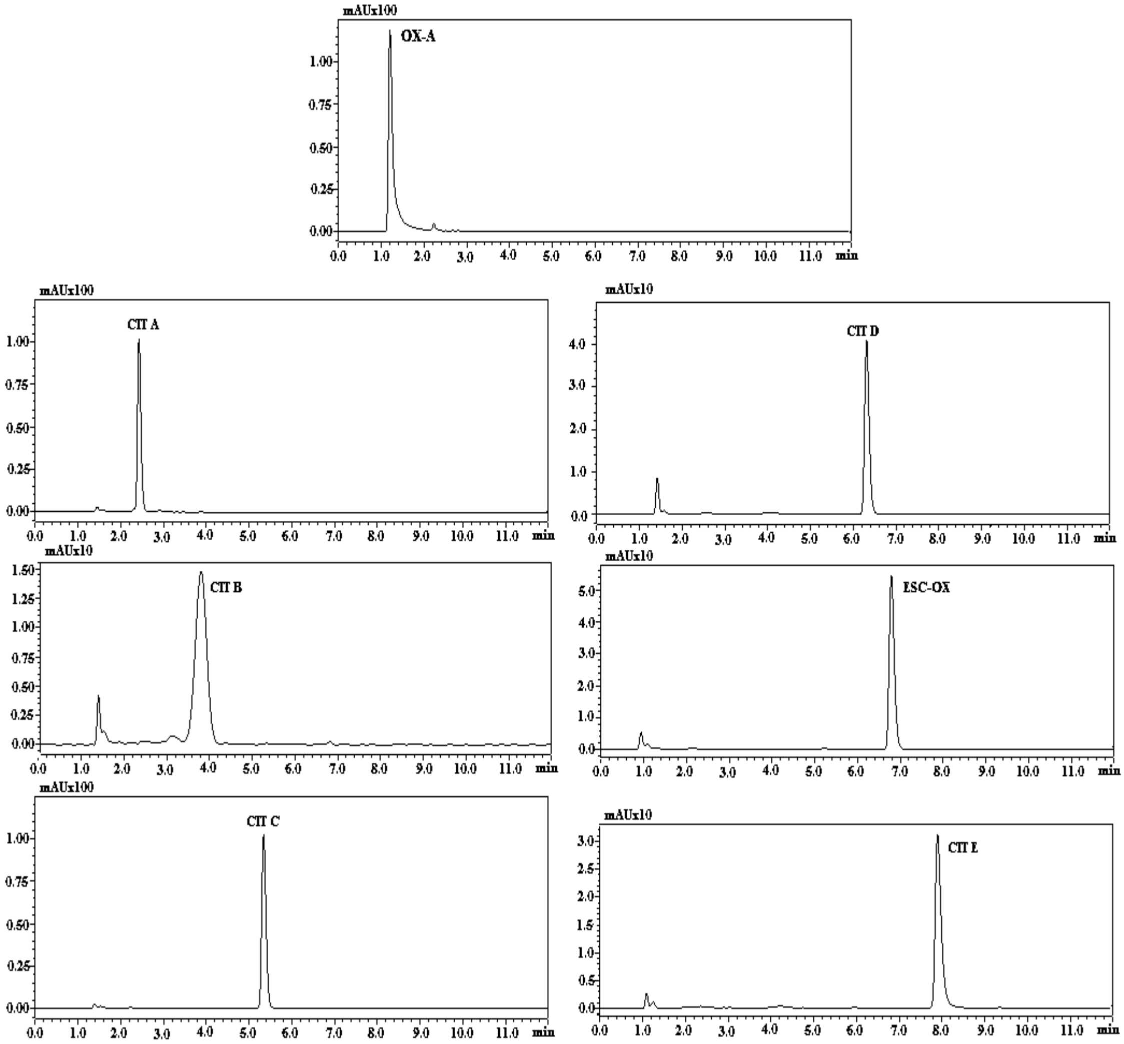


Figure 5.17. *Chromatogram of Each Standard Solution of ESC-OX (11.78 µg mL^{-1}), CIT A (12.05 µg mL^{-1}), CIT B (10.4 µg mL^{-1}), CIT C (10.1µg mL^{-1}), CIT D (10.4 µg mL^{-1}) and CIT E (10.3 µg mL^{-1}) by Kinetex ® Phenyl- Hexyl (2.6 µm, 150 × 4.6 mm) Core-Shell Column.*

After evaluation of all columns' performances via SST (Figure 5.12.-5.16), only six columns with different stationary phases (C_{18}, phenyl-hexyl and PFP) and different particle characteristics (conventional and core-shell) were selected for further examination studies. C_{18} is the most commonly available stationary phase in LC laboratories; this phase (C_{18}) was used to compare the results obtained from the other two phases. All columns were selected from the same manufacturer (http 6), in order to avoid any variation originating from the quality of silica or production method, except of conventional C_{18} column. They provide different chemically bond stationary phase and demonstrate some changes in the selectivity and resolution by using the same mobile phase composition.

The mechanism by which stationary phase hold the passing solute molecule is a complex phenomenon. This mechanism is affected by the several diverse intermolecular interactions between the separated solute, mobile phase and the stationary phase which include adsorption and partitioning (Kaczmarski, Prus and Kowalska, 2000, p. 57-58.). Also, column length, particle size and packing methods may affect the efficiency of separation.

The selection of PFP column was based on the presence of fluorine (F) in the stationary phase which contributes to improve in the retention and selectivity of the halogenated, polar compounds (http 6). Also, this stationary phase provides π-π interaction, steric and shape/size selectivity (Brindle and Albert, 1997, p. 3-20; Sadek and Carr, 1984, p. 25-41; Yamamoto and Rokushika,2000, p. 141–151). Since, ESC-OX and its selected impurities contain F atom in their structure, it was expected to get separation with good resolution and selectivity. PFP stationary phase is a Lewis acid (electron acceptor), it can contribute for the π-π interaction with electron donor analyte. In addition, dipole-dipole interaction between the solutes and the stationary phase, this interaction comes from the electronegative F atom of stationary phase and positively charged solutes (Przybyciel, 2005, p. 554-565). All these interactions result in increased retention relative to C_{18} stationary phase. Beside these interactions this stationary phase provides hydrogen bond and hydrophobic interaction. The elution time of the solutes may be related to the molecular size, increasing hydrophobicity and adsorptive interaction.

The selection of phenyl-hexyl stationary phase was based on having alternative selectivity for aromatic and hydrophobic interaction. This selectivity rise from the presence of six-carbon chain and aromatic phenyl group. Phenyl is considered as a Lewis base (electron donor) (http 6), a good source of selectivity for the amine and heterocyclic containing aromatic compound and π-π interaction (Goss, 1998, p. 267–271; http 11).

The elution order of all compounds was the same in all of the columns; only a slight superiority of phenyl-hexyl stationary phase was observed in term of resolution. This may be originated from different multisite interaction of the compounds with the phenyl ring. Relatively long retention in the PFP stationary phase in comparison with the other two stationary phase is due to presence of F atom, which can form dipole-dipole interactions and H-bonds with the solutes, resulting in slightly longer retention of the solutes. Also, the longer retention may be due to larger size of fluorinated-phenyl ring than C_{18} and phenyl-hexyl ligands of the stationary phase (Petruczynik et al, 2015, p. 943–950; Summers and Fountain, 2012, p. 1-4), which result in increased steric retention of the solutes. The peak symmetry was relatively better by using phenyl-hexyl stationary phase specially for CIT B.

In term of separation performance, system suitability parameters were compared for all of the compounds and six best-performing columns. The comparison is represented in Figure 5.18 to Figure 5.23. The values were calculated for each parameter, and they are shown in details in section 5.2.4.

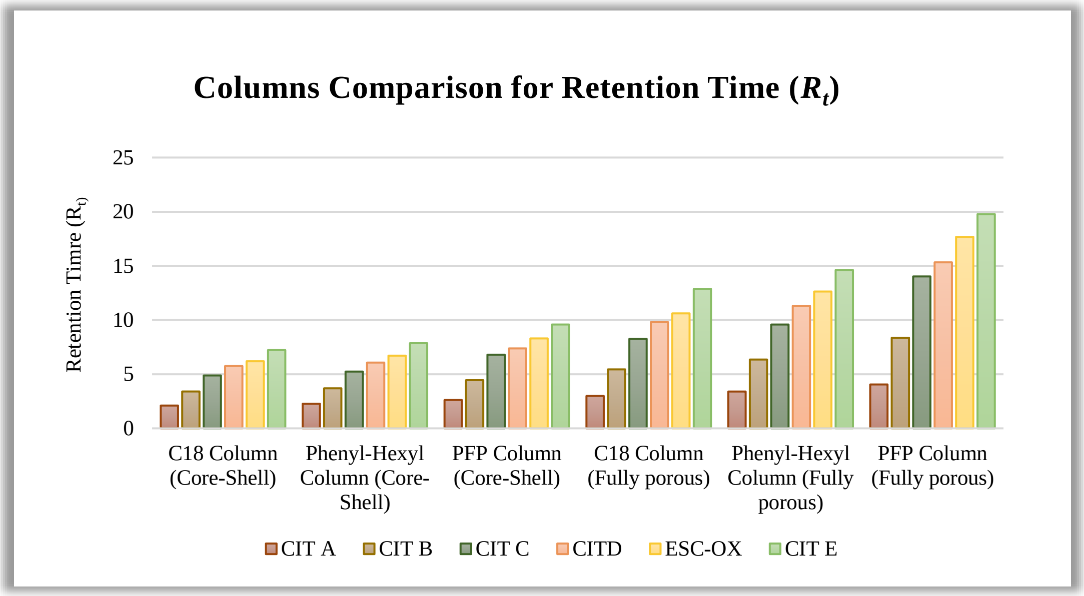


Figure 5.18. *Columns Comparison for Retention Time (R_t).*

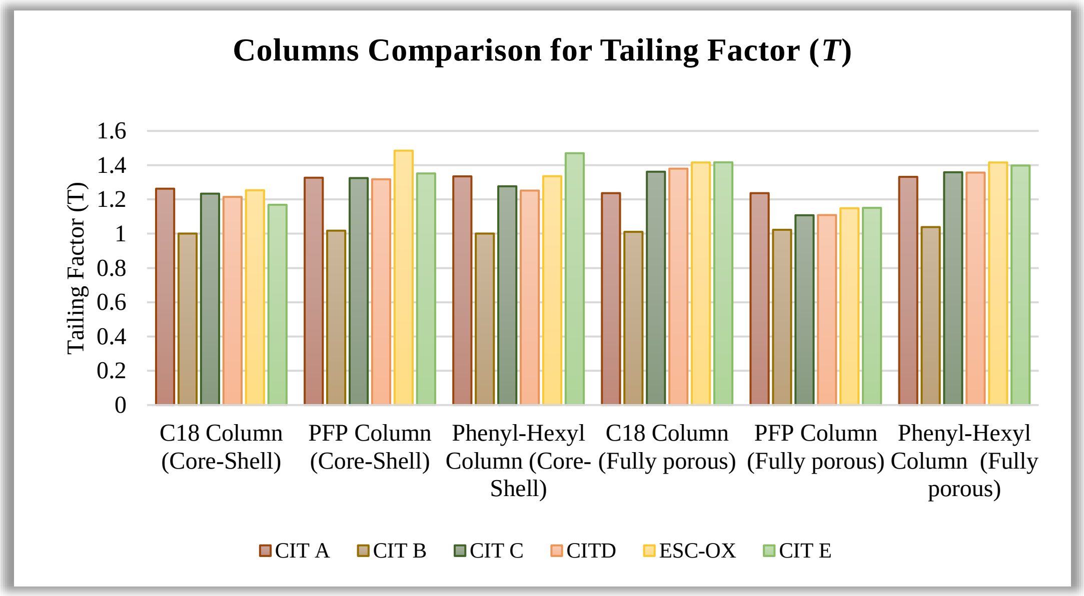


Figure 5.19. *Columns Comparison for ESC-OX Tailing Factor (T).*

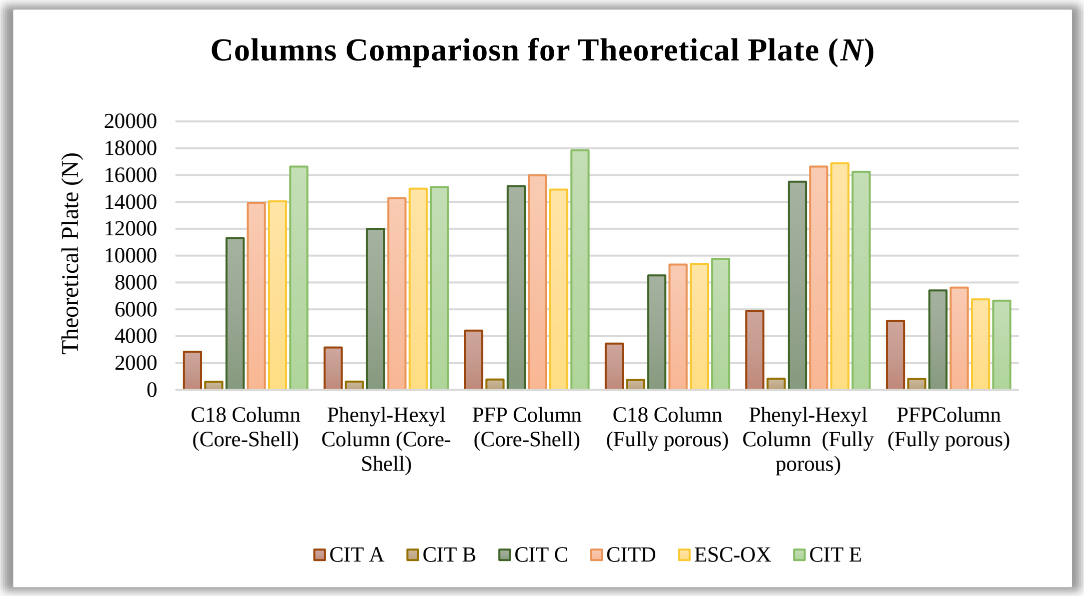


Figure 5.20. *Columns Comparison for Theoretical Plate (N).*

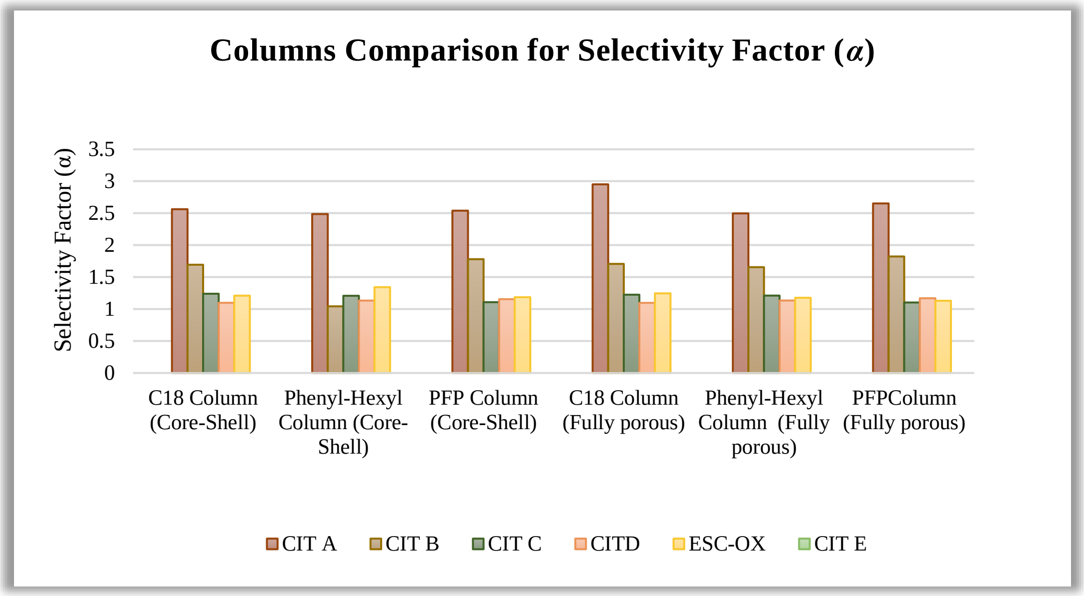


Figure 5.21. *Columns Comparison for Selectivity Factor (α).*

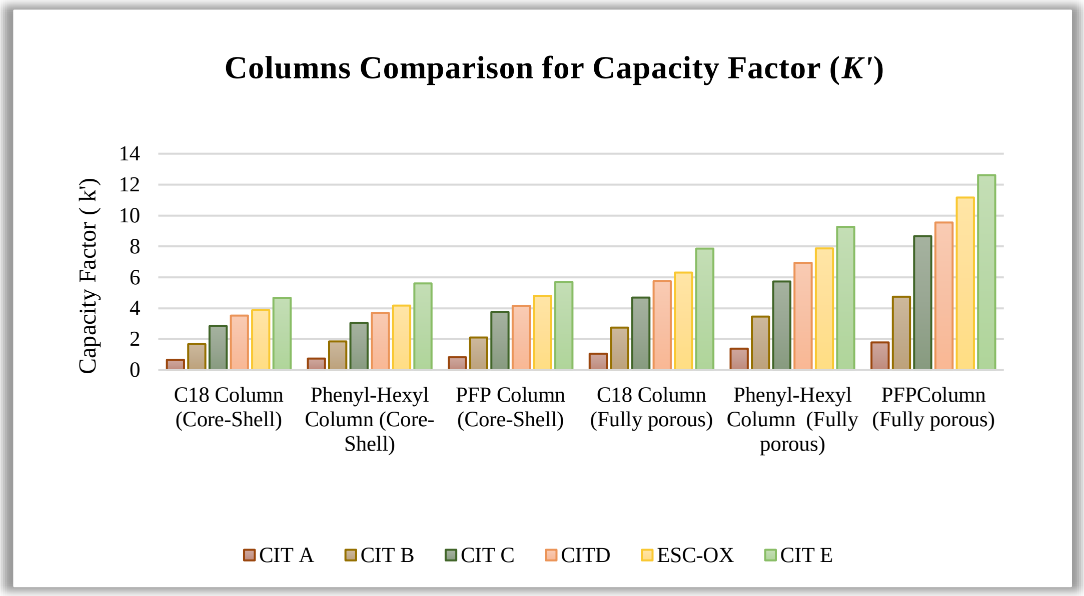


Figure 5.22. *Columns Comparison for ESC-OX Capacity Factor (K′).*

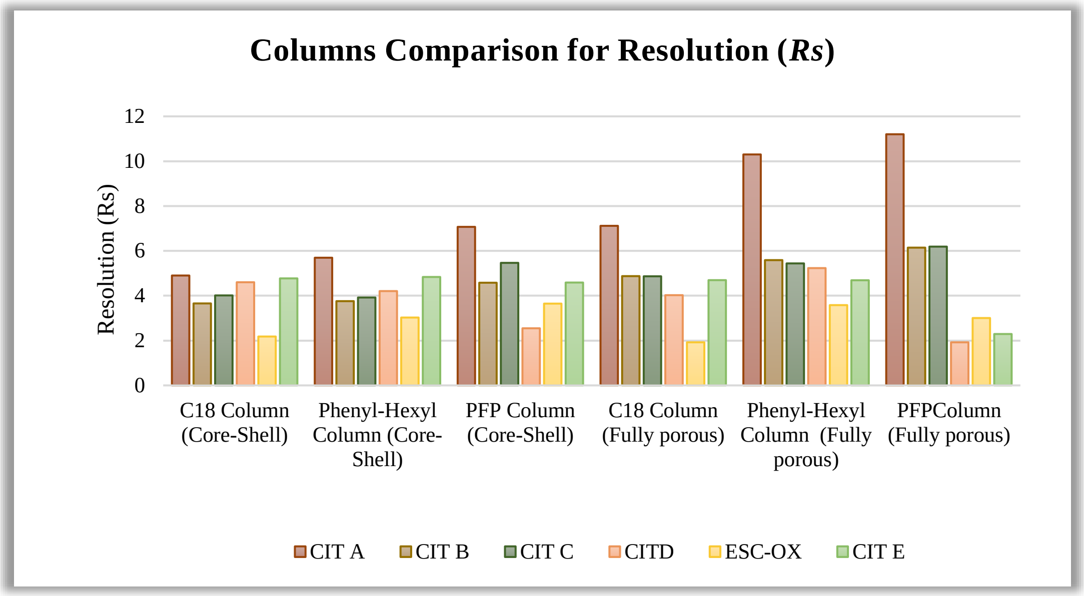


Figure 5.23. *Columns Comparison for Resolution* (*R_s*).

As shown above, the core-shell columns form the highest number of theoretical plates with preference of PFP column, the retention times were almost similar in the three core-shell columns. Comparing, the capacity factor (K') and selectivity of columns (α) the best results obtained by using fully porous columns that provide suitable analysis time with good selectivity but the peaks was slightly broader. The difference in the tailing factor of all columns was not great and all values were within the recommended value (T < 2). The resolution values between the CIT D, ESC-OX and CIT E were close to each other in most columns with the slight superiority of phenyl-hexyl column. In concern to the entire columns performance, all core-shell columns provide excellent separation of the ESC-OX and its impurities in regard to analysis time, theoretical plate and resolution.

The dissimilarity factor or orthogonality of core-shell column with the fully porous column was evaluated by using regression analysis and correlation coefficients (r) between the K' of all analytes. The low correlation coefficient indicates the high orthogonality and vice versa (Van Gyseghem et al, 2003, p. 77-93). A plot of capacity factors attained from Inertsil® C_{18} fully porous column versus the capacity factors attained from Kinetex ® C_{18} core-shell column indicates a high degree of correlation (r = 0.99959), i.e. high degree of similarity as shown in Figure 5.24 a. By comparing the correlation between capacity factors attained from Luna® PFP fully porous column versus the capacity factors attained from Kinetex ® PFP core-shell column and Luna® phenyl-hexyl fully porous column versus the *K*' attained from Kinetex ® phenyl-hexyl core-shell column the result shows that are (r = 99872) and (r = 0.99110), respectively (Figure 5.24 b, c). Accordingly, it can be concluded that the similarity in the selectivity between core-shell column and fully porous column for ESC-OX and its impurities order as following; C_{18} > PFP > Phenyl-Hexyl.

The orthogonality of stationary phase chemistry of conventional C_{18} with the PFP and phenyl-hexyl stationary phases was compared by the same way and the correlation coefficient between the C_{18} with the PFP and C_{18} with phenyl-hexyl stationary phases indicates that similarity of PFP column selectivity is near to the C_{18} more than the phenyl-hexyl column. The correlation coefficients are (r = 0.9970 and r = 0.9921) (Figure 5.25). The result of orthogonality confirms the slight superiority of phenyl-hexyl column in the selectivity for the ESC-OX and it impurities separation over the conventional C_{18} and PFP. However, there is insignificant dissimilarity between the C_{18} traditional column and the other two stationary phases.

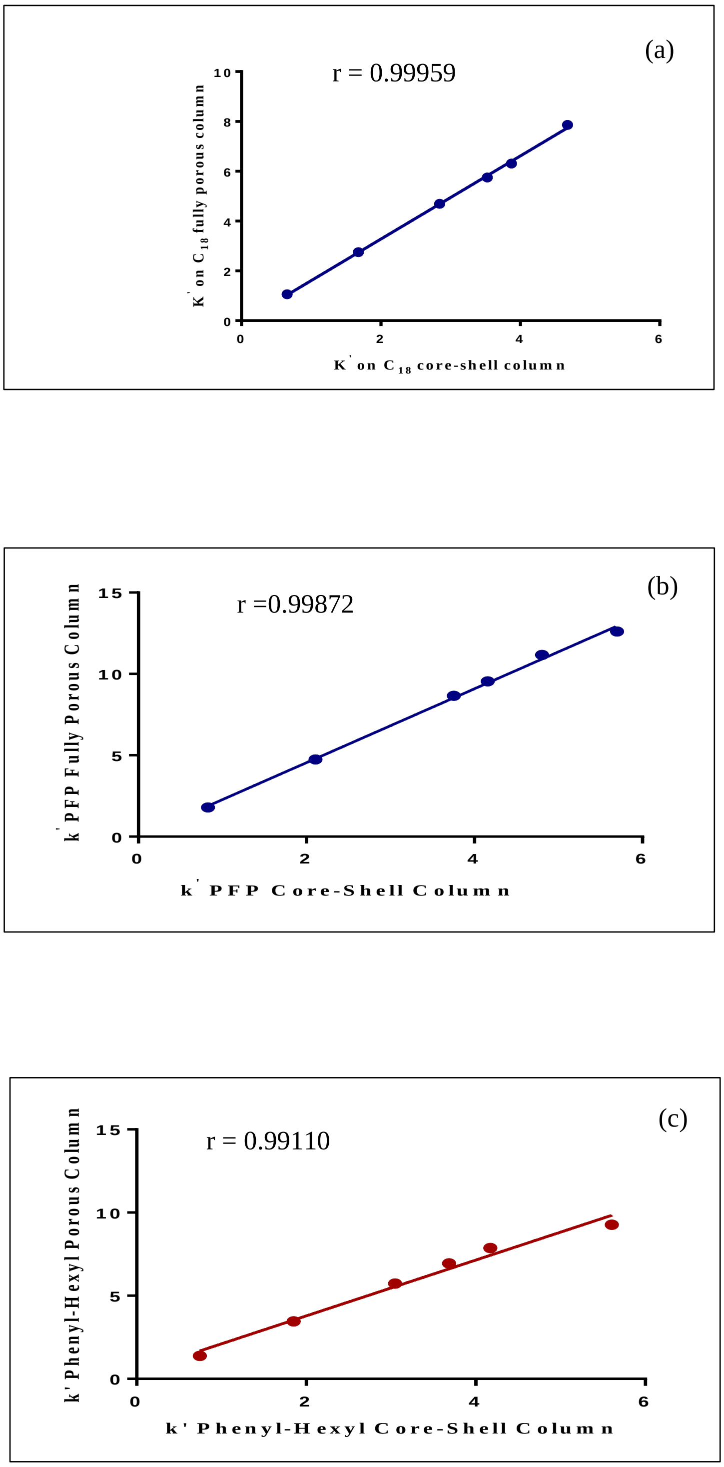


Figure 5.24. *K' of ESC-OX and Its Impurities on (a) C_{18} Fully Porous Column Versus C_{18} Core-Shell Column, (b) PFP Fully Porous Column Versus PFP Core-Shell Column, (c)Phenyl-Hexyl Fully Porous Column Versus Phenyl-Hexyl Core-Shell Column.*

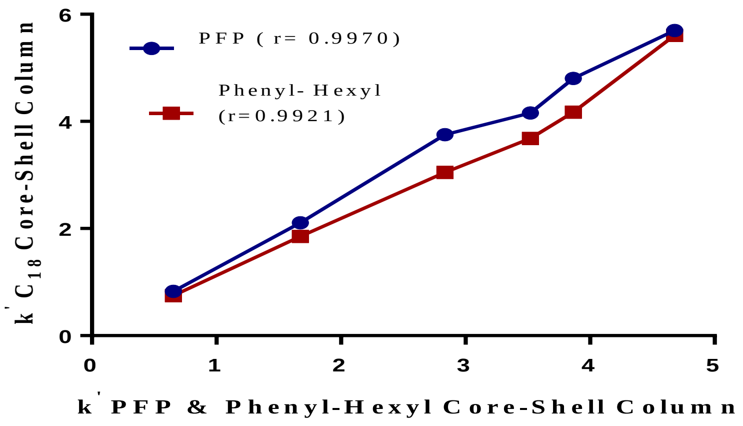


Figure 4.25. *K' of ESC-OX and Its Impurities on C_{18} Column Versus PFP and Phenyl-Hexyl Core-Shell Column Versus PFP Column.*

5.2.3. Effect of other parameters

The effect of flow rate is relatively small on the resolution, but it affects the overall analysis time and last peak's tailing. Flow rate 1.1, 1.2 and 1.3 mL min^{-1} were tried. The reasonable analysis time with best peak shape was acquired 1.2 mL min^{-1} flow rate.

Temperature should be kept constant during analysis time because it affects selectivity. Column oven temperature 35, 40 and 45 °C were changed. Column oven temperature kept at 40 °C because no valuable decrease in run time was observed at higher temperature. This temperature was best for maintaining low viscosity of the mobile phase and back pressure, providing fast flow rate and lower the analysis time. Relatively high temperature increases efficiency and mass transfer. In addition, two buffer concentrations were tried (25 and 50 mM. 50 mM) was selected because the peaks were sharper especially CIT B. The values and chromatograms related to the parameters changes were shown in the section 5.2.5.4. These optimized conditions were selected because all the peaks are symmetrical and well resolved in an acceptable run time. The selected conditions are summarized in Table 5.2.

Table 5.2. *Final Chromatographic Conditions for Analysis of ESC-OX and Its Impurities.*

Parameters	Value
Buffer and pH	Phosphate buffer pH 3.5 (50 mM)
Mobile phase composition	Buffer: ACN: MeOH: water (50:25:5:20) *v/v/v/v*
Column temperature	40°C
Flow rate	1.2 mL min^{-1}
Injection volume	10 µL
Wavelength	210 nm

5.2.4. SST

The analysis of ESC-OX and its impurities under optimized conditions resulted in good separation, according to SST results. System suitability parameters were determined to confirm the suitability of the developed method for application in the quality control laboratories. SST was calculated according to the USP 30, 2006 for the six columns. SST results are given in Table 5.3-5.8. The number of theoretical plates was lower than the recommended value only for CIT B for all of the columns. The recommended values for each parameter was taken from (USP 30, 2006, p. 2287-2288 and Thompson and LoBrutto, 2007, p. 658).

Table 5.3. *SST for Kinetex® C_{18} Core-Shell Column.*

Parameters	**CIT A**	**CIT B**	**CIT C**	**CIT D**	**ESC-OX**	**CIT E**	**Recommended value**
N	3189.13	803.68	12157.45	14817.70	14472.58	17438.25	>2000
t_R **(min)**	2.105	3.404	4.882	5.753	6.194	7.228	
T	1.263	1.001	1.234	1.215	1.254	1.169	< 2
A_s	1.255	1.004	1.217	1.202	1.239	1.160	0.95< A_S< 1.2
K'	0.655	1.678	2.843	3.526	3.872	4.678	1< K'< 10
R_s	4.901	3.656	4.013	4.601	2.180	4.773	>2
α	2.562	1.694	1.240	1.098	1.210	-	>1
RSD% of t_R	0.41	0.34	0.19	0.20	0.19	0.30	<1

Table 5.4. *SST for Inertsil ODS-3® C_{18} Fully Porous Column.*

Parameters	**CIT A**	**CIT B**	**CIT C**	**CIT D**	**ESC-OX**	**CIT E**	**Recommended value**
N	3682.75	1006.07	9758.81	10339.27	10900.16	11265.25	>2000
t_R ***(min)***	2.992	5.443	8.265	9.802	10.614	12.865	
T	1.237	1.011	1.362	1.380	1.416	1.417	< 2
A_s	1.230	1.012	1.326	1.343	1.370	1.364	0.95< A_S< 1.2
K'	1.060	2.748	4.691	5.75	6.309	7.859	1< K' < 10
R_s	7.117	4.874	4.866	4.025	1.922	4.695	>2
α	2.952	1.707	1.225	1.097	1.246	-	>1
RSD% of t_R	0.19	0.25	0.29	0.33	0.32	0.27	<1

Table 5.5. *SST for Kinetex® PFP Core-Shell Column.*

Parameters	**CIT A**	**CIT B**	**CIT C**	**CIT D**	**ESC-OX**	**CIT E**	**Recommended value**
N	4415.28	769.69	15178.22	15987.17	14922.74	17851.06	>2000
t_R ***(min)***	2.618	4.446	6.802	7.380	8.302	9.586	
T	1.327	1.018	1.325	1.318	1.485	1.352	< 2
A_s	1.297	1.023	1.293	1.290	1.447	1.314	0.95<A_S< 1.2
K'	0.829	2.107	3.754	4.159	4.805	5.700	1<K'< 10
R_s	7.071	4.580	5.465	2.547	3.648	4.588	>2
α	2.540	1.781	1.108	1.155	1.186	-	>1
RSD% of t_R	0.187	0.214	0.239	0.244	0.367	0.251	<1

Table 5.6. *SST for Luna® PFP Fully Porous Column.*

Parameters	CIT A	CIT B	CIT C	CIT D	ESC-OX	CIT E	Recommended value
N	5288.87	808.78	7407.11	7798.72	6884.89	6839.233	>2000
t_R ***(min)***	4.051	8.363	14.024	15.326	17.676	19.775	
T	1.332	1.0389	1.359	1.357	1.416	1.398	< 2
As	1.294	1.034	1.320	1.319	1.374	1.360	0.95<A_S< 1.2
K'	1.789	4.747	8.655	9.547	11.163	12.609	1< *K'*< 10
Rs	11.202	6.145	6.190	1.922	3.005	2.292	>2
α	2.653	1.823	1.103	1.169	1.130	-	>1
RSD% of t_R	0.324	0.360	0.435	0.422	0.469	0.445	<1

Table 5.7. *SST for Luna® Phenyl-Hexyl Fully Porous Column.*

Parameters	CIT A	CIT B	CIT C	CIT D	ESC-OX	CIT E	Recommended value
N	5878.93	834.011	15508.38	16635.45	16877.16	16249.16	>2000
t_R ***(min)***	3.399	6.353	9.585	11.307	12.632	14.620	
T	1.237	1.023	1.108	1.109	1.149	1.151	< 2
As	1.214	1.010	1.105	1.105	1.147	1.140	0.95< A_S < 1.2
K'	1.386	3.461	5.731	6.941	7.871	9.266	1< *K'* < 10
Rs	10.301	5.588	5.441	5.232	3.581	4.689	>2
α	2.497	1.656	1.211	1.134	1.177	-	>1
RSD% of t_R	0.158	0.286	0.354	0.336	0.355	0.348	<1

Table 5.8. *SST for Kinetex® Phenyl-Hexyl Core-Shell Column.*

Parameters	CIT A	CIT B	CIT C	CIT D	ESC-OX	CIT E	Recommended value
N	3155.52	614.36	11998.06	14284.38	14990.14	15107.56	>2000
t_R ***(min)***	2.272	3.697	5.242	6.072	6.712	7.861	
T	1.335	1.001	1.277	1.252	1.336	1.470	< 2
As	1.310	1.003	1.257	1.241	1.315	1.394	0.95<A_S< 1.2
K'	0.745	1.853	3.048	3.684	4.173	5.606	1< *K'*< 10
Rs	5.695	3.758	3.920	4.207	3.027	4.836	>2
A	2.487	1.043	1.208	1.133	1.343	-	>1
RSD% of t_R	0.067	0.041	0.029	0.025	0.031	0.048	<1

5.2.5. Validation of HPLC method

The validation of the developed method for the analysis of the target substances is based on the recommendations outlined in the ICH guideline on the validation of analytical methods. Validation was carried out in three core-shell columns because the best SST with suitable retention time.

5.2.5.1. Linearity

Linearity of the developed method was examined by using the three core-shell columns, ESC-OX in the range of (0.0753 - 117.80 µg mL^{-1}), CIT A (0.0603- 120.5 µg mL^{-1}), CIT B (0.0520- 104.0 µg mL^{-1}), CIT C (0.0505- 101.0 µg mL^{-1}), CIT D (0.052- 104.0 µg mL^{-1}) and CIT E (0.0515-103.0 µg mL^{-1}). The calibration curve for ESC-OX and its impurities was selected in following range ESC-OX (0.236- 70.70 µg mL^{-1}), CIT A (0.0241- 72.30 µg mL^{-1}), CIT B (0.208-62.40 µg mL^{-1}), CIT C (0.202- 60.60µg mL^{-1}), CIT D (0.208- 62.40 µg mL^{-1}) and CIT E (0.206- 61.80 µg mL^{-1}). The selection of calibration curve was depending on getting good peak shape and avoiding column overloading.

Linearity was investigated within intra-day and inter-day, and validation parameters such as slope, intercept, 95% CI limits and r were calculated by using statistical software. It has been observed that all of the compounds that are assayed gave linear results at the specified concentration range and obey the Lambert-Beer Law. LOD and LOQ values were calculated by using the intensity of the signal in the chromatograms and the closest noise intensity ratios. For each column, the (S/N) value obtained from the chromatograms is multiplied by 3 for the LOD and 10 for the LOQ. Statistical data on the intra-day and inter-day linearity of the method for the three core-shell columns are given in Table 5.9 - 5.14. In addition, possible differentiations between the analytical columns were examined by one way ANOVA test. Statistical calculations were done with Graph Pad Prism v 6.0 b software and the result show no significant statistical differences among the analytical columns for all analytes.

Table 5.9. *Statistical Evaluation of Linearity Analysis of CIT A Standard Solution by Using C_{18}, PFP and Phenyl-Hexyl Column.*

	Inter-days (n=27)		
Range (µg mL^{-1})	0.0241-72.30		
Column	**C_{18} column**	**PFP column**	**phenyl-hexyl column.**
Slope ± SD	41326 ± 32.75	40898 ± 54.94	36667 ± 52.60
Intercept ± SD	-4896 ± 995.1	1874 ± 1669	11214 ± 1598
r^2	1.000	1.000	0.9999
95 % CI of Slope	41258 to 41393	-1564 to 5312	36558 to 36775
95 % CI of Intercept	-6946 to -2847	-0.1301 to 0.0382	7922 to 14506
LOD (µg mL^{-1})	0.004	0.013	0.005
LOQ (µg mL^{-1})	0.014	0.038	0.016
ANOVA	F (1.004, 8.035) = 3.276, P= 0.1077		

Table 5.10. *Statistical Evaluation of Linearity Analysis of CIT B Standard Solution by Using C_{18}, PFP and Phenyl-Hexyl Column.*

	Inter-days (n=27)		
Range (µg mL^{-1})	0.208-62.40		
Column	**C_{18} column**	**PFP column**	**phenyl-hexyl column.**
Slope ± SD	23086 ± 91.56	25068 ± 93.82	24005 ± 165.8
Intercept ± SD	1435 ± 2401	-467.8 ± 2088	3304 ± 4346
r^2	0.9996	0.9996	0.9988
95 % CI of Slope	22897 to 23274	24874 to 25261	23664 to 24347
95 % CI of Intercept	-3511 to 6381	-4769 to 3833	-5649 to 12257
LOD (µg mL^{-1})	0.008	0.020	0.008
LOQ (µg mL^{-1})	0.023	0.061	0.025
ANOVA	F (1.011, 8.084) = 3.818, P= 0.0858		

Table 5.11. *Statistical Evaluation of Linearity Analysis of CIT C Standard Solution by Using C_{18}, PFP and Phenyl-Hexyl Column.*

	Inter-days (n=27)		
Range (µg mL^{-1})	0.202- 60.60		
Column	**C_{18} column**	**PFP column**	**phenyl-hexyl column.**
Slope ± SD	67498 ± 101.80	64661 ± 96.28	63780 ± 191.50
Intercept ± SD	-1067 ± 2592	-5340 ± 2452	4445 ± 4876
r^2	0.9999	0.9999	0.9998
95 % CI of Slope	67288 to 67708	64463 to 64860	63385 to 64174
95 % CI of Intercept	-6406 to 4272	-10391 to -289.6	-5598 to 14489
LOD (µg mL^{-1})	0.003	0.008	0.003
LOQ (µg mL^{-1})	0.008	0.024	0.009
ANOVA	F (1.010, 8.084) = 4.238, P= 0.0729		

Table 5.12. *Statistical Evaluation of Linearity Analysis of CIT D Standard Solution by Using C_{18}, PFP and Phenyl-Hexyl Column.*

	Inter-days (n=27)		
Range (µg mL^{-1})	0.208- 62.40		
Column	**C_{18} column**	**PFP column**	**phenyl-hexyl column.**
Slope ± SD	29412 ± 201.0	30168 ± 51.71	30544 ± 43.41
Intercept ± SD	3013 ± 5271	207.7 ± 1356	2547 ± 1138
r^2	0.9988	0.9999	0.9999
95 % CI of Slope	28998 to 29826	30062 to 30275	30455 to 30634
95 % CI of Intercept	-7844 to 13871	-2586 to 3001	201.9 to 4892
LOD (µg mL^{-1})	0.006	0.017	0.006
LOQ (µg mL^{-1})	0.018	0.050	0.019
ANOVA	F (1.019, 8.153) = 3.683, P= 0.0902		

Table 5.13. *Statistical Evaluation of Linearity Analysis of CIT E Standard Solution by Using C_{18}, PFP and Phenyl-Hexyl Column.*

	Inter-days (n=27)		
Range (µg mL^{-1})	0.206- 61.80		
Column	**C_{18} column**	**PFP column**	**phenyl-hexyl column.**
Slope ± SD	24007 ± 75.61	27162 ± 39.32	27851 ± 41.07
Intercept ± SD	274.4 ± 1964	2158 ± 1021	-5770 ± 1066
r^2	0.9998	0.9999	0.9999
95 % CI of Slope	23851 to 24163	27081 to 27243	27767 to 27936
95 % CI of Intercept	-3771 to 4319	54.51 to 4262	-7967 to -3573
LOD (µg mL^{-1})	0.007	0.018	0.007
LOQ (µg mL^{-1})	0.021	0.055	0.021
ANOVA	F (1,010, 8.080) = 4.166, P= 0.0750		

Table 5.14. *Statistical Evaluation of Linearity Analysis of ESC-OX Standard Solution by Using C_{18}, PFP and Phenyl-Hexyl Column.*

	Inter-days (n=27)		
Range (µg mL^{-1})	0.236- 70.70		
Column	**C_{18} column**	**PFP column**	**phenyl-hexyl column**
Slope ± SD	37468 ± 54.35	38535 ± 35.59	37942 ± 72.95
Intercept ± SD	35.50 ± 1615	-454.2 ± 1057	1672 ± 2167
r^2	0.9999	1.000	0.9999
95 % CI of Slope	37356 to 37580	38462 to 38609	37792 to 38092
95 % CI of Intercept	-3290 to 3361	-2632 to 1724	-2792 to 6137
LOD (µg mL^{-1})	0.006	0.017	0.006
LOQ (µg mL^{-1})	0.018	0.050	0.017
ANOVA	F (1.173, 9.384) = 4.026, P= 0.0702		

5.2.5.2. Accuracy

The accuracy of the method was tested via standard addition method; known amount of ESC-OX and its impurities were added into pre-analyzed tablet and oral solution samples. Three different concentrations, which represent 50% (low level), 100% (moderate level) and 150% (high level) were added and percentage of recovery was calculated. The tests at each level was carried out as three times for three days. The validation data related to accuracy studies are given in Table 5.15- 5.20.

Table 5.15. *Results of Accuracy Studies in Tablet Matrix by Using Phenyl-Hexyl Column.*

Compounds	Percentage of Concentration	Added (µg mL^{-1})	Found (µg mL^{-1})	Recovery %	Average Recovery
CIT A	50	2.41	2.36	98.04	98.87
	100	4.82	4.80	99.53	
	150	7.23	7.16	99.04	
CIT B	50	2.08	2.14	103.04	103.44
	100	4.16	4.34	104.23	
	150	6.24	6.43	103.06	
CIT C	50	2.02	2.02	99.80	100.2
	100	4.04	4.08	101.04	
	150	6.06	6.05	99.92	
CIT D	50	2.08	2.04	98.29	100.77
	100	4.16	4.32	103.86	
	150	6.24	6.25	100.18	
ESC-OX	50	2.35	2.33	98.73	100.17
	100	4.71	4.83	102.41	
	150	7.07	7.03	99.39	
CIT E	50	2.06	2.09	101.45	101.08
	100	4.12	4.22	102.42	
	150	6.18	6.14	99.39	

Table 5.16. *Results of Accuracy Studies in Oral Solution Matrix by Using Phenyl-Hexyl Column.*

Compounds	Percentage of Concentration	Added (µg mL^{-1})	Found (µg mL^{-1})	Recovery %	Average Recovery
CIT A	50	2.41	2.40	99.53	97.92
	100	4.82	4.66	96.65	
	150	7.23	7.055	97.58	
CIT B	50	2.08	2.11	101.24	103.10
	100	4.16	4.34	104.22	
	150	6.24	6.48	103.84	
CIT C	50	2.02	1.96	97.03	99.00
	100	4.04	4.06	100.48	
	150	6.06	6.03	99.50	
CIT D	50	2.08	2.04	97.86	98.94
	100	4.16	4.11	98.84	
	150	6.24	6.25	100.12	
ESC-OX	50	2.35	2.39	101.36	101.62
	100	4.71	4.82	102.38	
	150	7.07	7.45	105.36	
CIT E	50	2.06	2.08	100.97	101.07
	100	4.12	4.15	100.62	
	150	6.18	6.28	101.62	

Table 5.17. *Results of Accuracy Studies in Tablet Matrix by Using C18 Column.*

Compounds	Percentage of Concentration	Added (µg mL^{-1})	Found (µg mL^{-1})	Recovery %	Average Recovery
CIT A	50	2.41	2.54	105.27	104.17
	100	4.82	4.96	103.00	
	150	7.23	7.54	104.25	
CIT B	50	2.08	2.21	106.47	104.85
	100	4.16	4.35	104.45	
	150	6.24	6.47	103.62	
CIT C	50	2.02	1.99	98.29	97.06
	100	4.04	3.90	96.58	
	150	6.06	5.84	96.30	
CIT D	50	2.08	2.15	103.41	102.41
	100	4.16	4.21	101.27	
	150	6.24	6.40	102.56	
ESC-OX	50	2.35	2.38	100.84	98.72
	100	4.71	4.59	97.48	
	150	7.07	6.92	97.84	
CIT E	50	2.06	2.11	102.56	103.06
	100	4.12	4.28	103.79	
	150	6.18	6.35	102.82	

Table 5.18. *Results of Accuracy Studies in Oral Solution Matrix by Using C_{18} Column.*

Compounds	Percentage of Concentration	Added (µg mL^{-1})	Found (µg mL^{-1})	Recovery %	Average Recovery
CIT A	50	2.41	2.59	106.58	105.58
	100	4.82	5.02	104.21	
	150	7.23	7.66	105.96	
CIT B	50	2.08	2.16	103.82	105
	100	4.16	4.33	104.18	
	150	6.24	6.68	107.00	
CIT C	50	2.02	2.04	100.83	98.25
	100	4.04	3.92	96.98	
	150	6.06	5.87	96.93	
CIT D	50	2.08	2.08	99.76	100.00
	100	4.16	4.12	99.08	
	150	6.24	6.31	101.15	
ESC-OX	50	2.35	2.37	100.45	99.52
	100	4.71	4.63	98.33	
	150	7.07	7.05	99.78	
CIT E	50	2.06	2.18	105.89	103.57
	100	4.12	4.20	101.83	
	150	6.18	6.37	103.00	

Table 5.19. *Results of Accuracy Studies in Tablet Matrix by Using PFP Column.*

Compounds	Percentage of Concentration	Added (µg mL^{-1})	Found (µg mL^{-1})	Recovery %	Average Recovery
CIT A	50	2.41	2.44	101.14	99.99
	100	4.82	4.84	100.37	
	150	7.23	7.12	98.45	
CIT B	50	2.08	2.12	101.88	101.10
	100	4.16	4.15	99.73	
	150	6.24	6.33	101.36	
CIT C	50	2.02	2.06	101.74	99.77
	100	4.04	3.97	98.21	
	150	6.06	6.02	99.35	
CIT D	50	2.08	2.10	100.56	99.54
	100	4.16	4.12	99.15	
	150	6.24	6.17	98.90	
ESC-OX	50	2.35	2.34	99.50	101.49
	100	4.71	4.86	103.11	
	150	7.07	7.20	101.86	
CIT E	50	2.06	2.11	102.25	101.15
	100	4.12	4.21	102.11	
	150	6.18	6.19	100.18	

Table 5.20. *Results of Accuracy Studies in Oral Solution Matrix by Using PFP Column.*

Compounds	Percentage of Concentration	Added (µg mL^{-1})	Found (µg mL^{-1})	Recovery %	Average Recovery
CIT A	50	2.41	2.46	101.92	99.86
	100	4.82	4.77	99.02	
	150	7.23	7.13	98.63	
CIT B	50	2.08	2.08	100.22	100.79
	100	4.16	4.03	100.91	
	150	6.24	62.8	101.24	
CIT C	50	2.02	2.04	101.16	99.94
	100	4.04	4.02	99.49	
	150	6.06	6.01	99.17	
CIT D	50	2.08	2.12	101.74	100.30
	100	4.16	4.13	99.22	
	150	6.24	6.24	99.93	
ESC-OX	50	2.35	2.43	103.14	102.92
	100	4.71	4.85	102.96	
	150	7.07	7.25	102.65	
CIT E	50	2.06	2.11	102.25	101.71
	100	4.12	4.21	102.11	
	150	6.18	6.23	100.77	

5.2.5.3. Precision

To evaluate the developed method precision, repeatability (intra-day precision), intermediate precision (inter-day precision) were determined at the lowest concentration of the calibration curve. ESC-OX (0.236 µg mL^{-1}), CIT A (0.241 µg mL^{-1}), CIT B (0.208 µg mL^{-1}), CIT C (0.202 µg mL^{-1}), CIT D (0.208 µg mL^{-1}) and CIT E (0.206 µg mL^{-1}). The results were evaluated by a common statistical approach, including the calculation of SD, RSD, standard error of mean SEM and CI statistical results reveal that the method was analytically sufficient (RSD% < 2.0) in terms of precision. Precision results are given in Table 5.21-5.23.

Table 5.21. *Statistical Evaluation of Precision Studies for ESC-OX and Its Impurities Standard Solutions by Using C_{18} Core-Shell Column.*

		Intra-day			**Inter-days (n=18)**
		Day 1 (n=6)	**Day 2 (n=6)**	**Day 3 (n=6)**	
CIT A	Average	9906.50	10630.30	10306.83	10309.40
	Lower limit of CI	9836.59	10525.83	10230.54	10224.43
	Upper limit of CI	9976.41	10734.83	10383.12	10394.37
	SD	66.64	99.60	72.71	80.99
	SEM	27.20	40.66	29.68	33.06
	RSD%	0.67	0.94	0.71	0.79
CIT B	Average	5504.83	5389.17	5417.17	5437.06
	Lower limit of CI	5415.37	5296.31	5334.50	5338.41
	Upper limit of CI	5594.30	5482.03	5499.84	5535.70
	SD	85.27	88.51	78.79	94.02
	SEM	34.81	36.13	32.17	38.38
	RSD%	1.55	1.64	1.45	1.73
CIT C	Average	13135.33	12882.67	12678.17	12678.17
	Lower limit of CI	13059.27	12813.87	12580.95	12580.95
	Upper limit of CI	13211.40	12951.46	12775.38	12775.38
	SD	72.50	65.57	92.66	92.66
	SEM	29.60	26.77	37.83	37.83
	RSD%	0.55	0.51	0.73	0.73
CIT D	Average	5984.33	6303.67	6196.33	6207.33
	Lower limit of CI	5933.30	6218.33	6116.70	6130.66
	Upper limit of CI	6035.36	6389.01	6275.97	6284.01
	SD	48.64	81.34	75.90	73.08
	SEM	19.86	33.21	30.99	29.83
	RSD%	0.81	1.29	1.22	1.18
ESC-OX	Average	8856.16	8660.33	8764.00	8710.16
	Lower limit of CI	8777.91	8573.26	8679.86	8599.98
	Upper limit of CI	8934.42	8747.41	8848.14	8820.35
	SD	74.59	82.99	80.20	105.02
	SEM	30.45	33.88	32.74	42.87
	RSD%	0.84	0.96	0.92	1.21
CIT E	Average	6180.83	6161.00	6164.83	6175.66
	Lower limit of CI	6108.00	6085.28	6097.59	6058.20
	Upper limit of CI	6253.67	6236.72	6232.07	6185.80
	SD	69.42	72.17	64.09	60.81
	SEM	28.34	29.46	26.16	24.82
	RSD%	1.12	1.17	1.04	0.98

Table 5.22. *Statistical Evaluation of Precision Studies for ESC-OX and Its Impurities Standard Solutions by Using PFP Core-Shell Column.*

		Intra-day			**Inter-days (n=18)**
		Day 1 (n=6)	**Day 2 (n=6)**	**Day 3 (n=6)**	
CIT A	Average	11771.50	11492.00	11571.00	11611.50
	Lower limit of CI	11695.56	11400.86	11490.59	11497.94
	Upper limit of CI	11847.44	11583.14	11651.41	11760.40
	SD	72.38	86.87	76.64	141.92
	SEM	29.55	35.46	31.29	57.94
	RSD%	0.61	0.76	0.66	1.22
CIT B	Average	5521.33	5678.67	5655.67	5618.56
	Lower limit of CI	5459.10	5596.24	5570.75	5514.30
	Upper limit of CI	5583.56	5761.09	5740.59	5722.81
	SD	59.31	78.56	80.94	99.37
	SEM	24.21	32.07	33.04	40.57
	RSD%	1.07	1.38	1.43	1.77
CIT C	Average	11125.83	11144.00	11073.33	11114.39
	Lower limit of CI	11039.59	11081.01	11014.61	11040.80
	Upper limit of CI	11212.07	11206.99	11132.06	11187.98
	SD	82.20	60.04	55.97	70.14
	SEM	33.56	24.51	22.85	28.63
	RSD%	0.74	0.54	0.51	0.63
CIT D	Average	5658.50	5616.67	5687.50	5654.22
	Lower limit of CI	5578.85	5542.42	5625.32	5579.33
	Upper limit of CI	5738.15	5690.91	5749.68	5729.12
	SD	75.91	70.76	59.26	71.38
	SEM	30.99	29.55	24.19	29.14
	RSD%	1.34	1.26	1.04	1.26
ESC-OX	Average	9249.50	9360.50	9439.00	9349.67
	Lower limit of CI	9318.88	9271.73	9336.58	9232.46
	Upper limit of CI	9180.12	9449.27	9541.42	9466.88
	SD	66.13	84.61	97.62	111.71
	SEM	27.00	34.54	39.85	45.61
	RSD%	0.71	0.90	1.03	1.19
CIT E	Average	6605.5	6726.83	6717.33	6683.22
	Lower limit of CI	6533.74	6658.30	6634.97	6591.41
	Upper limit of CI	6677.26	6795.37	6799.70	6775.03
	SD	68.39	65.32	78.51	87.51
	SEM	27.92	26.67	32.05	35.72
	RSD%	1.04	0.97	1.17	1.31

Table 5.23. *Statistical Evaluation of Precision Studies for ESC-OX and Its Impurities Standard Solutions by Using Phenyl-Hexyl Core-Shell Column.*

		Intra-day			**Inter-days (n=18)**
		Day 1 (n=6)	**Day 2 (n=6)**	**Day 3 (n=6)**	
CIT A	Average	13783.00	13834	13889.67	13835.56
	Lower limit of CI	13710.73	13742.67	13767.92	13732.79
	Upper limit of CI	13855.27	13925.33	14011.41	13938.32
	SD	68.89	87.05	116.04	97.95
	SEM	28.12	35.54	47.37	39.99
	RSD%	0.50	0.63	0.84	0.71
CIT B	Average	4348.33	4360.17	4326	4344.83
	Lower limit of CI	4307.25	4338.09	4289.34	4301.93
	Upper limit of CI	4389.42	4382.24	4362.66	4379.84
	SD	39.16	21.04	34.94	37.13
	SEM	15.99	8.59	14.26	15.16
	RSD%	0.90	0.48	0.81	0.86
CIT C	Average	10308.33	10319.17	10281.33	10302.94
	Lower limit of CI	10232.78	10235.17	10320.01	10235.95
	Upper limit of CI	10383.89	10403.16	10242.66	10369.94
	SD	72.01	80.05	36.86	63.86
	SEM	29.40	32.68	15.05	26.07
	RSD%	0.70	0.78	0.36	0.62
CIT D	Average	6348.17	6311.67	6308.33	6322.72
	Lower limit of CI	6306.54	6241.39	6250.35	6265.01
	Upper limit of CI	6389.79	6381.95	6366.31	6380.43
	SD	39.68	66.99	55.26	55.01
	SEM	16.20	27.35	22.56	22.46
	RSD%	0.62	1.06	0.88	0.87
ESC-OX	Average	7208.00	7273	7266.5	7249.17
	Lower limit of CI	7171.78	7220.53	7203.76	7191.30
	Upper limit of CI	7244.22	7325.47	7329.24	7307.04
	SD	34.52	50.01	59.80	55.16
	SEM	14.09	20.42	24.41	22.52
	RSD%	0.48	0.69	0.82	0.76
CIT E	Average	3622.67	3629.67	3649.83	3631.72
	Lower limit of CI	3582.93	3601.89	3597.53	3587.85
	Upper limit of CI	3662.40	3657.45	3702.14	3675.59
	SD	37.87	26.48	49.85	41.81
	SEM	15.46	10.81	20.35	17.07
	RSD%	1.05	0.73	1.37	1.15

5.2.5.4. Selectivity

The selectivity of the developed method was evaluated by comparing the chromatograms obtained by using three core-shell HPLC columns. In addition, the chromatograms acquired by three columns was compared with the blank solution of the oral solution matrix and tablet matrix. The examination of blank chromatogram showed that there were no peak signals at the retention time of the examined analytes (Figure 5.26 (a, b, c)). Moreover, to confirm the selectivity, peaks of analytes were examined by DAD to examine the peak purity, the spectra showed the purity of all analytes. The peak purity spectra are illustrated in Figure 5.27.

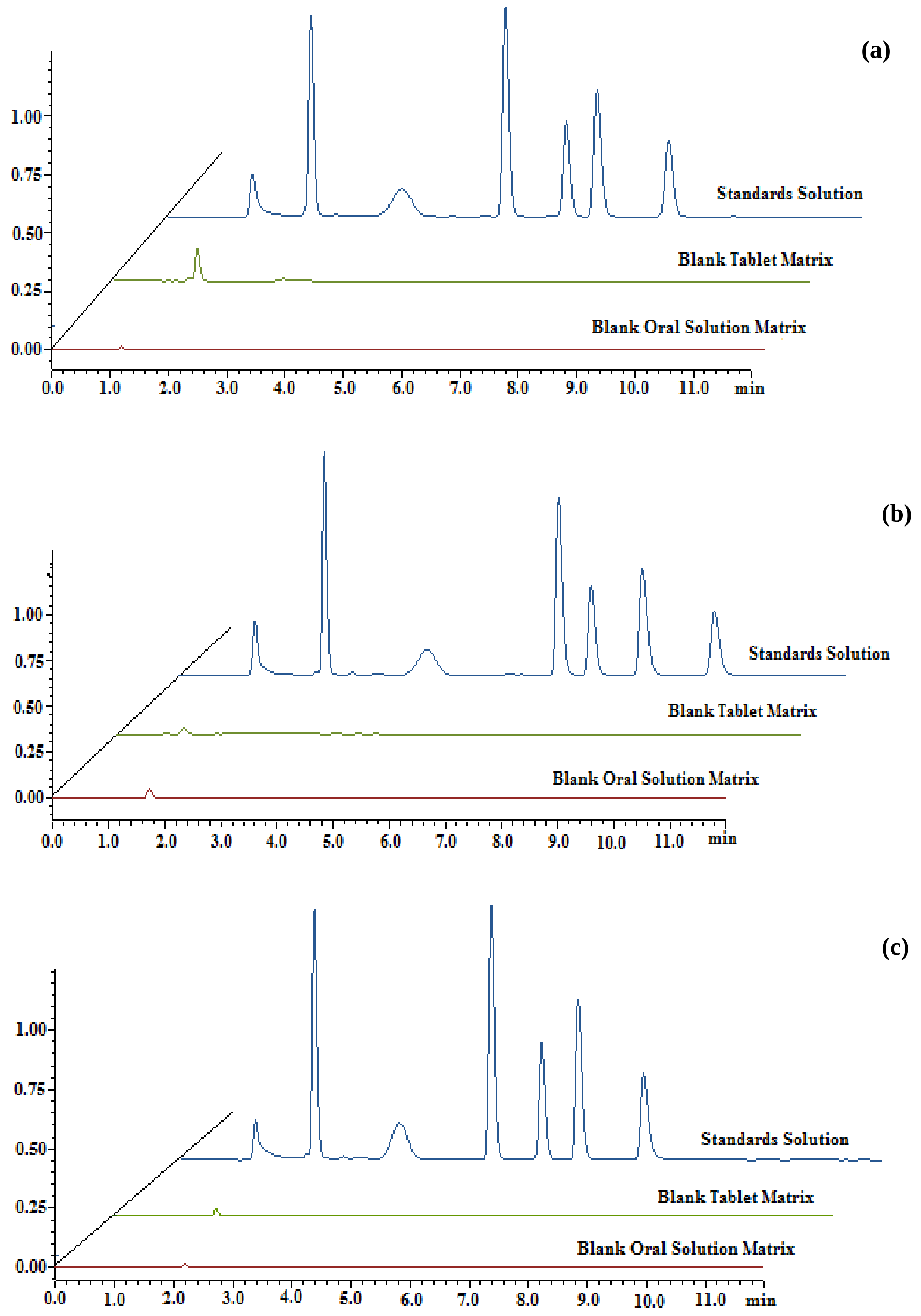


Figure 5.26. *Chromatograms Comparison for Blank with ESC-OX and it impurities (a) C_{18} Core-Shell Column, (b) PFP Core-Shell Column, (c) Phenyl-Hexyl Core-Shell Column.*

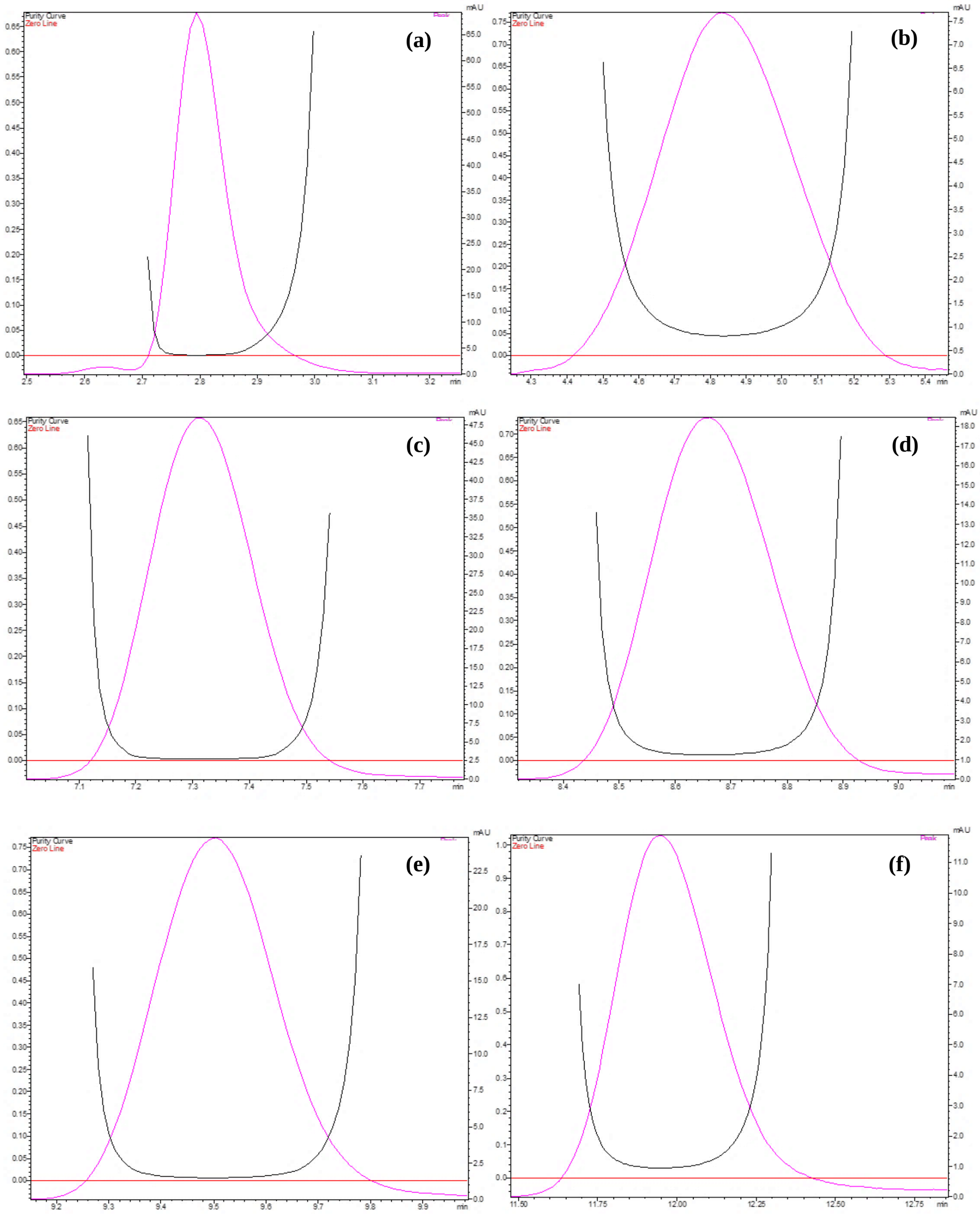


Figure 5.27. *The Peak Purity Spectrum of the (a) CIT A, (b) CIT B, (c) CIT C, (d) CIT D, (e) ESC-OX, (f) CIT E.*

4.2.5.5. Robustness and ruggedness

To evaluate robustness of the developed method, some instrumental parameters which have major effect on the retention were intentionally changed. Robustness of the method was examined by injecting standard solutions (n=3) of ESC-OX (11.78 µg mL^{-1}), CIT A (12.05 µg mL^{-1}), CIT B (10.4 µg mL^{-1}), CIT C (10.1µg mL^{-1}), CIT D (10.4 µg mL^{-1}) and CIT E (10.3 µg mL^{-1}). The effect of these parameters on the final result was investigated. The effect of pH was studied by varying ±0.2 pH units (at 3.3 and 3.7 buffer pH). The effect of column temperature on the resolution was studied by studying at two temperatures (35°C and 45°C). The effect of buffer, ACN and MeOH percentages was studied by changing by ±2 units (at 48 and 50) (at 3 and 7 for MeOH) and (23 and 27 for ACN). The influence of wavelength was also evaluated by two different wavelengths of detection (205 and 215 nm). (Heyden et al, 2001, p. 723–753). The result of the robustness study is represented in Table 5.24. The chromatograms of the robustness study are shown in Figure 5.28- 5.29.

The robustness study finding indicate that increasing in the ACN % and pH value have a slight effect on the repeatability of the developed method. The RSD% value is below 2% which indicate the robustness of the developed method. Also, the ruggedness of the method was evaluated by using different HPLC system and Synergi® Hydro-RP C_{18} column. The chromatograms of the two HPLC systems are shown in Figure 5.30. The result obtained proved that there is not any significant difference in the results. Consequently, the proposed method is both robust and rugged.

Table 5.24. *Data of the Robustness Study of Developed HPLC Method (*n*=3).*

Compound	Parameter	Condition	Area ± SD	% Change of Area
CIT A	***Standard solution***	***Standard condition***	***445815.66***	------
	Flow rate change	1.1 mL min^{-1}	472874 ± 822.35	0.0606
		1.3 mL min^{-1}	416509 ± 1317.00	0.0657
	Buffer pH	3.3 pH	444035 ± 3390.32	0.0039
		3.7 pH	522369 ± 3204.58	0.1717
	Buffer %	48 %	455056 ± 521.92	0.0207
		52 %	451175 ± 714.55	0.0120
	ACN %	23 %	491572 ± 1928.91	0.1026
		27 %	569123 ± 5068.51	0.2766
	MeOH%	3 %	472595 ± 1587.77	0.0601
		7 %	462994 ± 853.72	0.0385
	Temperatures	35℃	461748 ± 2506.97	0.0357
		45℃	458309 ± 1955.55	0.0280
	Wavelengths	205 nm	591160 ± 8145.91	0.3260
		210 nm	457983 ± 551.77	0.0273
CIT B	***Standard solution***	***Standard condition***	***249162***	------
	Flow rate change	1.1 mL min^{-1}	271298 ± 1963.76	0.0888
		1.3 mL min^{-1}	224197 ± 1390.77	0.1002
	Buffer pH	3.3 pH	247970 ± 1373.70	0.1097
		3.7 pH	285594 ± 2066.31	0.2695
	Buffer %	48 %	252537 ± 1786.89	0.1097
		52 %	254495 ± 1306.33	0.2695
	ACN %	23 %	276501 ± 2776.42	0.1097
		27 %	316316 ± 1085.81	0.2695
	MeOH%	3 %	260355 ± 1113.73	0.1097
		7 %	248061 ± 541.18	0.2695
	Temperatures	35℃	249192 ± 2222.21	0.0001
		45℃	249875 ± 1827.54	0.0029
	Wavelengths	205 nm	433305 ± 2703.51	0.7390
		210 nm	251197 ± 1241.20	0.0082
CIT C	***Standard solution***	***Standard condition***	***661739***	------
	Flow rate change	1.1 mL min^{-1}	720626 ± 2073.36	0.0890
		1.3 mL min^{-1}	620292 ± 734.58	0.0626
	Buffer pH	3.3 pH	666415 ± 9111.35	0.0071
		3.7 pH	757590 ± 1531.06	0.1448
	Buffer %	48 %	649687 ± 1411.62	0.0182
		52 %	666217 ± 4004.42	0.0068
	ACN %	23 %	712434 ± 2506.01	0.0766
		27 %	829513 ± 4001.88	0.2535
	MeOH%	3 %	679022 ± 854.80	0.0261
		7 %	664229 ± 2248.53	0.0038
	Temperatures	35℃	668092 ± 1388.57	0.0096
		45℃	661881 ± 1199.84	0.0002
	Wavelengths	205 nm	563236 ± 3113.96	0.1489
		210 nm	664024± 2399.65	0.0035

Table 5.24. *(continued) Data of the Robustness Study of Developed HPLC Method (*n*=3).*

CIT D	***Standard solution***	***Standard condition***	***329454***	-------
	Flow rate change	1.1 mL min^{-1}	363029 ± 2875.00	0.1019
		1.3 mL min^{-1}	308254 ± 2306.57	0.0643
	Buffer pH	3.3 pH	334140 ± 1516.85	0.0142
		3.7 pH	382662 ± 4967.41	0.1615
	Buffer %	48 %	326230 ± 4266.61	0.0098
		52 %	334420 ± 1556.17	0.0151
	ACN %	23 %	355101 ± 509.22	0.0778
		27 %	411097 ± 3242.55	0.1052
	MeOH%	3 %	338933 ± 1741.58	0.0778
		7 %	331135 ± 779.69	0.1676
	Temperatures	35℃	331079 ± 1939.74	0.0049
		45℃	325149 ± 2021.97	0.0131
	Wavelengths	205 nm	429309 ± 2749.03	0.3031
		210 nm	327367 ± 767.13	0.0063
ESC-OX	***Standard solution***	***Standard condition***	***459439***	-------
	Flow rate change	1.1 mL min^{-1}	504508 ± 1713.56	0.0981
		1.3 mL min^{-1}	430516 ± 2411.73	0.0623
	Buffer pH	3.3 pH	455688 ± 3534.70	0.0082
		3.7 pH	523836 ± 4967.41	0.1402
	Buffer %	48 %	440878 ± 1092.96	0.0404
		52 %	463802 ± 4028.13	0.0095
	ACN %	23 %	498450 ± 2366.05	0.0849
		27 %	573935 ± 3242.55	0.2492
	MeOH%	3 %	467628 ± 3095.74	0.0178
		7 %	461548 ± 1166.48	0.0046
	Temperatures	35℃	464583 ± 2911.87	0.0112
		45℃	460283 ± 281.10	0.0018
	Wavelengths	205 nm	601162 ± 2322.86	0.3084
		210 nm	452508 ±	0.0151
CIT E	***Standard solution***	***Standard condition***	***304054***	-------
	Flow rate change	1.1 mL min^{-1}	336630 ± 3246.85	0.1071
		1.3 mL min^{-1}	288424 ± 408.11	0.0514
	Buffer pH	3.3 pH	314654 ± 1327.45	0.0349
		3.7 pH	354191 ± 2700.88	0.1649
	Buffer %	48 %	304731 ± 1092.96	0.0022
		52 %	312823 ± 1349.33	0.0288
	ACN %	23 %	330723 ± 4102.34	0.0877
		27 %	392716 ± 5104.19	0.2916
	MeOH%	3 %	309989 ± 674.22	0.0195
		7 %	304581 ± 3830.72	0.0017
	Temperatures	35℃	307366 ± 4637.71	0.0109
		45℃	308228 ± 651.66	0.0137
	Wavelengths	205 nm	393856 ± 2846.95	0.2953
		210 nm	303647 ± 2631.16	0.0013

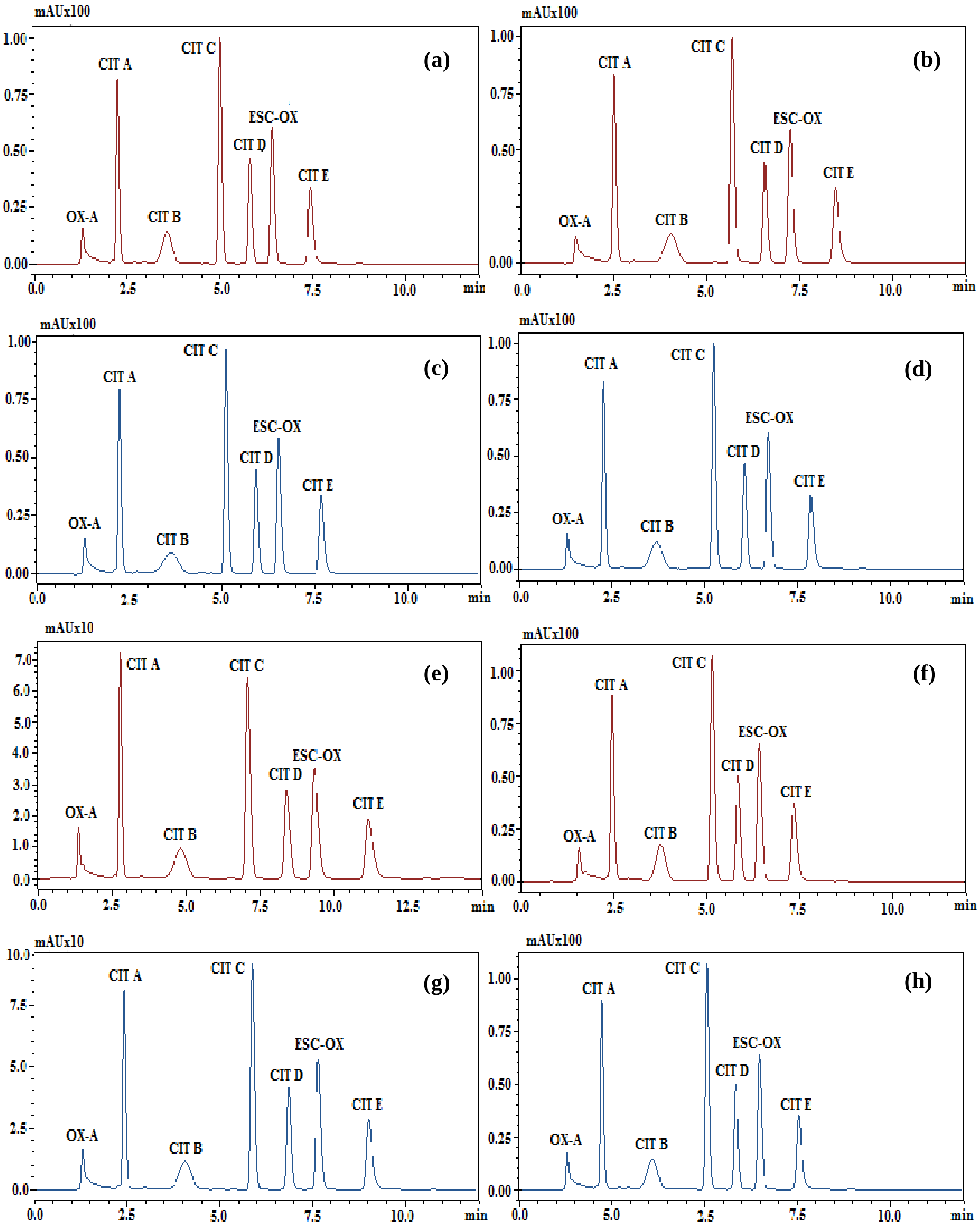


Figure 5.28. *The Chromatogram of Robustness Study by Using (a) pH 3.3, (b) pH 3.7, (c) Buffer 48%, (d) Buffer 52%, (e) ACN 23%, (f) ACN 27%, (g) MeOH 3%, (h) MeOH 7%.*

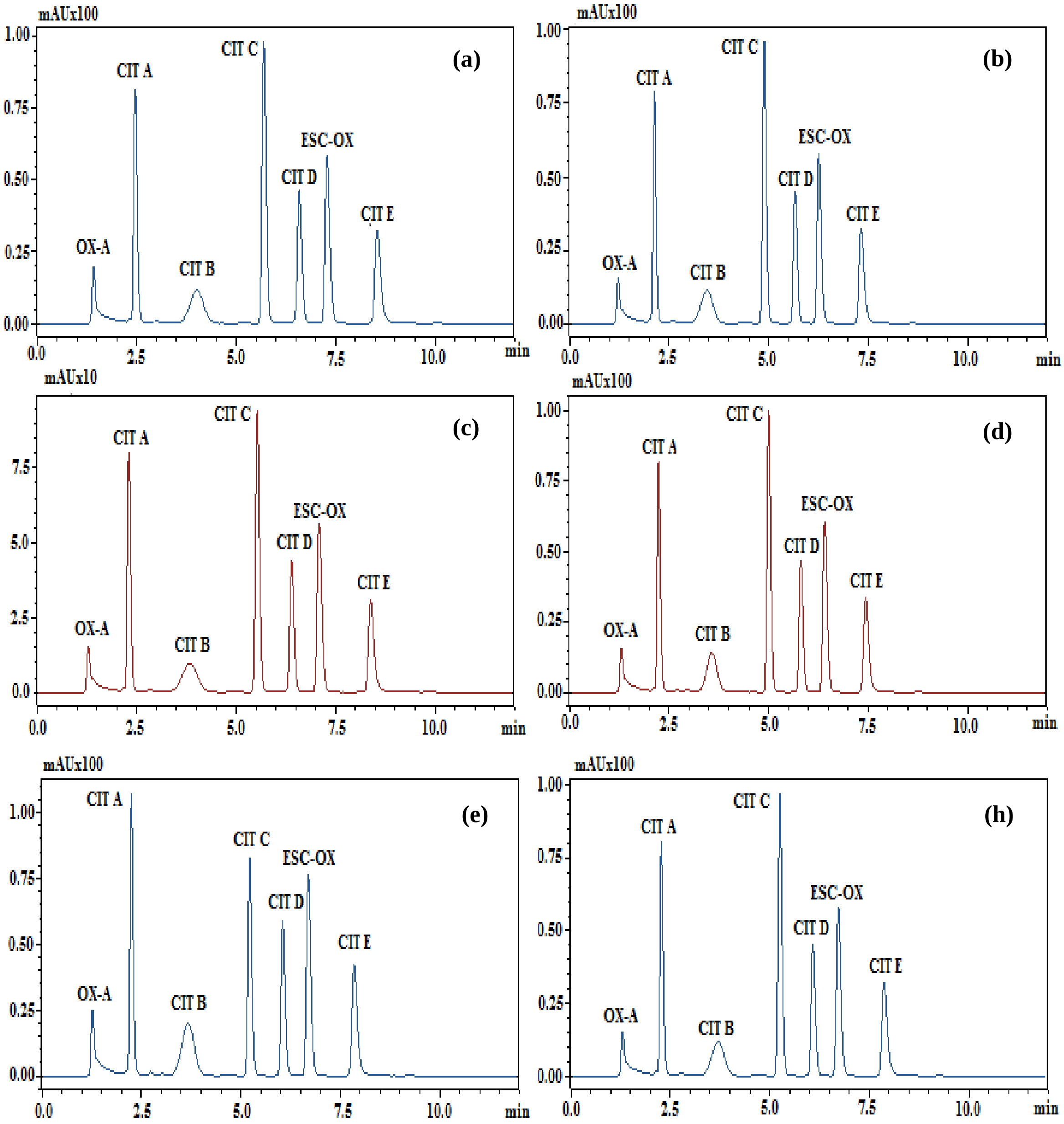


Figure 5.29. *The Chromatogram of Robustness Study by Using (a) Flow Rate 1.1 min mL*$^{-1}$*, (b) Flow Rate 1.3 min mL*$^{-1}$*, (c) Temperature 35℃, (d) Temperature 45℃, (e) Wavelength 205 nm (f) Wavelength 215 nm.*

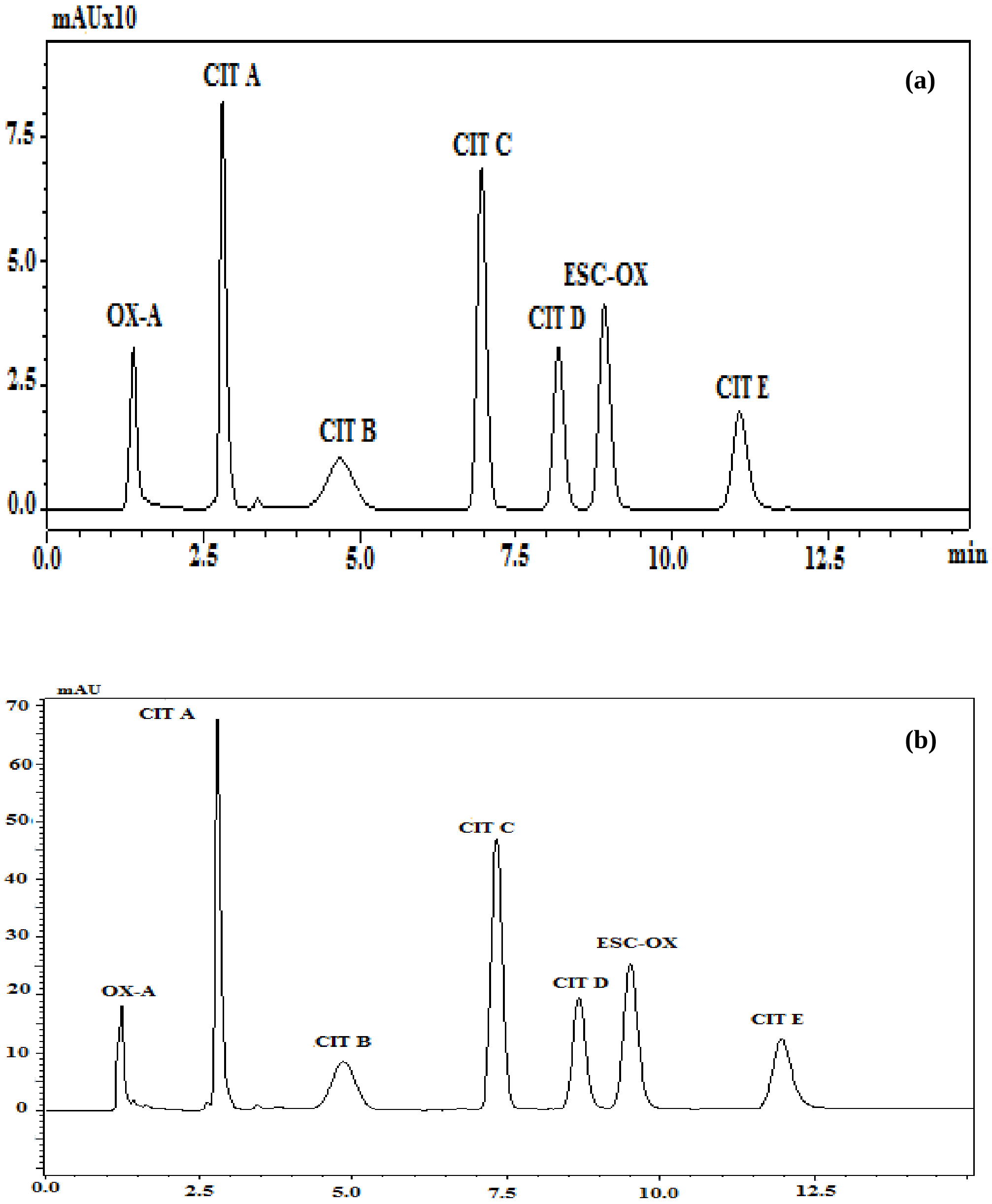


Figure 5.30. The Ruggedness Study by Using Synergi® Hydro-RP C_{18} Column (a) by Using HPLC System A, (b) by Using HPLC System B.

5.2.5.6. *Stability study*

The standard solutions of ESC-OX (11.78 µg mL^{-1}), CIT A (12.05 µg mL^{-1}), CIT B (10.4 µg mL^{-1}), CIT C (10.1 µg mL^{-1}), CIT D (10.4 µg mL^{-1}) and CIT E (10.3 µg mL^{-1}). were examined for the short term (1 day), freeze and thaw and long term (30 days) stability studies and the monitoring of the stability showed that all compounds were stable at all studied conditions. The study was carried out by using Kinetex C_{18} core-shell column. Table 5.25. and Figure 5.31. represent the change in the peak area of the compounds during stability study.

Table 5.25. *Stability study of ESC-OX and its Impurities.*

Compound		**Peak Area**			
		First Day	**Short Term Stability**	**Freeze and Thaw Stability**	**Long Term Stability**
CIT A	Average	457511.33	453076.67	454896.67	425751.00
	SD	2307.94	1309.18	1511.07	2534.89
	RSD %	0.50	0.29	0.33	0.57
CIT B	Average	244733	243096.67	240399.33	236222.33
	SD	1307.70	875.47	435.08	1402.01
	RSD %	0.53	0.36	0.18	0.59
CIT C	Average	659891.33	655443.00	658593.67	568085.33
	SD	1307.71	932.69	525.26	774.97
	RSD %	0.53	0.14	0.08	0.14
CIT D	Average	324996.00	321343.00	324216.67	309054.33
	SD	962.39	1597.08	1389.72	3607.87
	RSD %	0.30	0.50	0.43	1.17
ESC-OX	Average	462514.33	455192.67	459427.00	444883.67
	SD	3171.95	278.83	1612.17	1021.24
	RSD %	0.69	0.06	0.35	0.23
CIT E	Average	321050.67	316857.67	315669.00	309219.33
	SD	1487.75	3116.02	446.52	1065.38
	RSD %	0.46	0.98	0.14	0.34

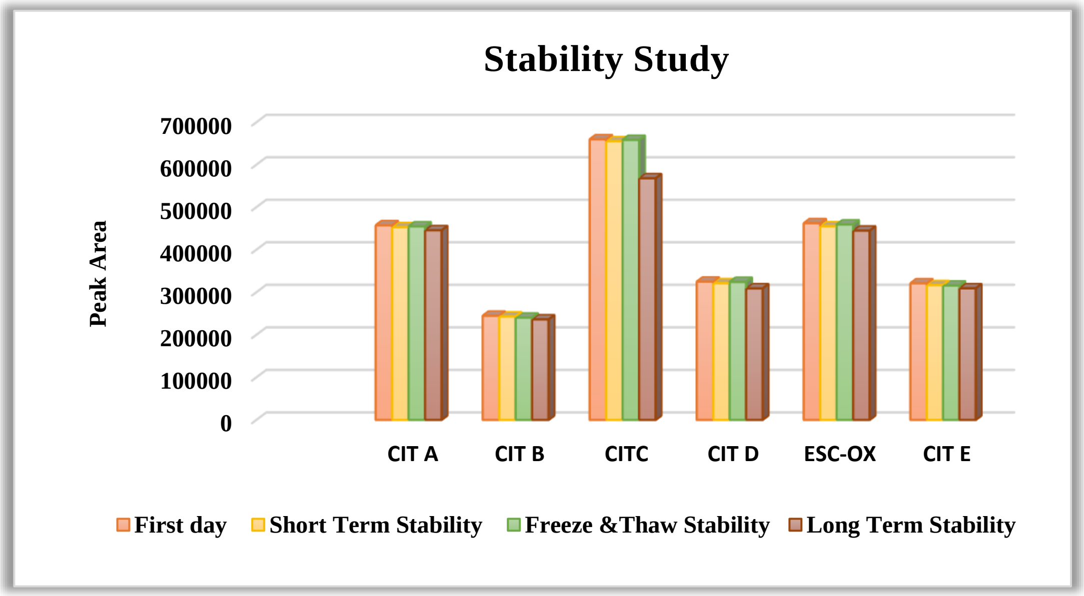


Figure 5.31. *Stability Study of ESC-OX and its Impurities.*

5.2.6. Analysis of ESC-OX in the pharmaceutical formulations

The applicability of the developed method was assessed by using it for determination of ESC-OX and its impurities (if any) in different pharmaceutical dosage forms (tablet and oral solution). The results for assay of dosage form are evaluated in Table 5.26-5.28.

At the same time, an expired oral solution (Expiration date: 04. 2015) and tablet dosage form (Expiration date: 08. 2016) were examined to determine the presence of impurities. The results obtained from different pharmaceutical dosage forms indicates the presence of CIT C impurity in all dosage form with very low quantity. This impurity CIT C is considered one of the critical impurities that can arise during cyclization step and it may be remained after conventional purification steps (Vedantham et.al, 2012, p. A-G). In the tablet dosage form CIT C and CIT A were detected. Impurity CIT A may be resulted from hydrolysis (Sharma et al, 2011, p. 836-848) and also monitored during synthesis of the ESC-OX (Gangula et al, 2011, p.1-5).

In the evaluation of expired oral solution dosage form impurity CIT D was detected alongside impurity CIT C. This CIT D was detected and identified as degradation product of CIT (racemic mixture). (Tadic, Nikolic and Agbaba, 2012, p. 733-743). The examination of the expired tablet identifies only impurity CITA (Figure 5.32- 5.33). The detected quantities of all impurities were below the maximum allowed limit of USP 2016.

The chromatograms of ESC-OX in dosage forms for the three columns are represented in Figure 5.34-5.36.

Intercolumn differences was examined by one- way ANOVA. The result shows that was no significant difference in the efficiency of the three columns from the analytical point of view.

Table 5.26. *Assay Results of ESC-OX 20 mg Film Tablet (n=6) by Using C18, PFP and Phenyl-Hexyl Column.*

	C_{18} column	**PFP column**	**phenyl-hexyl column**
Average (mg)	20.06	20.07	20.12
Minimum (mg)	20.00	20.00	20.07
Maximum (mg)	20.10	20.10	20.19
SD (mg)	0.032	0.034	0.046
RSD %	0.16%	0.17%	0.23%
SEM (mg)	0.0104	0.0107	0.0189
Bias%	-0.30	-0.35	-0.60
95% CI of Average	20.03 to 20.08	20.04 to 20.09	20.07 to 20.17
% Content Uniformity	100.3	100.35	100.6
ANOVA	F (2, 27) = 3.066, P = 0.0631		

Table 5.27. *Assay Results of ESC-OX 10 mg Film Tablet (*n*=6) by Using C18, PFP and Phenyl-Hexyl Column.*

	C_{18} column	PFP column	phenyl-hexyl column
Average (mg)	10.34	10.30	10.29
Minimum (mg)	10.27	10.16	10.20
Maximum (mg)	10.39	10.42	10.33
SD (mg)	0.042	0.072	0.037
RSD %	0.41%	0.70%	0.36%
SEM (mg)	0.0133	0.0228	0.0117
Bias%	- 3.4	- 3.0	- 2.9
95% CI of Average	10.31 to 10.37	10.25 to 10.35	10.27 to 10.32
% Content Uniformity	100.3	100.35	100.2
ANOVA		F (2,27) = 2.009, P = 0.1537	

Table 5.28. *Assay Results of ESC-OX Oral Solution (10 mg mL^{-1}) (*n *=6) by Using C18, PFP and Phenyl-Hexyl Column.*

	C_{18} column	PFP column	phenyl-hexyl column
Average (mg)	10.23	10.21	10.25
Minimum (mg)	10.18	10.14	10.21
Maximum (mg)	10.33	10.27	10.29
SD (mg)	0.054	0.043	0.025
RSD %	0.53%	0.42%	0.24%
SEM (mg)	0.0170	0.0135	0.0079
Bias%	-2.3	-2.1	-2.5
95% CI of Average	10.19 to 10.27	10.18 to 10.24	10.23 to 10.27
% Content Uniformity	102.3	102.1	102.5
ANOVA		F (2,27) = 1.835, P = 0.1789	

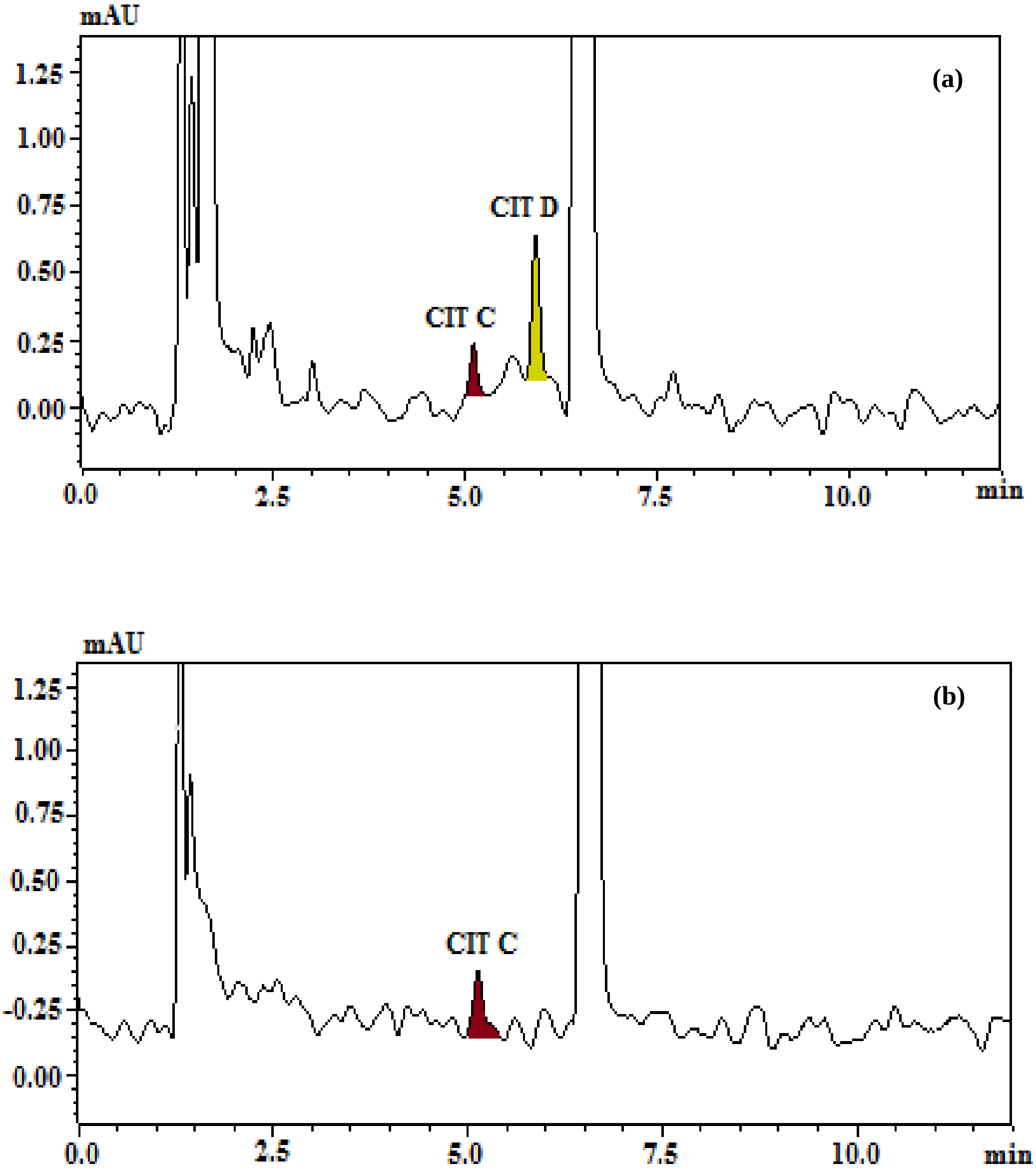


Figure 5.32. *Chromatograms Represent Impurities in (a) An Expired Oral Solution, (b) Oral Solution by Using Phenyl-Hexyl Column.*

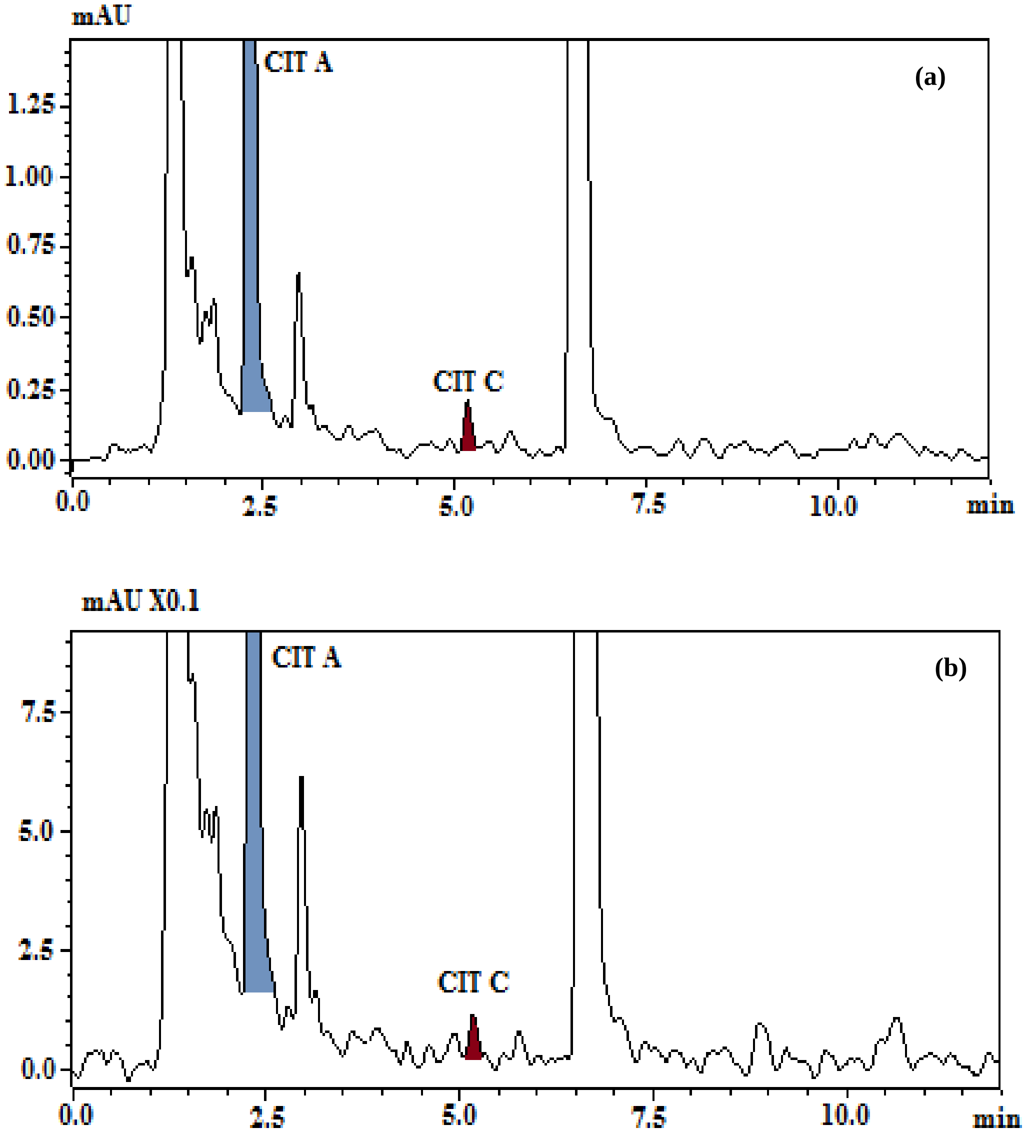


Figure 5.33. *Chromatograms Represent Impurities in (a) An Expired Tablet Dosage Form 20 mg, (b) Tablet Dosage Form 20 mg by Using Phenyl-Hexyl Column.*

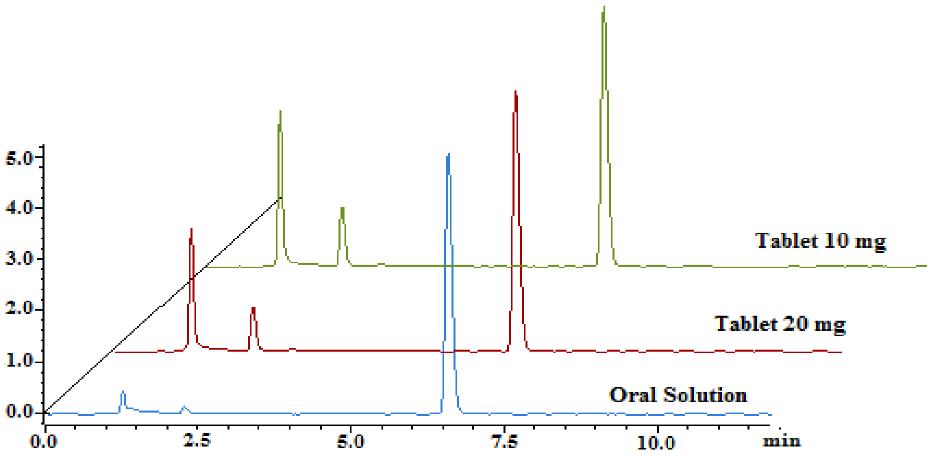


Figure 5.34. *Chromatograms Represent ESC-OX in (a) Tablet Dosage Form 10 mg, (b) Tablet Dosage Form 20 mg(c) Oral Solution 10 mg mL^{-1} by Using Kinetex ®Phenyl-Hexyl Core Shell Column.*

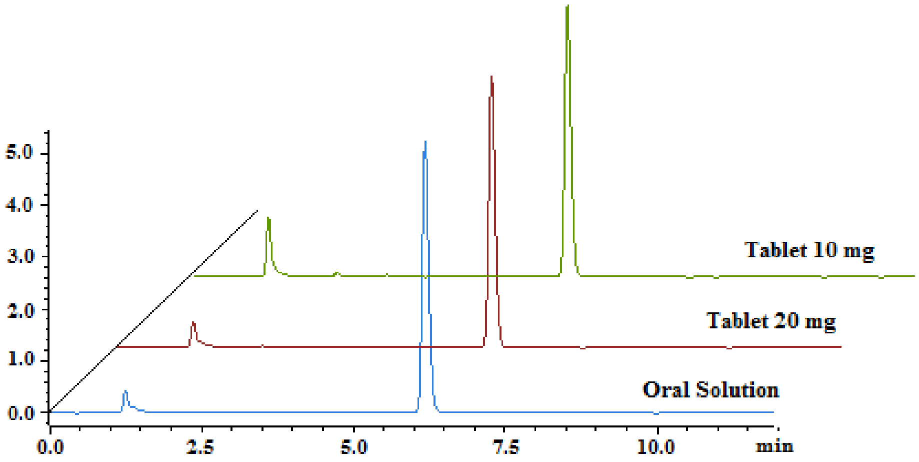


Figure 5.35. *Chromatograms Represent ESC-OX in (a) Tablet Dosage Form 10 mg, (b) Tablet Dosage Form 20 mg(c) Oral Solution 10 mg mL^{-1} by Using Kinetex ® C$_{18}$ Core Shell Column.*

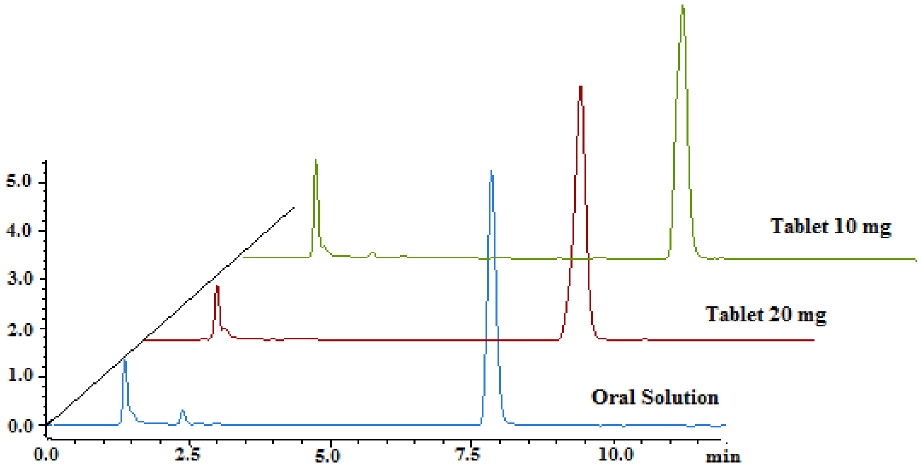


Figure 5.36. *Chromatograms Represent ESC-OX in (a) Tablet Dosage Form 10 mg, (b) Tablet Dosage Form 20 mg (c) Oral Solution 10 mg mL^{-1} by Using Kinetex ® PFP Core Shell Column.*

5.2.7. General evaluation of HPLC method

The purity of the pharmaceutical dosage formulation from the impurities became a mandatory demand by the most pharmaceutical agencies. The presence of impurities in dosage form has a hazard potential on the health of human being. Subsequently, there is a regular need to develop analytical method which fulfils the requirement from the quality control laboratories.

In this thesis, a simple isocratic, reversed phase HPLC method with a run time of 12 min was developed for quantitative determination ESC-OX and its impurities moreover, successfully applied for detection of impurities in the pharmaceutical dosage forms. The developed method was applied by using 12 different columns. Thereafter, six different columns (core-shell and fully porous) with three different stationary phases. The best separation was obtained from core-shell columns with good peak symmetry, resolution and short analysis time. The phenyl hexyl stationary phase with middle hydrophobic selectivity in comparison with the C_{18} and PFP stationary phase provides the best resolution. The PFP fully porous stationary phase proved to be more retentive with poor resolution and peak shape, however, the developed method can be utilized by using C_{18} fully porous column if they are the only available choice with relatively longer run time about 15 min.

The ESC-OX and its impurities are basic compounds, the desirable separation was by using phosphate buffer pH 3.5 (50 mM) and mobile phase content ACN: MeOH:

water: Buffer solution (25:5:20:50 *v/v/v/v*). The developed method met all acceptance recommended values for SST. The method was fully validated and the result obviously shows that the current method is robust, precise with low LOD, which makes it the preferable alternative from the official USP method. By the development of this method, two of this thesis objectives were achieved with satisfactory results.

5.3. Development and Optimization of the Electrochemical Methods

The reported methods so far in the electrochemical analysis required special electrodes such as construction of novel sensors for determination of ESC-OX in dosage forms and biological fluids (Al-Amri, Alarfaj and A. Aly, 2013, p. 10044 – 10058; Jain, Dhanjai and Sharma, 2013, p. 178–184). However, these methods are complicated and need special requirements and electrodes. Based on the literature review there is no electroanalytical studies based on the mercury electrode for ESC-OX.

The current study initially concentrates on the investigation of the redox behavior of ESC-OX drug at HMDE; secondly, establishment of a rapid, economic, easy and selective electrochemical analysis for analysis of ESC-OX in different matrices, like pharmaceutical formulations and human urine.

5.3.1. Optimization of LLE for urine sample preparation

Sample preparation is an important step in analytical procedures, because it affects the accuracy of the developed method. LLE was used as the extraction technique because it considers one of the simplest sample preparation methods, available in any laboratory and doesn’t require an expert technician. In addition, the log P of ESC-OX is 3.5, so it is hydrophobic and using LLE for urine sample preparation is a favorable choice.

Several factors affect the efficiency of LLE including polarity properties of the analytes and interfering compounds in the matrix, pH of the solutions, selection of organic solvents, addition of modifiers such as acid or base to alter the ionization of analyte. Taking these factors into consideration results in better recovery in the extraction procedure.

The best solvent for extraction of ESC-OX is chloroform or a similar solvent that can form hydrogen bond with the analyte. Chloroform is considered as a carcinogenic solvent and it is relatively soluble in water; it has higher density that makes it difficult to remove it precisely by pipette. Thus, it is excluded from use. Hexane was selected as an organic solvent because it is considered as non-carcinogenic, non-polar and low-cost.

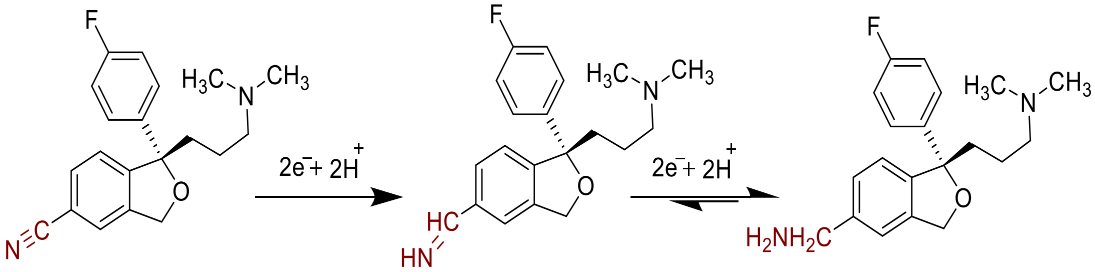
Hexane can undergo hydrophobic interaction with the propyl group of ESC-OX structure, that enhance the extraction of analyte into the organic layer. Also, using a solvent with a low polarity index is preferable to reduce the extraction of another matrix components. Hexane has low density and float over water and can be easily removed by pipette after extraction (Hansen, Pedersen-Bjergaard and Rasmussen, 2012 c, p. 80-89).

NH_4OH and NaOH were tested as the extraction modifier. The percentage recovery was higher in the case of NH_4OH. The extraction modifier enhances the solubility of analyte in the organic phase by altering the pH of the solution. ESC-OX is a basic salt and by addition of NH_4OH it becomes partially non-ionized and pass to the organic solvent.

Successive extraction was not carried out due to formation of emulsion. The emulsion is one of the LLE problem that can be happened due to presence of phospholipids (Lewandrowski and Pincus, 2016). The emulsion layer was reduced by performing centrifugation at 4°C. The percentage of recovery was high, so it is satisfactory to perform one step LLE. However, successive extraction is recommended to get higher recovery percentage.

5.3.2. Electrochemical behavior of ESC-OX

CV, DPV and OSWV were applied in order to recognize the electrochemical behavior of ESC-OX on HMDE. One well-defined reduction peak was obtained by applying CV technique which was resulted from the reduction of the unsaturated nitrile group. The cathodic reduction of the nitrile group can be occurred either through two-steps via an imine group or single four-electron reduction into an amine group (Lund, 1991, p. 466-470). A single four-electron reduction peak was observed due to the reduction of the aromatic nitriles at about - 0.6 V versus Ag/AgCl reference electrode with formation the corresponding amine. The proposed mechanism for electrochemical reduction of ESC-OX is given in Scheme 5.1.



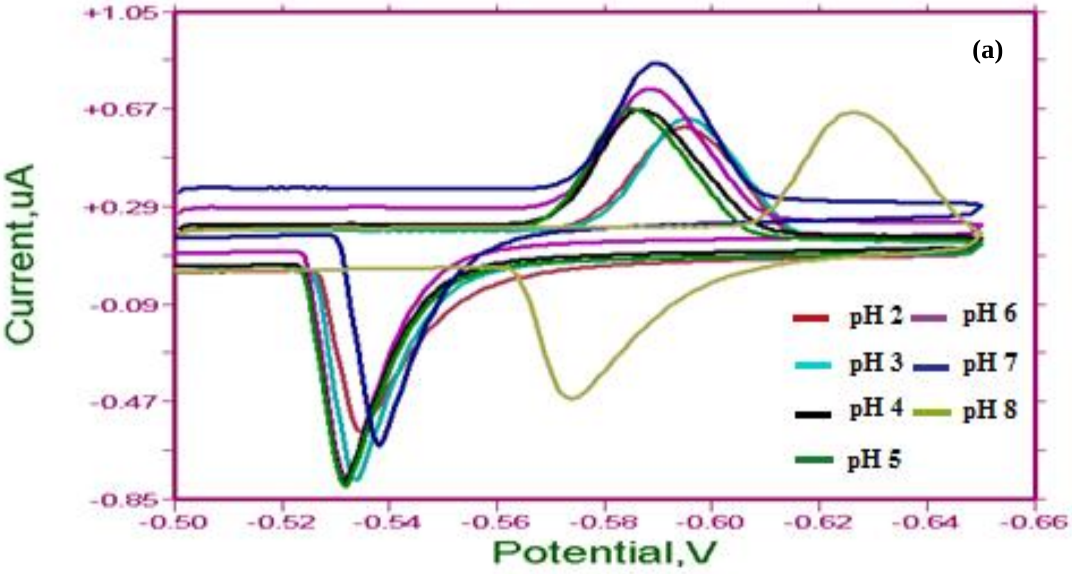
Scheme 5.1. *Proposed Reduction Mechanism of ESC-OX.*

In this study, the CV technique was applied to get more information for the electrochemical behavior of the antidepressant drug ESC-OX on HMDE. The CV technique facilitate understanding the essential characteristic concerning the kind of electrode reactions, their kinetic specification, and electrochemical reversibility (Özkan, Uslu and Aboul-Enein, 2003, p. 155–181). Consequently, the impact of experimental and instrumental conditions was studied extensively. The cyclic voltammograms of the blank were recorded in the potential range from - 0.5 to -1.2 V. There was no redox peak in the absence of ESC-OX at the entire working potential range, and a low background current on HMDE was observed.

5.3.3. Impact of supporting electrolyte composition and its pH

The pH of the medium influences the electrode reaction. The reduction of ESC-OX (41.44 μg mL^{-1}) was investigated over pH range 2-12 in BR buffer by the CV technique at scan rate of 100 mV s^{-1}. BR buffer was chosen because it can be used over a wide pH range and electrochemically inactive in a broad potential window. This buffer includes boric acid, which can react with various organic compounds (Ozkan, Kauffmann and Zuman, 2015 b, p. 27-28).

The solution pH impacts the peak current considerably. As it is shown in Figure 4.37 (a), there was no peak recognized before the ESC-OX peak, which indicates that the redox product was not adsorbed on the electrode surface at different pH values (2 to 8). Moreover, there was no peak recognized after ESC-OX peak indicated that the analyzed ESC-OX was not strongly adsorbed on the electrode surface. But at pH 9, there was a small peak after the ESC-OX peak which may indicate to the adsorption of the intermediate on the electrode surface (Wopschall and Shain, 1967, p. 1514–1527). Figure 5.37 (b) represents the effect of pH on the CV signal.



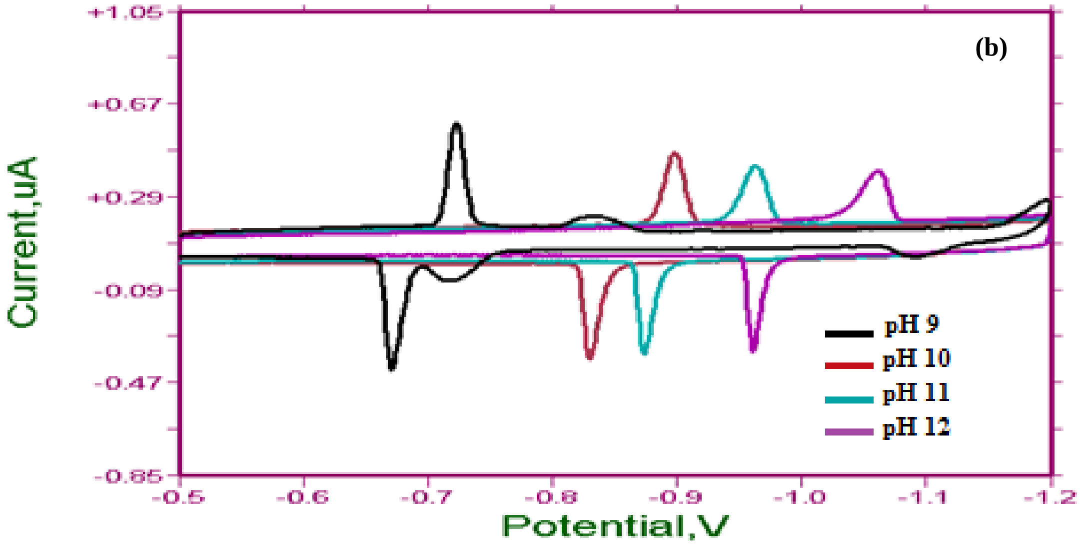


Figure 5.37. *(a)The effect of pH on the CV Signal of 41.44µg mL^{-1} ESC-OX Recorded in the BR Buffer Solutions (pH 2.0-8.0); (b) The Effect of pH on the CV Signal of 41.44µg mL^{-1} ESC-OX Recorded in the BR Buffer Solutions (pH 9.0-12.0).*

A correlation of peak current versus peak potential at different pH values shows that ESC-OX is reduced in the pH range of 2.0 to 12.0 by utilizing an equivalent number of electrons. A negative shift in cathodic peak potential (*E*pc) with increasing pH was observed. This pH dependence of *E*pc suggests the involvement of protons in the rate-limiting step (Figure 5.37). The relationship between the pH and the peak potential follow the following equation (5.1):

*E*pc (v) = 63.06 pH - 230.48 (r^2= 0.8377) (5.1)

The slope of this equation was found to be 63.06 mV pH^{-1}. The value of slope is near the conventional theoretical value of 59 mV pH^{-1} which proposes that the number of electrons transferred is equal to that of the hydrogen ions involving in the electrode reaction (Figure 4.38) (Smith, 2006, p. 259–264).

The peak potential was depended on pH; above pH 9.0, the potential shifted to the negative values, but remained constant between pH 2 to 7 (Figure 5.38). The region of the break is near to the pKa value of ESC-OX, which was at about 9.4 (Cipralex$^{®,}$ 2012, p. 3).

The plot of *I*pc versus pH (Figure 4.38) shows that the highest intensity value was obtained at pH 7.0 and the peak was broad; but at pH 6.0, the peak current shape was well-defined with a current value of 0.67 µA. Therefore, pH 6.5 was selected as the optimum medium pH for further experiments.

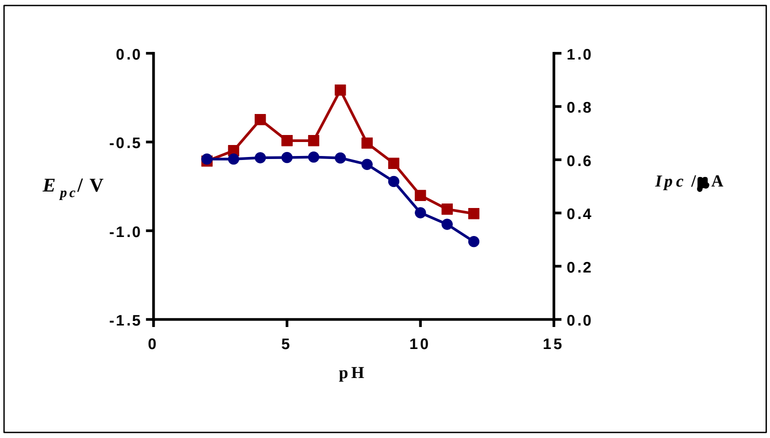


Figure 5.38. *The Dependence of the CV Peak Currents (Ipc) (Right y Axis) and Peak Potentials (Epc) (Left y Axis) on pH.*

To investigate the type of the electrochemical mechanism of ESC-OX either it is diffusion or adsorption-controlled, the CV behavior of ESC-OX (at 41.44 µg mL^{-1}) in buffer (at pH 6.5) in the potential range from -0.5 to -1.2 V was studied at various (ν), starting from 10 mV s^{-1} to 1000 mV s^{-1}. ESC-OX displayed two peaks; one sharp and well-shaped cathodic peak at the potential of -0.6 V at different (ν); and one peak is recognized in the anodic potential range of the reverse scans. The cyclic voltammograms in the presence of ESC-OX in BR buffer (pH 6.5) at various scan rates on HMDE are represented in Figure 5.39 (a). The slight negative shift of the reduction potentials with increasing the scan rate represent the quasi-reversibility of the electrode reaction. In the reversed scan, the signals showed current intensities less than the cathodic peaks which also confirms the quasi-reversibility nature of the electrode process (Diculescu et al, 2010, p. 1-7).

The relationship between the (*I*pc) of the ESC-OX and the square root of the scan rate ($\nu^{1/2}$) shows good linearity. This indicated that the process is mainly diffusion controlled on the electrode surface. The relationship is shown in Figure 5.39 (b). There is a slight shift of peak potential with the increase of the scan rate, which is distinctive for quasi-reversible and irreversible processes (Marken, Neudeck, and Bond, 2010, p. 83-87). The interrelation can be represented by this equation (5.2):

$$I_{pc}\,(\mu A) = 0.155xV^{1/2}\,(mV\ s^{-1})^{1/2} - 0.462 \qquad (r^2 = 0.9830) \tag{5.2}$$

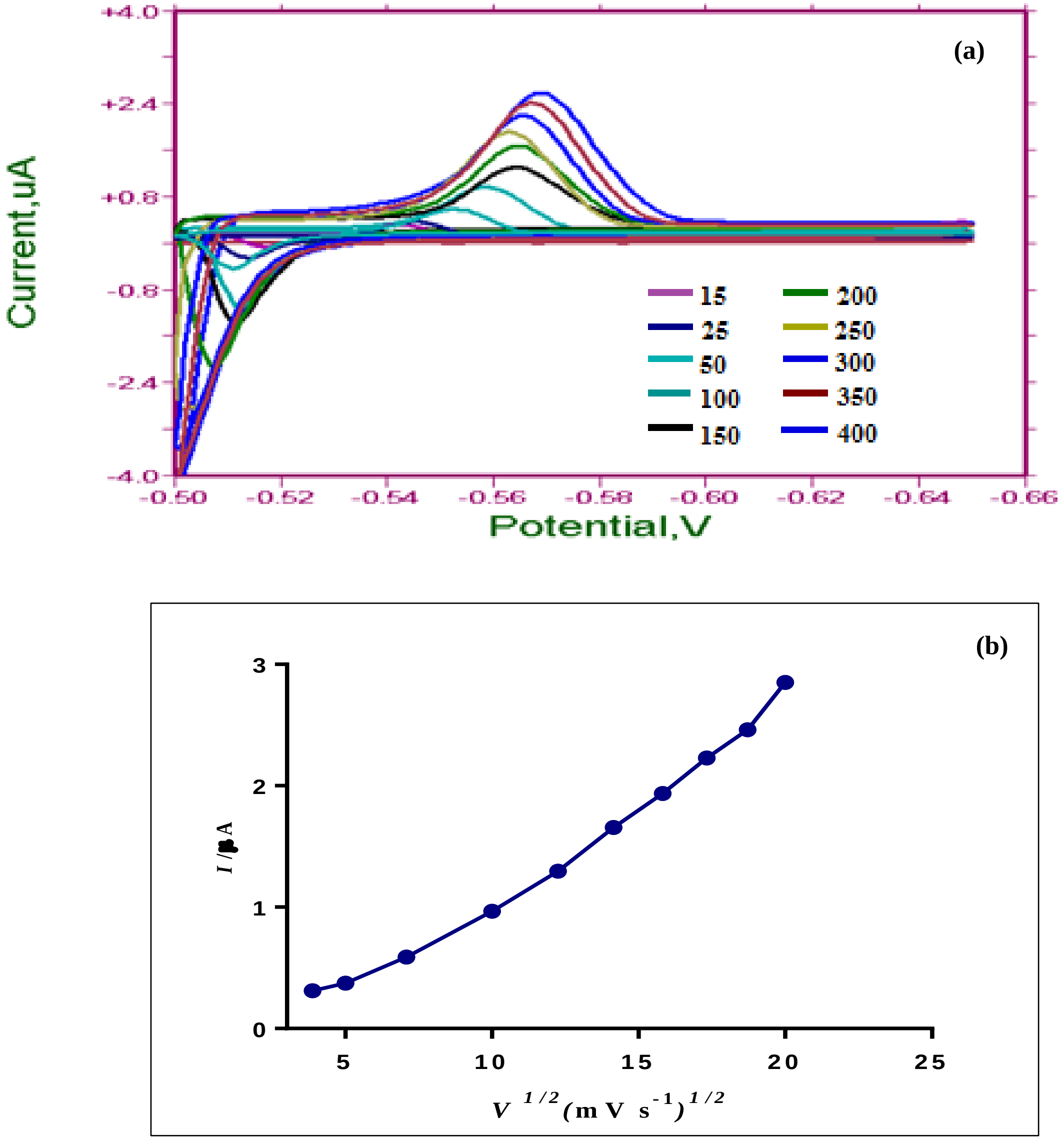


Figure 5.39. *(a) Cyclic Voltammograms of ESC-OX (41.44 $\mu g\ mL^{-1}$) Recorded in the BR Buffer (pH 6.5), KCl (0.3 M) and 5% MeOH on HMDE at Various Scan rate, (b) the Linear Dependence of the (Ipc) of ESC-OX on the Square Root of the Potential Scan Rate* ($\nu^{1/2}$).

This information can be supported by evaluation of the relationship between the logarithm of the peak current (log *Ipc*) and the logarithm of the scan rate (log ν). The plot of relationship between logarithm of the peak current (log Ipc) and the logarithm of the scan rate (log ν) is illustrated in Figure 5.40 (a), and the relationship can be represented by this equation (5.3):

$$\log Ipc\ (\mathrm{A}) = 0.691 \log \nu\ (\mathrm{mV\ s^{-1}}) - 7.37 \qquad (r^2 = 0.9941) \qquad (5.3)$$

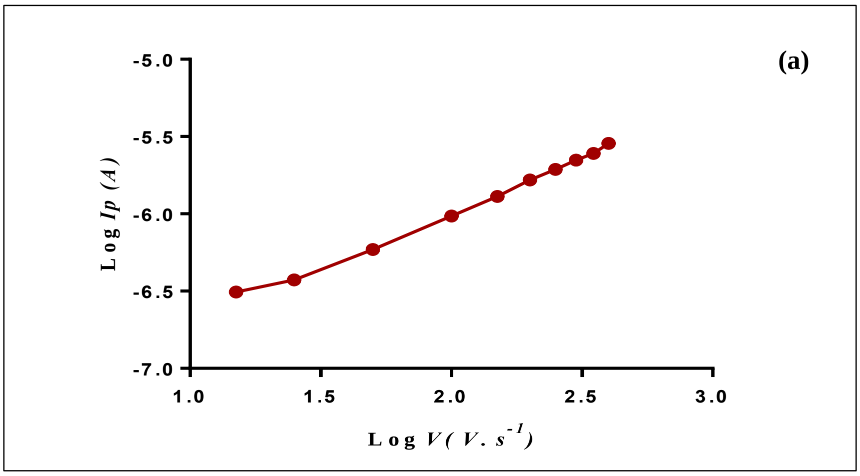
The slope value, which was close to 0.5, the theoretical value for diffusion controlled reactions, confirms that the reduction peak current of ESC-OX was mainly a diffusion controlled (Bard and Faulkner, 2001a, p. 137-153), however, the slope value was between 0.5-1.0 which indicated that the reaction also adsorption controlled diffusion in a limited value (Gosser. Jr, 1993, p. 43). The general electrode process is essentially diffusion-controlled with a slight adsorption of the drug compound at the electrode surface. The negative value of intercept on the current axis was obtained. This supports the information about diffusion controlled process of cathodic reaction of the HMDE electrode with the electroactive species of ESC-OX, in addition, other electroactive substances take part to towards the formation of the current (Stankovic et al, 2015, p. 95-100).

Additionally, the number of electrons involved in the reduction of the ESC-OX was evaluated. According to the Laviron equation (Laviron, Roullier and Degrand, 1980, p. 11-23) for quasi-reversible and an irreversible electrode process, *E*pc is represented by the equation (5.4)

$$E\rho c = \mathrm{E}° + \left(\frac{2.303\mathrm{RT}}{\alpha \mathrm{nF}}\right) \log\left(\frac{RTK°}{\alpha \mathrm{nF}}\right) + \left(\frac{2.303\mathrm{RT}}{\alpha \mathrm{nF}}\right) log v \qquad (5.4)$$

where α is the symbol of transfer coefficient, k° is the standard heterogeneous rate constant of the reaction, n is the number of electron transferred, v is the scan rate, and E° is the formal redox potential. T is temperature and F is faradic constant. Consequently, value of αn can be readily measured from the slope of the *E*pc versus log v. In this electrochemical process, the slope was -20.143; when using T = 298 K, R = 8.314 J $\mathrm{K^{-1}}$ $\mathrm{mol^{-1}}$, and F= 96480 C $\mathrm{mol^{-1}}$, the αn was calculated to be 2.936×10^{-3}. Corresponding to Bard and Faulkner (Bard and Faulkner, 2001b, p. 97), α can be given as between 0.5- 0.7

for quasi-reversible and an irreversible reaction. By using 0.7 for α value, the number of electrons transferred in the ESC-OX reduction was calculated as ~4.19. This value confirms the theoretical assumption of number of electron transfer as mentioned above. The dependence of the (*E*pc) of ESC-OX on the (Log ν) is shown in Figure 5.40 (b).



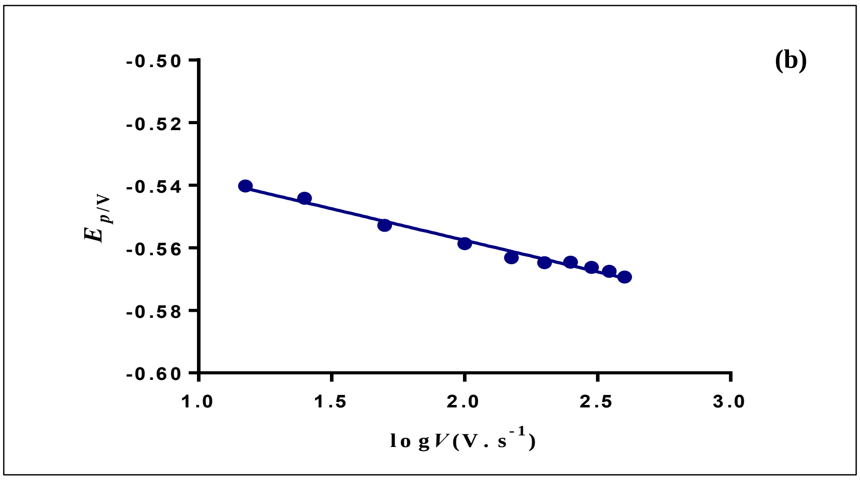


Figure 5.40. *(a) The Linear Dependence of the Logarithm of the Peak Current (Log Ipc) of ESC-OX on the Logarithm of the Potential Scan Rate (Log ν); (b) the Reliance of the Peak Potential (Ep) of ESC-OX on the Logarithm of Potential Scan Rate (Log ν).*

5.3.4. Optimization of experimental conditions

Development of a new method for the analysis of the pharmaceuticals or in the biological fluids requires the evaluation of its reliability in routine use. This can be achieved by studying the experimental and analytical variables that affect the robustness of the developed method. For the voltammetric assay, ESC-OX was investigated by using OSWV and DPV techniques.

In the OSWV technique, the peak shape was not well-defined and unrepeatable. The reduction of the ESC-OX pass through two steps as mentioned above. Since the OSWV technique is a large-amplitude differential technique, its reverse pulse causes the reverse reaction of the product and the current is sampled twice: Once at the end of the forward pulse and once at the end of the reversed pulse technique. Because of the speed and the rejection of background currents, complete voltammogram can be recorded within a couple of seconds. However, in this analysis two steps of reduction may be combined and resulted in an unstable peak. Also, OSWV technique is very sensitive, and possibly due to adsorption of intermediate product on the HMDE, a rise of the ill-shaped peak was observed in the voltammogram.

In DPV technique; potential pulse is fixed and of small amplitude (10 to 100 mV), and it is superimposed on a slowly changing base potential. Current is measured at two points for each pulse: The first point is just before the application of the pulse, and the second one is at the end of the pulse. These sampling points are selected to allow for the decay of the non-faradaic current. The difference between current measurements at these points for each pulse is determined and plotted versus the base potential. So, the technique proposes an improved resolution between the species with similar potential. In this study, when the DPV method was used, the electrode polarization was relatively slow and a single, well-defined shaped reduction signal of ESC-OX was observed. Since stable analyte response is a key point for success of any analytical method, the estimation of ESC-OX in pharmaceutical formulations and human urine was carried out by DPV.

During the method development, the most critical step is selection of the supporting electrolyte. Its contents influence the interface between the electrode and the solution, and changes the mechanism and kinetics of the reaction procedure (Wang, 2000, p. 117).

5.3.5. Impact of supporting electrolyte composition and pH

The pH is one of the most critical factors that influence the electrochemical behavior of electroactive compounds; thus, its impact on the peak potential and peak current should be investigated. In accordance, to define optimum analysis parameters for the DPV technique, the impact of the supporting electrolyte composition and its pH on ESC-OX reduction peak was studied.

ESC-OX (41.44 μg mL^{-1}) shows one sharp and well-defined cathodic peak in the potential range from -0.5 to -1.2 V, at various pH (2 to 12). The impact of the pH on the peak potentials and currents is presented in Figure 5.41. A negative shift in (*E*pc) with the increase of pH reveals participation of H^+ ions in the electron transfer process. The pH dependent shift of (*E*pc) was in accordance with the results obtained in CV studies. The pH dependency of the reduction process is a proof of the involvement of protons; in addition, the same value of pKa was derived from the graph of pH versus peak potential.

The peak current of ESC-OX increases with increase of pH, reaches its maximum at pH 7, and it decreases monotonously as solution gets to alkaline conditions. The relationship between pH and peak potential for pH between 4 and 12 can be expressed by following equation:

$$Ep\ (v) = -59.73pH - 260.8 \qquad (r^2 = 0.9282) \qquad (5.5)$$

The slope is near to conventional theoretical value of 59 mV pH^{-1}, that is in accordance with CV results; this also indicates the irreversibility of the electrode reaction.

The maximum peak current was obtained at its highest value at pH 7.0. However, the peak was broad at this pH, which could be explained by the effect of electrochemical interferences. At pH 6.0 the peak was sharp and well-defined with a current value of 109 μA, and accordingly pH 6.5 was chosen as the optimum pH for further experiments.

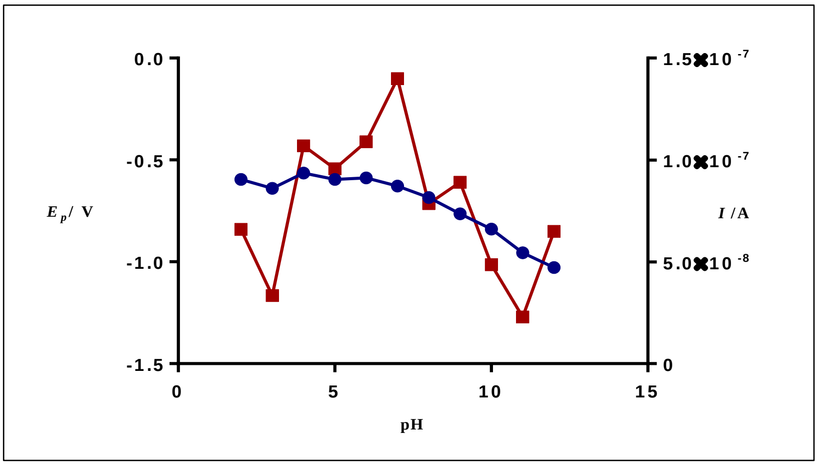


Figure 5.41. *The Dependence of the DPV (Ipc) (Right y Axis) and (Ep) (Left y Axis) on pH.*

Supporting electrolytes lower the resistance of the solution, reduce the electro-migration effects, and ensure a stable ionic strength. In this study, KCl was selected for this purpose because it is very effective and can be obtained as a pure substance with affordable costs; in addition, its ions have similar activities in aqueous solutions. Besides, the mercury electrode in contact with a de-aerated potassium chloride solution supports the behavior of ideal polarized electrode (Bard and Faulkner, 2001c, p.11). The molarity of KCl (0.10-0.15-0.20-0.25-0.30 M) was examined for ESC-OX (41.44 µg mL^{-1}) and KCl of 0.3M was selected as the final concentration in the voltammteric cell, because the current was stable and high at 0.3 M KCl.

The effect of MeOH percentage on the height and shape of DPV peaks was studied. The MeOH percentages of 30, 20, 10, and 5% were tested, and the highest peak intensity was obtained when 5% of MeOH was used. An increase of MeOH percentage resulted in negative shift of the potential, which may be due to slight increase in the acidity of solution because MeOH can donate H$^+$ to the solution. Also, the subsequent reduction in peak current was also noticed, which may be due to an increase in the dissociation constant of protonated species (Raddy and Raddy, 1991, p. 7-12). The best supporting electrolytes for ESC-OX regarding the highest current magnitude, low background current and the best repeatability were obtained with BR buffer at pH 6.5, KCl at 0.3 M and 5% MeOH.

5.3.6. Instrumental parameters

The reduction of the ESC-OX was carried out in acidic medium. The electro-reduction activity arises from the reduction of nitrile group by passing in two steps as mentioned before. DP voltammograms recorded from -0.40 V to -1.10 V for ESC-OX concentration lower than 5 µg. mL^{-1} consist of two cathodic reduction peaks at about -0.60 V and -0.70 V (Figure 5.42). When a higher concentration of the ESC-OX was applied, the signals integrated and only one cathodic peak was detected. This behavior of the ESC-OX may be explained by a reduction in the rate of chemical stage by the concentration decreased. Two peaks were appeared due to the transfer of electrons at the electrode surface may be taking place via two successive stages due to the first and the second electron transfer step consequently. In addition, the formation of thermodynamically stable intermediate with little adsorption at the electrode surface (Polcyn and Shain, 1966, p. 370-375). At higher concentration, the rate of reaction also increases, with formation of unstable intermediate, a single voltammetric peak appears. Since the splitting behavior is concentration, scan rate, frequency and other instrumental parameters dependent. The DPV parameters were changed in order to avoid peak splitting at low concentration. (Meunier-Prest and Laviron, 1996, p. 133-143; Mirceski and Gulaboski, 2003, p. 37-48).

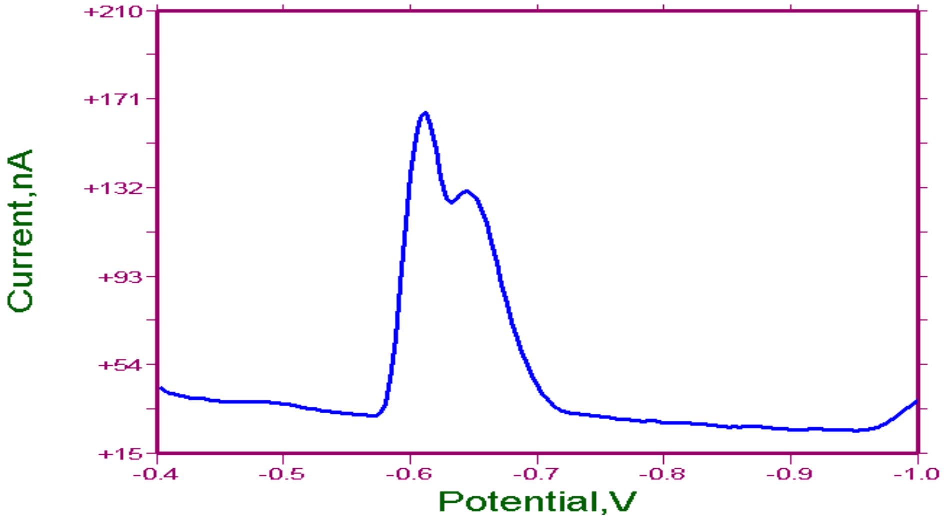


Figure 5.42. *The Voltammogram Representing Peak Splitting in DPV Technique*.

During the optimization of the instrument parameters, the investigated parameter was altered at the same time the other was retained constant at 4.14 μg mL^{-1} concentration of the ESC-OX. The effect of (ν) was investigated in the range of 15 to 50 mV s^{-1}. The well-shaped signal was accessed for ν=20 mV s^{-1}, and this value was selected as optimal for consecutive experiments. The peak current increased linearly with increasing pulse amplitude from 25 to 100 mV. Further, increase of pulse amplitude result in boarding and distortion of the peak. The most suitable peak, observing peak current, shape and width, was obtained at the value of 50 mV. Next, the impact of the pulse period on ESC-OX was investigated in the range from 100 ms to 250 ms. It was found that the peak current of ESC-OX decreased together with increasing pulse period. In optimization of sample width from 5 to 30 ms, the analytical signal was high with good shape peak at 25 ms. By changing the pulse width from 50 to 100 ms., the influence of pulse width on current peak was minor. The resolving of the peak splitting by changing of parameter can be explained, by increasing of pulse amplitude the rate of reaction is increased. As it is known the electrochemical reactions have rate constants that depend upon the potential applied to the electrode, with increasing potential leading to an increased rate. i.e. increase the rate of conversion of intermediate to the product. Therefore, the scan rate 20 mV s^{-1}, pulse amplitude 50 mV, sample width 25 ms., pulse width 175 ms., and pulse period 350 ms. were used in further analytical studies. These parameters have interrelated effects that can solve the problem of peak splitting. Furthermore, the effect of Hg drop size, stirring rate and purge time on the peak current was also studied. The selected working parameters were: drop size of 16 au, stirring rate of 400 rpm and purge time of 5 min.

The pulse period and pulse width were increased which lead to decrease in peak current to the values comparable to current obtained with the pervious DPV parameters. The reduction of current was due to increase in the pulse period. Also, at concentrations higher than 29.00 μg mL^{-1} there was no further increase in the peak current, which can be explained by the formation of a layer of product on the surface of the electrode that prevent the further diffusion of analyte to the electrode surface for reduction, thereby causing no further increase in current with increasing the concentration. By changing the parameters, there is a negative shift of peak and the peak showed broadness at low concentration indicating the merging of two cathodic peaks. The overall selected conditions are summarized in Table 5.29.

Table 5.29. *The Selected Conditions for Electrochemical Analysis.*

Parameters	Value
Buffer and pH	BR buffer pH 6.5 (0.04 M)
Electrolyte composition	KCl (0.3 M) + 5% MeOH
Cell temperature	20℃
Drop size	16 au
Scan rate	20 mV s^{-1}
Sample width	25 ms.
Pulse width	175 ms.
Pulse amplitude	50 mV
Pulse period	350 ms.

5.3.7. Validation of the developed method

To emphasize the performance of the propose method, validation of the developed method should be done. All validation parameters were estimated as stated to the ICH Q2 (R1), 2005 guidelines.

5.3.7.1. Linearity

Linearity of the calibration curve was examined by plotting the (*I*pc) (measured in nA) versus the concentration of ESC-OX (c) in triplicate (n=3). It was found that the reduction peaks increase linearly with the concentration of the ESC-OX (4.14-29.00 µg mL^{-1}). The linearity was checked by using the least square regression equation. Calibration plots were chosen in this range and concentrations were analyzed for three consecutive days. The parameters of the calibration curve for ESC-OX standard solution and spiked human urine obtained using DPV are outlined in Table 5.30 and 5.31. The voltammograms relating to the calibration curve of standard ESC-OX solution and spiked human urine are represented in Figure 5.43 (a, b).

Table 5.30. *Statistical Evaluation of Intra and Inter-Days Linearity Analysis of ESC-OX Standard Solution.*

	Intra-day (n=7)			**Inter-days (n=21)**
Range (μg mL^{-1})	4.14 - 29.00			
Slope ± SE	5.752 ± 0. 101	5.731 ± 0.094	5.641 ± 0.997	5.708 ± 0.052
Intercept ± SE	24.79 ± 1.881	25.59 ± 1.732	26.26 ± 1.847	25.55 ± 0.97
r^2	0.9984	0.9987	0.9984	0.9984
95% CI of Slope	5.491 to 6.013	5.490 to 5.971	5.424 to 6.012	5.598 to 5.818
95% CI of Intercept	19.96 to 29.63	21.14 to 30.05	19.96 to 30.85	23.51 to 27.58
LOD (μg mL^{-1})	1.16	1.073	1.162	1.152
LOQ (μg mL^{-1})	3.87	3.578	3.88	3.490
ANOVA	F (1.625, 9.748) = 0.7575, P = 0.4683			

Table 5.31. *Statistical Evaluation of Intra and Inter-Days Linearity Analysis of Urine Sample Spiked with ESC-OX Standard solution.*

	Intra-day (n=7)			**Inter-days (n=21)**
Range (μg mL^{-1})	4.14 - 29.00			
Slope ± SE	4.628 ± 0.084	4.451 ± 0.081	4.494 ± 0.073	4.524 ± 0.047
Intercept ± SE	52.74 ± 1.554	54.63 ± 1.506	53.58 ± 1.361	53.65 ± 0.877
r^2	0.9984	0.9983	0.9987	0.9979
95% CI of Slope	4.412 to 4.844	4.242 to 4.659	4.305 to 4.682	4.425 to 4.623
95% CI of Intercept	48.75 to 56.74	50.75 to 58.50	50.08 to 57.08	51.82 to 55.48
LOD (μg mL^{-1})	1.31	1.32	1.18	1.31
LOQ (μg mL^{-1})	3.97	4.00	3.58	3.97
ANOVA	F (1.012, 6.070) = 2.143, P = 0.1932			

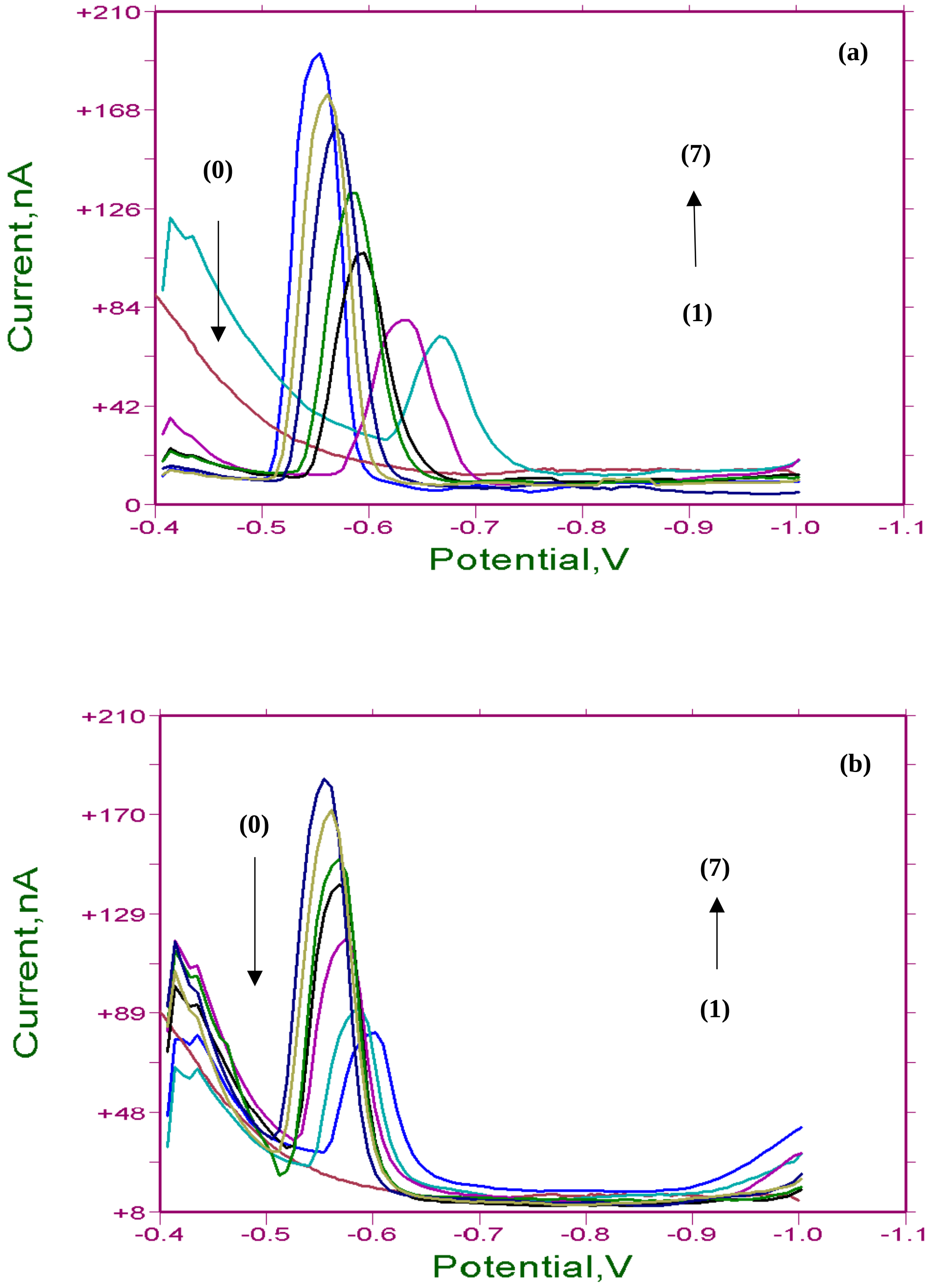


Figure 5.43. *DP Voltammograms of ESC-OX Recorded in BR buffer, pH 3.5, 3M KCl, 5% MeOH. The Concentrations of the ESC-OX Increase in the Following Manner: (0) Blank, (1) 4.14, (2) 8.28, (3) 12.43, (4) 16.56, (5) 20.72, (6) 24.86, (7) 29.00μg mL^{-1}. (b) DP voltammograms of ESC-OX recorded in spiked human urine.*

5.3.7.2. Accuracy

Regarding the study of the accuracy of the developed method, recovery study was applied for 4.14 μg mL^{-1}, 16.5 μg mL^{-1}, and 29.00 μg mL^{-1} ESC-OX standard solutions IN pharmaceutical dosage forms matrix and spiked human urine samples (n=6) for three consecutive days. The recovery percentages were close to 100% with RSD %less than 2% for the standard solution. The closeness of the result to 100% shows that accuracy of the current voltammetric method is reasonable (Shabir, 2003, p. 57-66). The RSD% from the spiked urine is below the 15%. The data is represented in Table 5.32 and 5.33.

5.3.7.3. Precision

The measurement of repeatability (intra-day) and intermediate precision (inter-days) was done to evaluate the precision of the developed method. Standard solution of ESC-OX and spiked human urine sample at 4.14, 16.5 and 29.00 μg mL^{-1} was examined on three consecutive days, six times in a day. The calculated RSD% value is lower than 2% deviation from the nominal value of precision for both standard and urine sample. This indicates that the current method is highly precise and analytically acceptable. The statistical evaluation is demonstrated in Table 5.34.

Table 5.32. *Accuracy Data for ESC-OX Standard Solution.*

	ESC-OX standard			
Day 1 (n = 6)	Added (µg. mL^{-1})	4.14	16.5	29.00
	Found (mean ± SD)	4.239 ± 0.0549	16.778 ±0.176	29.155 ± 0.404
	Recovery%	102.39	101.69	100.54
	SD	1.33	1.06	1.39
	SEM	0.54	0.44	0.57
	RSD%	1.29	1.05	1.39
Day 2 (n = 6)	Added (µg mL^{-1})	4.14	16.5	29.00
	Found (mean ± SD)	4.2433± 0.04793	16.613 ±0.274	30.074 ±0.526
	Recovery%	102.50	100.68	101.56
	SD	1.158	1.55	1.81
	SEM	0.47	0.63	0.74
	RSD%	1.13	1.64	1.79
Day 3 (n = 6)	Added (µg mL^{-1})	4.14	16.5	29.00
	Found (mean ± SD)	4.195 ± 0.074	16.711± 0.189	29.365± 0.336
	Recovery%	101.32	101.28	101.26
	SD	1.79	1.21	1.16
	SEM	0.73	0.49	0.47
	RSD%	1.77	1.13	1.14
Inter-days (n =18)	Added (µg mL^{-1})	4.14	16.5	29.00
	Found (mean ± SD)	4.226 ± 0.059	16.70 ± 0.213	29.531 ± 0.422
	Recovery%	102.07	101.62	101.12
	SD	1.47	1.31	1.458
	SEM	0.60	0.53	0.60
	RSD%	1.44	1.29	1.44

Table 5.33. *Accuracy Data for ESC-OX Spiked Urine Sample.*

		Spiked urine		
Day 1 (n=6)	Added (µg mL^{-1})	4.14	16.5	29.00
	Found (mean ± SD)	4.349 ± 0.1309	16.665 ±0.329	26.698 ±0.627
	Recovery%	105.04	100.64	92.06
	SD	3.16	1.98	2.16
	SEM	1.29	0.81	0.88
	RSD%	3.011	1.972	2.350
Day 2 (n=6)	Added (µg mL^{-1})	4.14	16.5	29.00
	Found (mean ± SD)	4.309 ± 0.114	16.958 ±0.455	27.930± 0.925
	Recovery%	104.08	102.40	96.31
	SD	2.77	2.75	3.19
	SEM	1.13	1.12	1.30
	RSD%	2.656	2.683	3.311
Day 3 (n=6)	Added (µg mL^{-1})	4.14	16.5	29.00
	Found (mean ± SD)	4.292 ± 0.088	28.908 ± 0.627	26.638 ± 0.772
	Recovery%	103.69	101.37	91.86
	SD	2.14	2.77	2.66
	SEM	0.87	1.13	1.09
	RSD%	2.061	2.730	2.900
Inter-days (n=18)	Added (µg mL^{-1})	4.14	16.5	29.00
	Found (mean ± SD)	4.316 ± 0.109	16.803 ± 0.412	27.089 ± 0.958
	Recovery%	104.27	101.47	93.41
	SD	2.62	2.49	3.31
	SEM	1.07	1.02	1.35
	RSD%	2.51	2.45	3.54

Table 5.34. *Precision Data for ESC Standard and Spiked Urine.*

Medium	Parameters	Lower Concentration (n=18)	Medium Concentration (n=18)	High Concentration (n=18)
ESC-OX Standard Solution	Average (nA)	49.49	121.20	193.40
	Lower limit CI	49.32	120.6	192.20
	Upper limit CI	49.67	121.80	194.60
	SD	0.35	1.16	2.43
	SEM	0.082	0.27	0.57
	RSD%	0.70	0.96	1.25
ESC-OX Spiked Urine Sample	**Parameters**	**Lower Concentration (n=18)**	**Medium Concentration (n=18)**	**High Concentration (n=18)**
	Average (nA)	73.13	129.6	175.1
	Lower limit CI	72.89	128.70	173.50
	Upper limit CI	73.38	130.60	176.70
	SD	0.49	1.86	3.18
	SEM	0.115	0.439	0.751
	RSD%	0.67	1.44	1.81

5.3.7.4. Specificity and selectivity

The selectivity and specificity of the current method for the analysis of human urine samples was evaluated by the determination of investigating drug in spiked human urine and different pharmaceutical dosage forms with satisfactory results. There were no interfering peaks recognized near the peak potentials of examining drug neither in the human urine samples after LLE nor from the different pharmaceutical dosage forms after simple pretreatment. Figure 4.43. (a) illustrates the blank tablet and oral solution matrix in comparison to the ESC-OX signal from different dosage form. Figure 5.44. (b) shows the blank urine sample and urine sample spiked with ESC-OX.

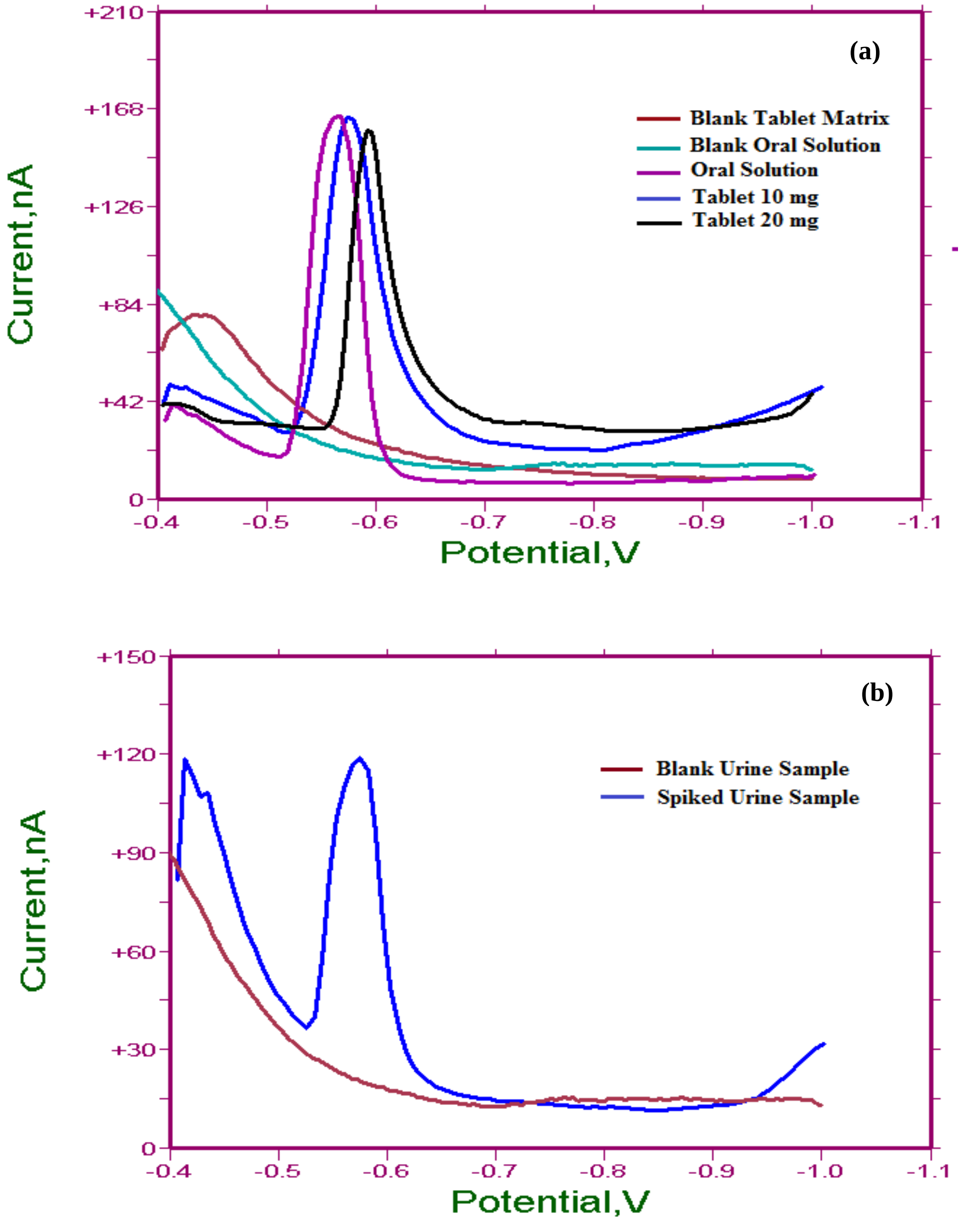


Figure 5.44. *The DP Voltammogram of (a) the Blank Tablet Matrix, Blank Oral Solution Matrix, Standard Solution, Tablet 20 mg, Tablet 10 mg and Oral Solution, (b) Blank Urine Sample and Spiked ESC-OX Standard Solution.*

5.3.7.5. Stability

The study of standard solutions stability was performed by analyzing prepared standard solution of ESC-OX in supporting electrolyte kept at +4 °C in the dark and comparing the result with the freshly prepared sample. The analysis results showed that there is no significant change in the working standard solutions over a period of one month.

For the urine sample, it was noticed that peak currents and potentials have not changed significantly in three successive analysis of the sample over an interval of approximately 24 hrs. which indicates the stability of urine samples. This period is enough for applying analysis.

5.3.8. Analysis of the human urine spiked with the ESC-OX solution

To prove the validity of the developed DPV techniques, the determination of the ESC-OX in human urine was tested. Urine represents a simple biological fluid for analysis because of lacking protein and another cellular constituent. Although, it contains urea, which may adsorb at the mercury electrode (Parsons, Peat and M. Reeves, 1975, p. 151-161). Also, ions and organic compounds which are sources of high conductivity and may result in the nonfaradic current. LLE was performed to get a net electroactive sample profile, high sensitivity and good reproducibility for quantification of ESC-OX in human urine. The optimization of the LLE discussed in detail in section 5.5.1. The validation relating to the spiked urine sample is mentioned in Table above.

5.3.9. Analysis of ESC-OX in pharmaceutical formulations

The current method was used for the quantitative determination of ESC-OX in different pharmaceutical dosage forms. The ESC-OX content of commercially available tablets (10 mg, 20 mg) and oral solution (10 mg mL^{-1}) was determined after simple pretreatment as mentioned in the section 4.3.6. The results indicate that the proposed DPV technique was effectively applied for the quantitative determination of the ESC-OX in two film tablets and an oral solution with no interference from the excipient (Table 5.35). The mean of the obtained results was very close to the stated declared value of 10 mg, 20 mg which indicates adequate precision and accuracy of the developed method for determination of ESC-OX in different pharmaceutical formulations without any interference from the excipients. The voltammograms related to the analysis of the different dosage forms are shown in Figure 5.45.

Table 5.35. *Statistical Evaluation of Three Different Pharmaceutical Dosage Forms.*

	Tablet (20 mg)	**Tablet (10 mg)**	**Oral Solution (10 mg mL^{-1})**
Average (mg)	20.39	10.17	10.28
Minimum (mg)	20.21	10.02	10.13
Maximum (mg)	21.01	10.28	10.47
SD (mg)	0.3079	0.1003	0.1221
RSD %	1.51	0.986	1.19
SEM (mg)	0.1257	0.04093	0.04983
Bias%	-1.95	-1.70	-2.80
95% CI of Average	20.07 to 20.72	10.07 to 10.28	10.15 to 10.41
% Content Uniformity	101.95	101.7	102.8

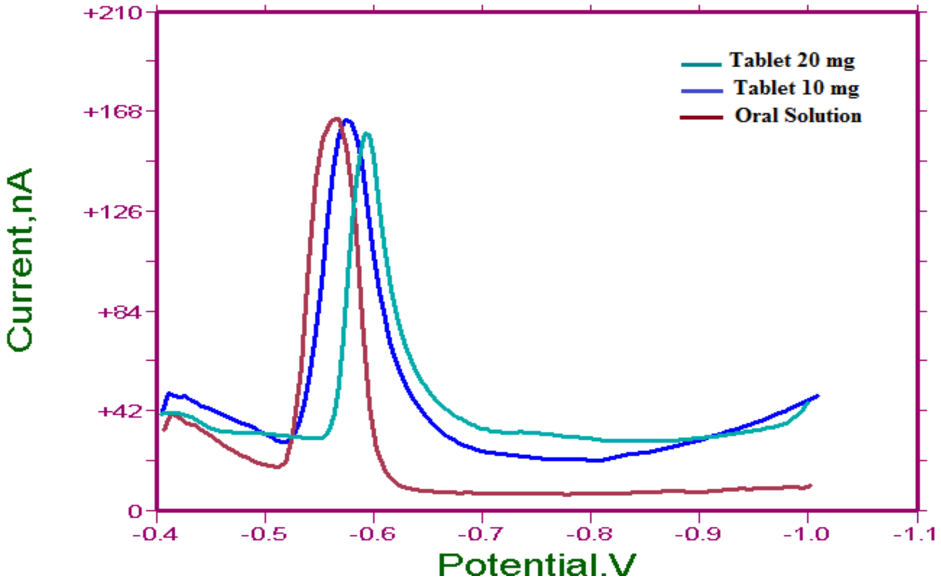


Figure 5.45. *The DP Voltammogram of the Three pharmaceutical preparations.*

5.3.10. General evaluation of DPV method

According to the literature survey, this is the first report investigating the electrochemical behavior of the ESC-OX using mercury electrode. Analysis of drugs in biological fluids usually is tedious procedure, but LLE which was used in this study is simple, fast and use small amount of organic solvent and other chemicals. The results showed above for determination of ESC-OX in bulk form, pharmaceuticals and human urine samples elucidate that the developed method offers a sensitive and selective method of ESC-OX analysis. Despite the fact that, the sensitivity and (LOD) have been enhanced in some of the previously developed methods (Al-Amri, Alarfaj and A. Aly, 2013, p. 10044 – 10058; Jain, Dhanjai and Sharma, 2013, p. 178–184), they might have some limitations such as longtime of analysis, using special electrodes and sophisticated instrumentation, complicated pretreatment steps for extraction as mentioned before. On the contrary, the present study has been shown to be appropriate for the determination of cited drug in diverse matrices, using of simple LLE for the urine sample, rapid, economical, sensitive as well as robust. Moreover, the developed method is fast, demanding not more than 5 min to run the sample, and require only simple pretreatment for preparation of pharmaceutical dosage forms. Therefore, the developed method has a high possibility for being a satisfactory alternative to the HPLC and GC, techniques in quality control and clinical laboratories where time and cost are important factors. In addition, the redox behavior of ESC-OX at mercury electrode could provide an interpretation to the pharmacokinetics profile and pharmacological action of drug in body (Kauffmann and Vire, 1993, p. 329-334).

5.4. Development and Optimization of the CE Method

According to literature search, there are some limited studies in which CE technique has been used. Among these, separation of the R- enantiomer of ESC-OX was accomplished by using sulfated-β-CD was used as an enatioseparator in the BGE (Deng et al., 2012, p. 1648–1651). In another study, the determination of the R- enantiomer of ESC-OX and one of the impurities, namely citadiol was also realized (Sungthong, Jac and Scriba 2008, p. 959–965). However, methods for the analysis of ESC-OX in different pharmaceutical dosage forms and urine samples are very limited and till to date there is no CE analysis method that has been developed for its determination in urine sample.

5.4.1. IS

In order to perform highly precise bioanalytical CE method, it is recommended to use IS which can reduce the variation that may come from sample matrix, manipulation, preparation, or from the volume of injection (Dose and Guiochon, 1991, p.1154-1158). Some compounds that can be used as an IS was tested, such as hydrochlorothiazide, (MET) and granisetron; among these only MET produced good peak shape with reasonable resolution at the selected conditions. The chemical structure of MET is illustrated in Figure 5.46. The pKa of IS is equal to 9.6 (http 1).

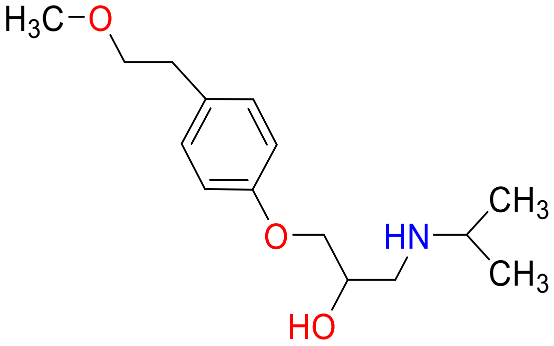


Figure 5.46. *The Chemical Structure of Metoprolol.*

5.4.2. Detection wavelength

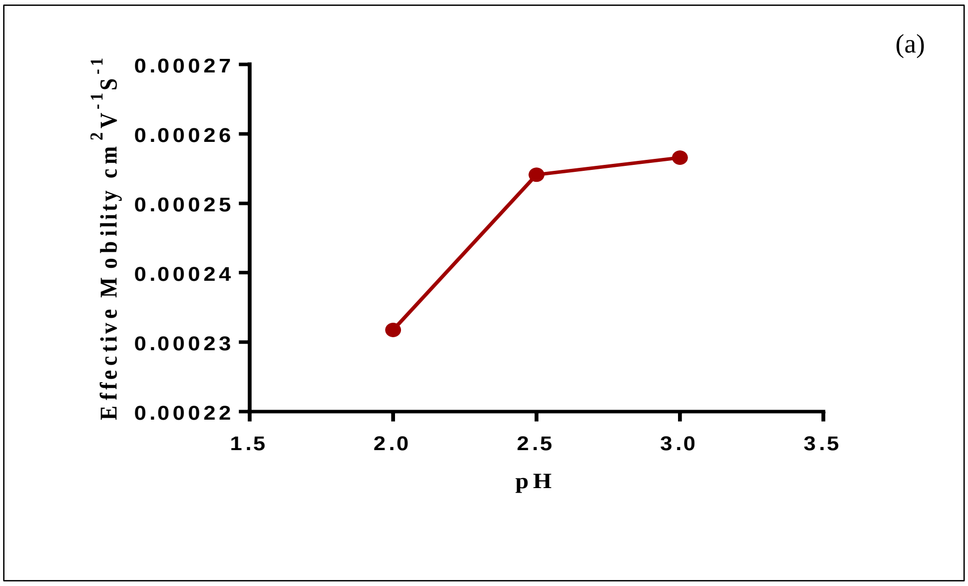
Absorption characteristics of ESC-OX and MET was monitored in the range of 200–400 nm, and a utilizable absorption band for both compounds were found to be about 200 nm.

5.4.3. BGE

BGE pH is a significant parameter in controlling and optimizing CE separation. Acidic, neutral and basic buffers (i.e., phosphate pH 3.0, phosphate pH 7.0, phosphate 11.0 and borate buffer at pH 10.0) were evaluated to achieve optimum efficiency. There was no peak at both basic and neutral buffers. Thereafter, the effect of the pH was examined by using phosphate buffer at pH 2.0, 2.5 and 3.0; besides, the buffer concentration was also verified. There was peak boarding when pH 3.0 was used, that can be explained by adsorption of basic cationic analyte to the negatively charged silanol groups at the capillary wall (Altria, 2003, p. 447-453).

The plots of buffer concentration and buffer pH versus effective electrophoretic mobility are illustrated in Figure 5.47 (a, b). The electropherogram of buffer pH effect on migration time and peak shape is shown in Figure 5.48. Reasonable retention time and well-shaped peak appeared with the use of pH 2.5. As it is known, the ESC pKa value is 9.50, at this pH the ESC is completely ionized and moves along the capillary with electrophoretic velocity. Although theoretically at very low pH values, the silanol groups are no longer ionized, meaning that the EOF is suppressed and the migration of ESC was dominated by the electrophoretic mobility. Suppression of EOF is favorable because small change in the EOF result in changing analyte mobility which in turn result in deviation in migration time (Van De Griend, 2008, p.133-134)

Initially, phosphate buffer was investigated in the concentration range of 10 to 25 mM and 15 mM phosphate buffer provided the optimum results. Use of higher buffer concentration was avoided here because it causes excessive Joule heating in CE capillary, which may affect analytes’ stability and resolution. The electropherograms recorded using various buffer concentrations are illustrated in Figure 5.49.



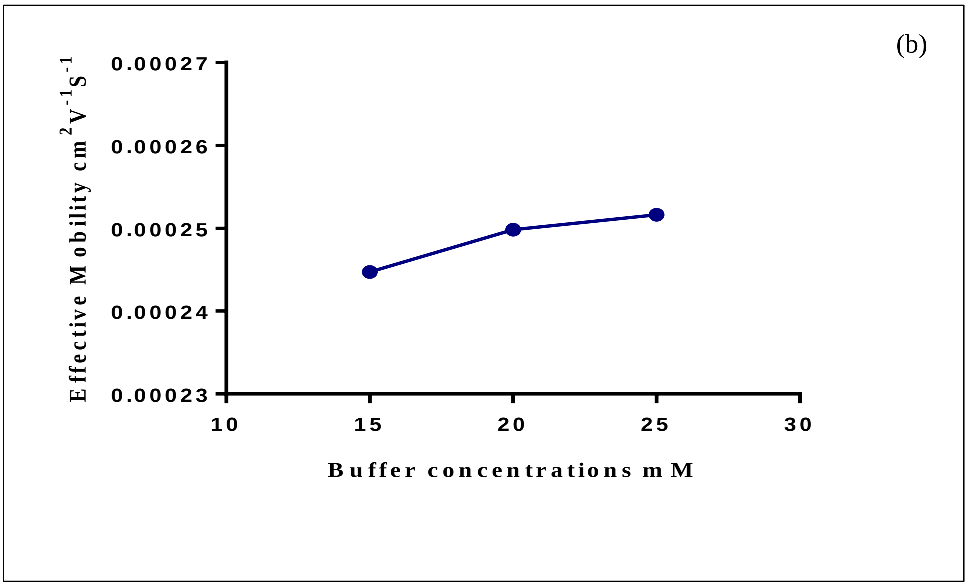


Figure 5.47. *The plots of buffer concentration and buffer pH versus effective electrophoretic mobility: (a) pH 2, 2.5, 3, and (b) Buffer Concentration 10, 15, 20, 25 mM.*

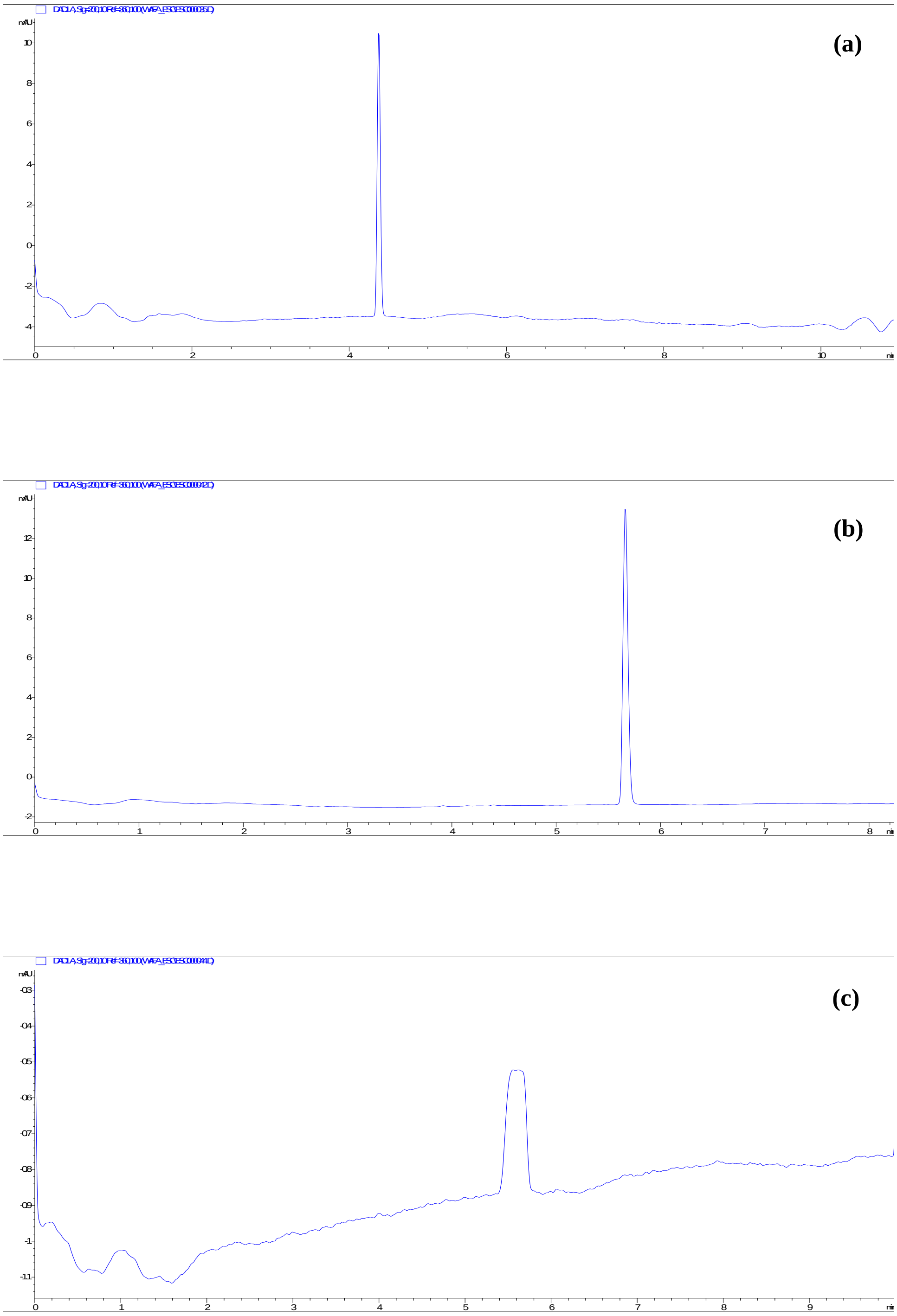


Figure 5.48. *The Effect of Buffer pH on the Peak Characterizations of ESC-OX (a) pH 2, (b) pH 2.5, (c) pH 3.*

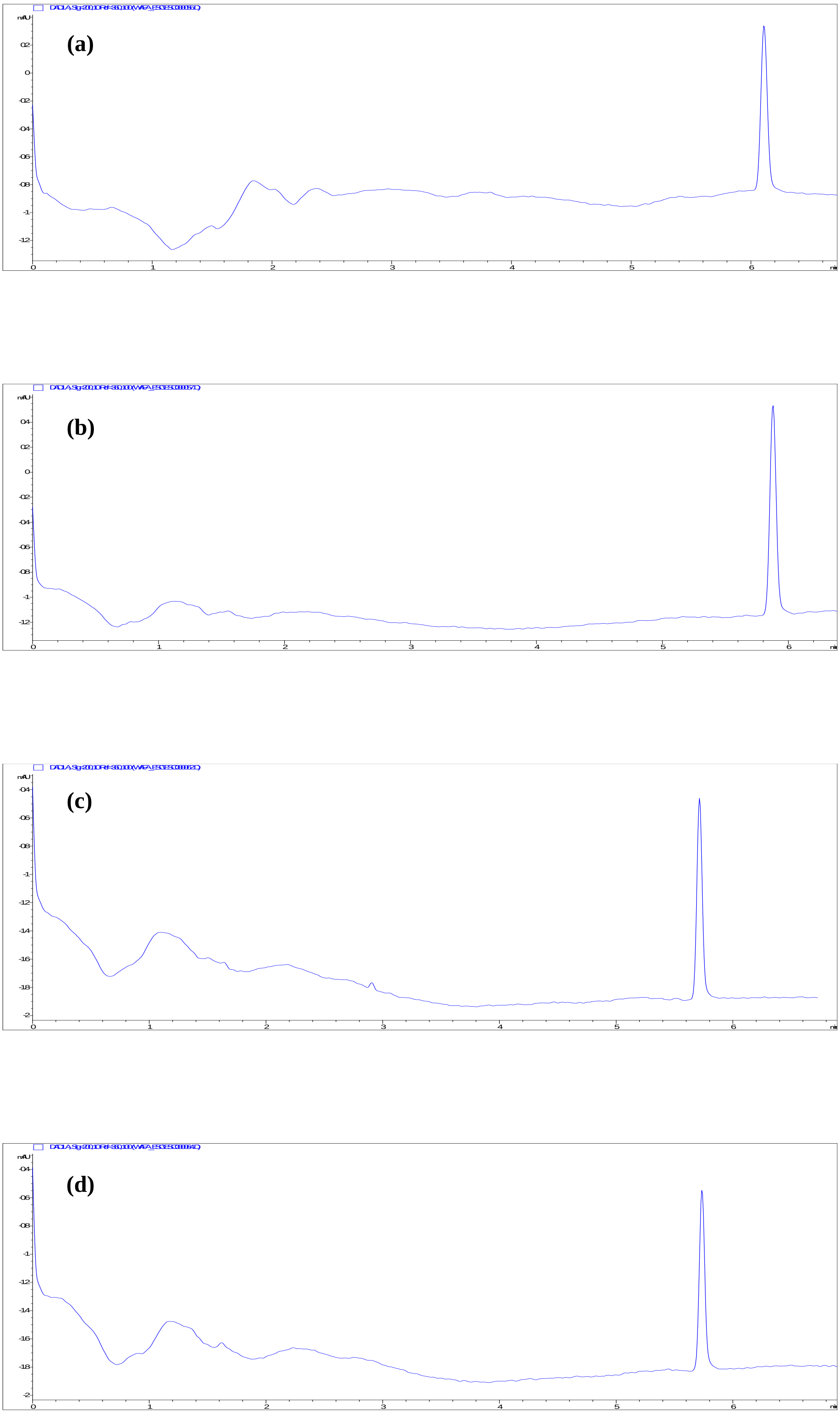


Figure 5.49. *The Effect of Buffer Concentration on the Peak Characteristics of ESC-OX (a) 10 mM, (b) 15 mM, (c) 20 mM, (d) 25 mM.*

5.4.4. Organic modifier

Addition of organic solvent has an unpredictable effect on the CE separation because it affects many parameters such as the BGE viscosity, dielectric constant and zeta potential (Baker, 1995 b, p. 29). The effect of organic solvent such as MeOH and ACN was also studied because in some studies the addition of an organic solvent improves mobility of the analyte, separation and selectivity (Lee, Price and Sheil, 1995, p. 2689-2694). In the case of ACN 10% in BGE, there was neither reasonable effect on the electrophoretic behavior of ESC nor on the resolution and the peak areas. However, the peak shape was unacceptable by the addition of MeOH 10%. The addition of MeOH result in increasing the viscosity of the BGE, which reduce the electrophoretic mobility of the analyte (Fujiwara and Honda, 1987, p. 2773-2776). Besides ESC-OX and MET were dissolved in distilled water, no modifier was used in this study. The electropherograms of ACN 10% and MeOH 10% are given in Figure 5.50.

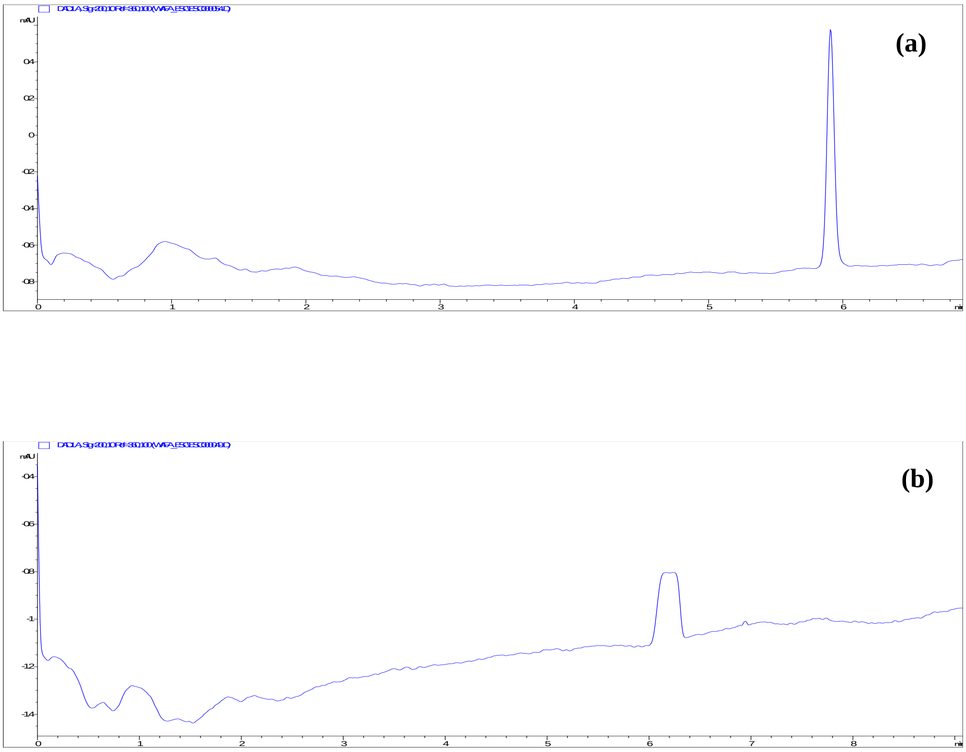


Figure 5.50. *The Effect of Organic Modifier on the Peak Characteristics of ESC-OX (a) ACN 10%, (b) MeOH 10%.*

5.4.5. Separation voltage

The effect of the applied voltage (+27.5 kV, +25.0 kV and +22.5 kV) on the CE separation of ESC-OX was investigated. Application of higher voltage results in reduction of migration time and increasing theoretical plate number but reduce selectivity and resolution. In addition, utilization of higher voltage result in increasing current which subsequently produce Joule heating in the capillary. The appropriate voltage can be determined from the Ohm's law plot (Issaq et al., 1991, p. 155-161). The plot of applied voltage versus current is illustrated in Figure 5.51 (a). However, higher voltage result in the generation of excessive Joule heating and degrade the separation efficiency. So, +22.5 kV was selected as optimal separation voltage as it produces well-shape peak with good separation efficiency and a reasonable run time. The plots of separation voltage on the migration time is illustrated in Figure 4.51 (b). The effect of applied voltage on the migration time is illustrated in the electropherograms in Figure 5.52.

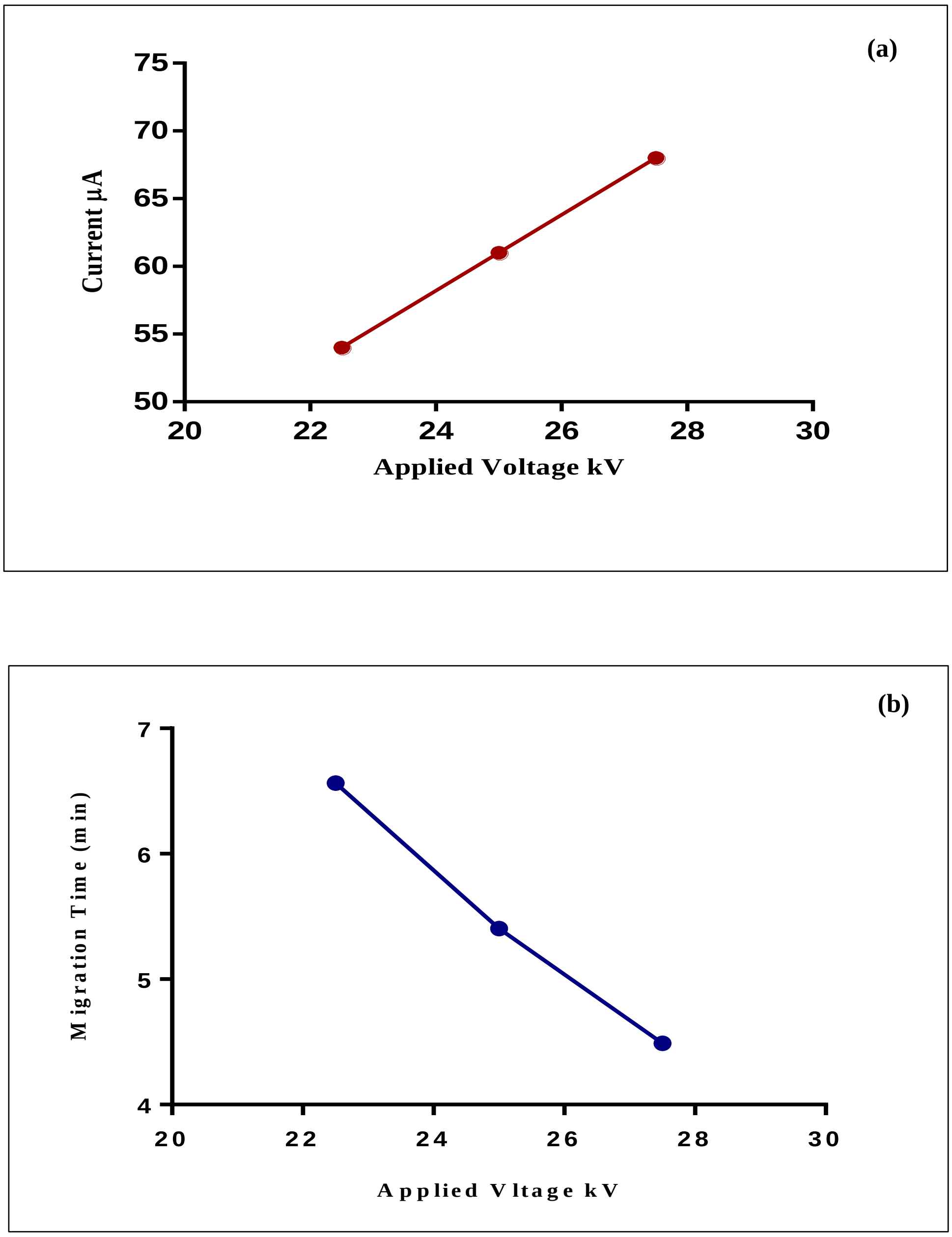


Figure 5.51. *(a) Ohm's Law Plot. Applied Voltage Versus Current, (b) The plot of Applied Voltage versus Migration Time.*

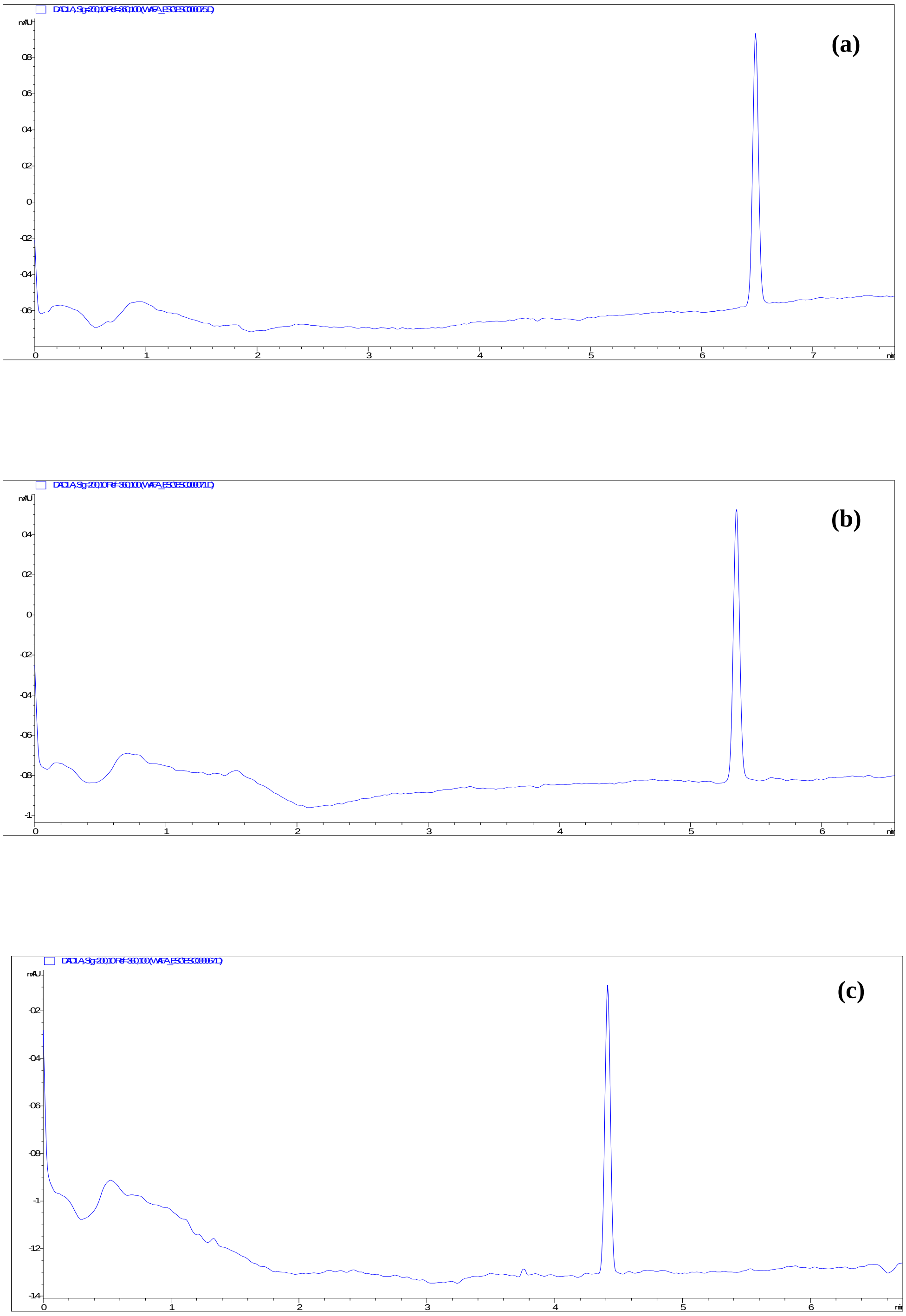


Figure 5.52. *The Effect of Applied Voltage on the Characteristics of ESC-OX (a) +22.5 kV, (b) +25 kV, (c) +27.5 kV.*

5.4.6. Injection pressure and time

In this work, hydrodynamic injection was used by application of different pressure at different time. The injection pressure and time were also studied by applying 50 mbar for 5 sec., 50 mbar for 10 sec. and 100 mbar for 5 sec.; symmetrical peak shape with reasonable time and intensity was obtained at 50 mbar and 10 sec. The effect of injection pressure and time on peak characterization are shown in Figure 5.53.

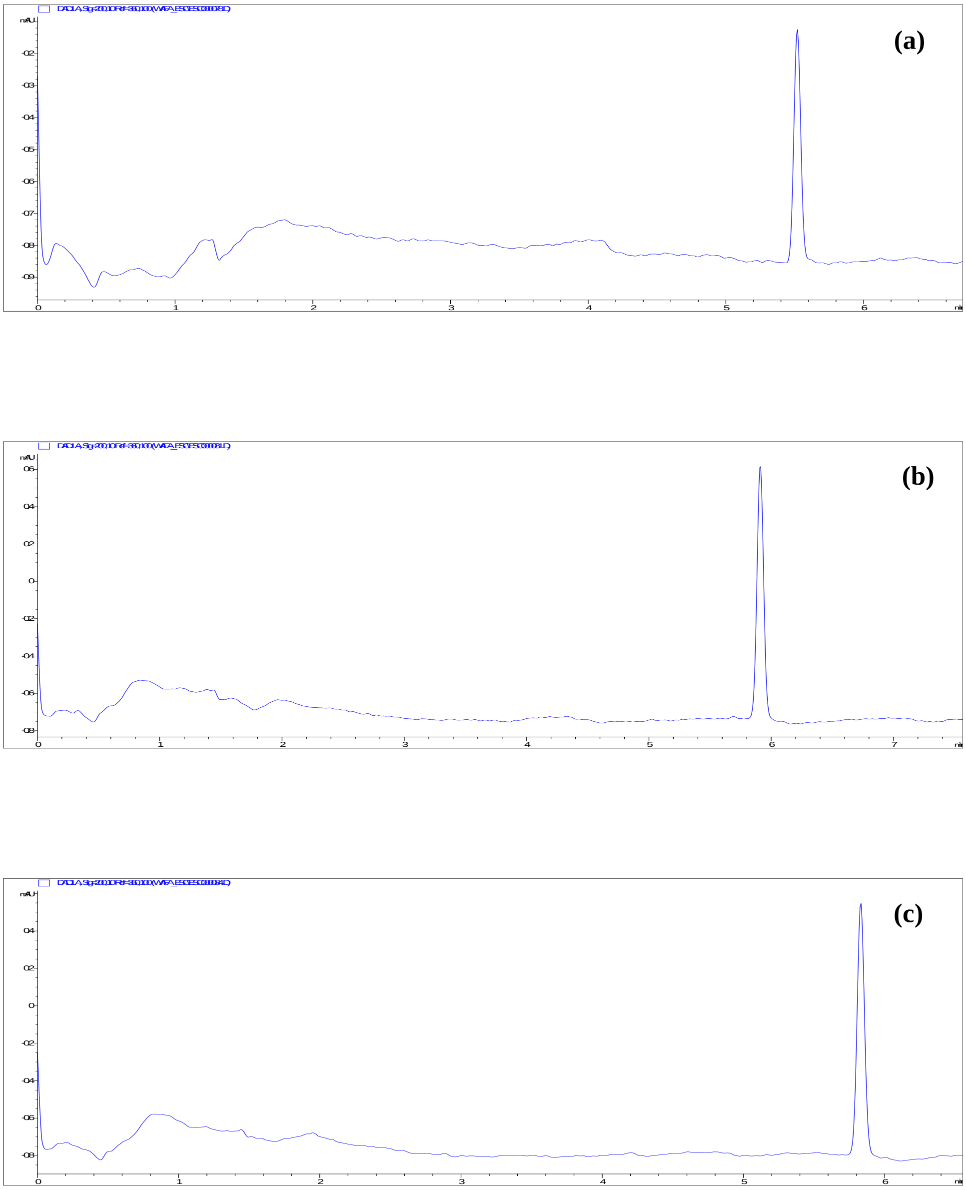


Figure 5.53. *The Effect of Injection Pressure and time on Peak Characteristics of ESC-OX (a)50 mbar for 5 sec., (b)50 mbar for 10 sec. and (c)100 mbar for 5 sec.*

The optimum analytical and instrumental conditions for CE method are given in Table 5.36.

Tablet 5.36. *Optimum Conditions for Analysis of ESC-OX using CE Method.*

Parameters	**Value**
Capillary	Fused silica capillary with 40 cm effective (48.5 cm total, 75 µm i. d.) length.
Buffer pH and concentration	Phosphate buffer pH 2.5 (15 mM)
Applied voltage	+22.5 kV
Injection pressure / time	50 mbar / 10 sec.
Detection wavelength	200 nm
Ambient temperature	25℃
Internal standard	MET
Total run time	7 min (6.51 ± 0.07 and 6.731 ± 0.08 min. for ESC-OX and IS, respectively).

Electroosmotic mobility decreases in low-pH media, and the analytes mostly migrate electrophoretically. Properties of the compounds could, therefore, easily be calculated from the migration time data and the electropherogram. The apparent electrophoretic mobility of IS and ESC were calculated by using the following equation (Weinberger, 1999, p. 31):

$$\mu_{EP} = ({}^{l}/_{t_m} - {}^{l}/_{t_{nm}})(L/V) \tag{5.6}$$

μ_{EP} = Electrophoretic mobility.
l = Effective capillary length.
t_m = Migration time.
t_{nm} = Migration time of neutral marker.
L = Total capillary length
V = Voltage.

According to the Equation 4.6 the electrophoretic mobilities of ESC-OX and IS were calculated as 2.2074 × 10^{-4}cm^{2}V^{-1} S^{-1} and 2.1348X 10^{-4} cm^{2}V^{-1} S^{-1}, respectively.

5.4.7. SST

Evaluation of system suitability is a very important criterion which confirm that the developed method and the instrument used give acceptable analytical data. The SST parameters were calculated by using Agilent ChemStation software which are summarized in the Table 5.37. All parameters were within the recommended values of USP 30 NF 25, 2006.

Table 5.37. *SST for the Developed CE Method.*

Parameters	ESC-OX	MET	Recommended value
N	66655	70080	>2000
Mt *(min)*	6.51	6.731	
T	1.013	0.994	< 2
As	0.99	1.00	$0.95<A_S< 1.2$
Rs	-	2.12	>2
Mt, RSD%	0.07	0.08	<1

5.4.8. Validation of the CE method

The analytical performance of the CE method was evaluated in terms of linearity, sensitivity, LOD, LOQ, accuracy, repeatability and selectivity according to the ICH guideline 2005.

5.4.8.1. Linearity

The linearity of the method was examined in the range of 0.016 to 32.25 µg mL^{-1} and the calibration plots were chosen in this range 0.0264 to 2.75 µg mL^{-1} because the linearity with good regression was at this range. LOD and LOQ values were calculated by using the intensity of the signal in the electropherogram by taking S/N ratio of 3 for LOD and 10 for LOQ. Good correlation for the calibration curve with regression near to 0.9999. The intercepts of the plots were very close to zero in case of analysis of reference solutions. The statistical evaluation of linearity is shown in Table 5.38; correlation between groups was calculated by one way ANOVA.

In case of urine, the calibration plots were chosen in the range of 0.0269 to 4.351 µg mL^{-1}. It is lower than the concentration expected to be secreted in urine, the straight-line equation correlation coefficient is 0.9987. The statistical evaluation of linearity is shown in Table 5.39.

Table 5.38. *Statistical evaluation of Intra and Inter-Days Linearity Analysis of ESC-OX Standard Solution.*

	Intra- day (n=6)			**Inter-days (n=18)**
Range (µg. mL^{-1})	0.0264 - 2.750			
Slope ± SE	5.218 ± 0.0360	5.157 ± 0.03056	5.124 ± 0.02716	5.166 ± 0.02904
Intercept ± SE	-0.07158 ± 0.04341	-0.06407 ± 0.03683	-0.03458 ± 0.03273	-0.05674 ± 0.03500
r^2	0.9998	0.9999	0.9999	0.9999
95% CI of Slope	5.118 to 5.318	5.072 to 5.242	5.048 to 5.199	5.085 to 5.247
95% CI of Intercept	-0.1921 to 0.04892	-0.1663 to 0.03817	-0.1254 to 0.05627	-0.1539 to 0.04041
LOD (µg mL^{-1})	0.00422	0.00381	0.00352	0.00385
LOQ (µg mL^{-1})	0.014052	0.01270	0.0117	0.0128
ANOVA	F (1.161, 5.804) = 0.6722, P = 0.4671			

Table 5.39. *Statistical Evaluation of Intra and Inter-Days Linearity Analysis of Urine Sample Spiked with ESC-OX Standard solution.*

	Intra- day (n=7)			Inter-days (n=21)
Range (µg mL^{-1})	0.0269- 4.351			
Slope ± SE	5.368 ± 0.0646	5.409 ± 0.1143	5.409 ± 0.1143	5.385 ± 0.08525
Intercept ± SE	-0.04859 ± 0.1281	-0.02776 ± 0.2265	-0.04143 ± 0.1538	-0.03926 ± 0.1689
r^2	0.9993	0.9978	0.9990	0.9987
95% CI of Slope	5.202 to 5.534	5.115 to 5.703	5.180 to 5.579	5.166 to 5.605
95% CI of Intercept	-0.3779 to 0.2808	-0.6100 to 0.5545	-0.4368 to 0.3539	-0.4735 to 0.3950
LOD (µg mL^{-1})	0.00524	0.00549	0.00477	0.00517
LOQ (µg mL^{-1})	0.01747	0.01830	0.01592	0.01723
ANOVA	F (1.000, 6.002) = 0.8288, P = 0.3977			

5.4.8.2. Accuracy

The accuracy of the method was verified through the recovery test. The accuracy was carried out for the standard solution and for spiked ESC-OX in the urine matrix. Three concentrations (0.0264, 0.3302 and 2.7517 µg mL^{-1}) for standard solution and (0.0269, 0.3343 and 4.3515 µg mL^{-1}) for spiked urine sample were examined six times a day for three days. The result shows that all recoveries are within 100 ± 5%. The results are summarized in Table 5.40 and 5.41.

The accuracy for the pharmaceutical products was performed by comparing the results of the sample analysis results with those of obtained in the HPLC method, as recommended in the ICH Q2(R1) guideline.

Table 5.40. *Accuracy Data for ESC-OX Standard Solution.*

		ESC-OX standard		
Day 1 (n=6)	Added (µg mL^{-1})	0.0264	0.3302	2.7517
	Found (mean ± SD)	0.0276 ± 0.00031	0.3443 ± 0.00452	2.7469 ± 0.02085
	Recovery%	104.48	104.29	99.82
	SD	1.18	1.37	0.76
	SEM	0.48	0.56	0.31
	RSD%	1.13	1.31	0.76
Day 2 (n=6)	Added (µg mL^{-1})	0.0264	0.3302	2.7517
	Found (mean ± SD)	0.0277 ± 0.00029	0.3394 ± 0.00270	2.7381 ± 0.03063
	Recovery%	105.07	102.77	99.50
	SD	1.11	0.82	1.11
	SEM	0.45	0.33	0.45
	RSD%	1.06	0.80	1.12
Day 3 (n=6)	Added (µg mL^{-1})	0.0264	0.3302	2.7517
	Found (mean ± SD)	0.0276 ± 0.00030	0.3341 ± 0.00331	2.777 ± 0.03743
	Recovery%	104.51	101.17	100.93
	SD	1.14	1.00	1.36
	SEM	0.46	0.41	0.56
	RSD%	1.09	0.99	1.35
Inter-days (n=18)	Added (µg mL^{-1})	0.0264	0.3302	2.7517
	Found (mean ± SD)	0.0277 ± 0.00030	0.3392 ± 0.00351	2.754 ± 0.02963
	Recovery%	104.69	102.74	100.09
	SD	1.11	1.66	1.21
	SEM	0.45	1.62	0.50
	RSD%	1.06	0.68	1.21

Table 5.41. *Accuracy Data for ESC-OX Spiked Urine Sample.*

Spiked urine				
Day 1 (n=6)	Added (µg mL^{-1})	0.0269	0.3343	4.3515
	Found (mean ± SD)	0.0237 ± 0.00039	0.3061± 0.00433	4.2599 ± 0.0866
	Recovery%	88.11	92.02	97.89
	SD	1.46	1.54	1.99
	SEM	0.596	0.629	0.812
	RSD%	1.66	1.67	2.03
Day 2 (n=6)	Added (µg mL^{-1})	0.0269	0.3343	4.3515
	Found (mean ± SD)	0.0231 ± 0.00040	0.3077 ± 0.005151	0.30695 ± 0.00496
	Recovery%	86.05	91.53	97.05
	SD	1.49	1.30	1.68
	SEM	0.608	0.529	0.684
	RSD%	1.73	1.42	1.73
Day 3 (n=6)	Added (µg mL^{-1})	0.0269	0.3343	4.3515
	Found (mean ± SD)	0.02353 ±0.00039	0.3069 ± 0.004960	4.1631 ± 0.044954
	Recovery%	87.51	91.77	95.66
	SD	1.46	1.48	1.03
	SEM	0.597	0.605	0.422
	RSD%	1.67	1.61	1.07
Inter-days (n=18)	Added (µg. mL^{-1})	0.0269	0.3343	4.3515
	Found (mean ± SD)	0.02346 ±0.00039	0.30695 ± 0.00481	4.2155 ± 0.06816
	Recovery%	87.22	90.35	96.87
	SD	1.64	1.37	1.79
	SEM	0.670	0.56	0.73
	RSD%	1.88	1.52	1.85

5.4.8.3. Precision

The precision of the proposed CE method was assessed for (intra-day) and intermediate precision (inter-days) in three concentrations (0.0264, 0.3302 and 2.7517 μg mL^{-1}). Statistical evaluation of the precision results for standard showed that RSD% values were below 2% and in the case of urine sample were below 3% for both intra-day and inter-days. These results indicate that method precision is analytically acceptable for both bioanalysis and pharmaceutical analysis according to ICH guidelines. The results are summarized in Table 5.42.

Table 5.42. *Precision Data of ESC Standard and Spiked Urine.*

Medium	Parameters	Lower Concentration (n=18)	Medium Concentration (n=18)	High Concentration (n=18)
ESC-OX Standard Solution	Mean	0.08613	1.696	14.17
	Lower limit CI	0.08538	1.682	14.09
	Upper limit CI	0.08688	1.710	14.26
	SD	0.00152	0.02839	0.1725
	SEM	0.00036	0.006691	0.04067
	RSD%	1.76	1.67	1.22
	Parameters	**Lower Concentration (n=18)**	**Medium Concentration (n=18)**	**High Concentration (n=18)**
ESC-OX Spiked Urine Sample	Mean	0.08708	1.614	22.66
	Lower limit CI	0.08590	1.601	22.45
	Upper limit CI	0.08826	1.626	22.87
	SD	0.002380	0.02469	0.4190
	SEM	0.0005610	0.005821	0.09875
	RSD%	2.73	1.53	1.85

5.4.8.4. Selectivity

The developed method was examined for selectivity by comparing the blank tablet matrix and blank oral solution matrix with standard solution and the pharmaceutical preparations (Figure 5.54). In addition, the blank urine sample was compared with the urine sample spiked with MET and urine sample spiked with ESC-OX and MET (Figure 5.55). It was verified that there was no peak interfering with the ESC-OX and MET peaks. It can be concluded that the established method is specific for detection of ESC-OX both for the urine sample and pharmaceutical dosage forms.

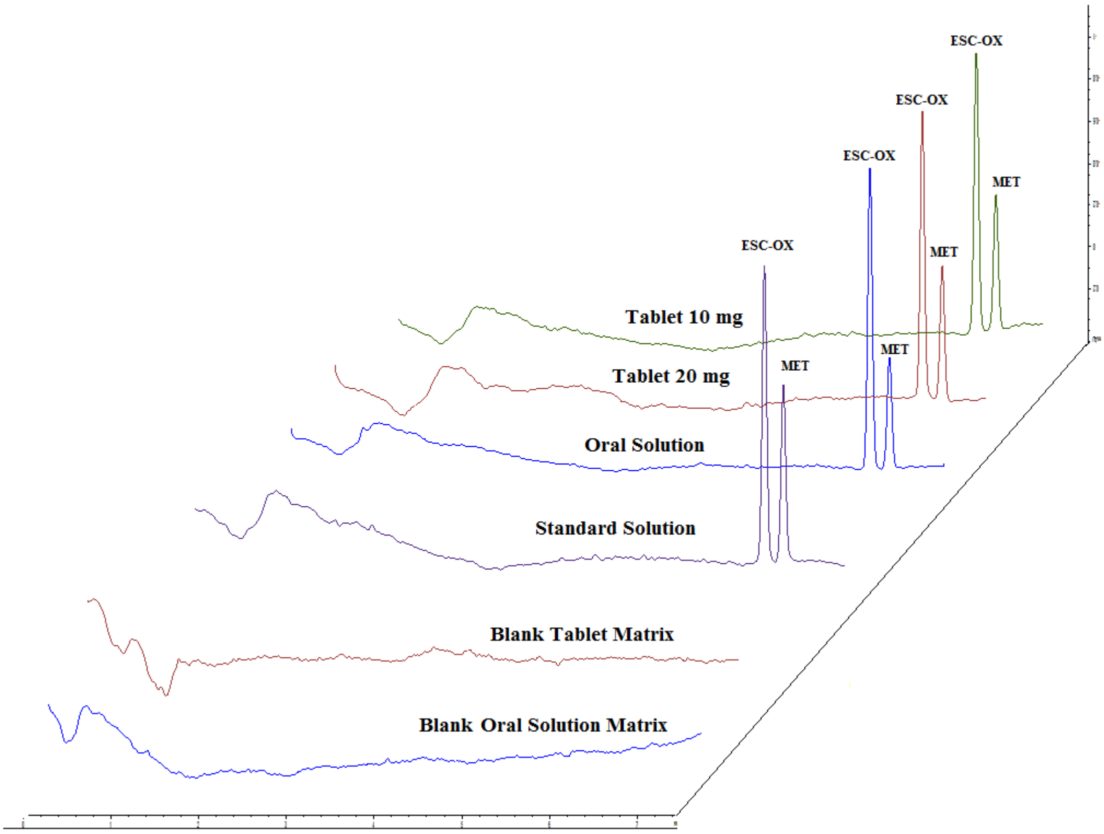


Figure 5.54. *The Electropherograms of the Blank Tablet Matrix, Blank Oral Solution Matrix, Standard Solution, Tablet 20 mg, Tablet 10 mg and Oral Solution.*

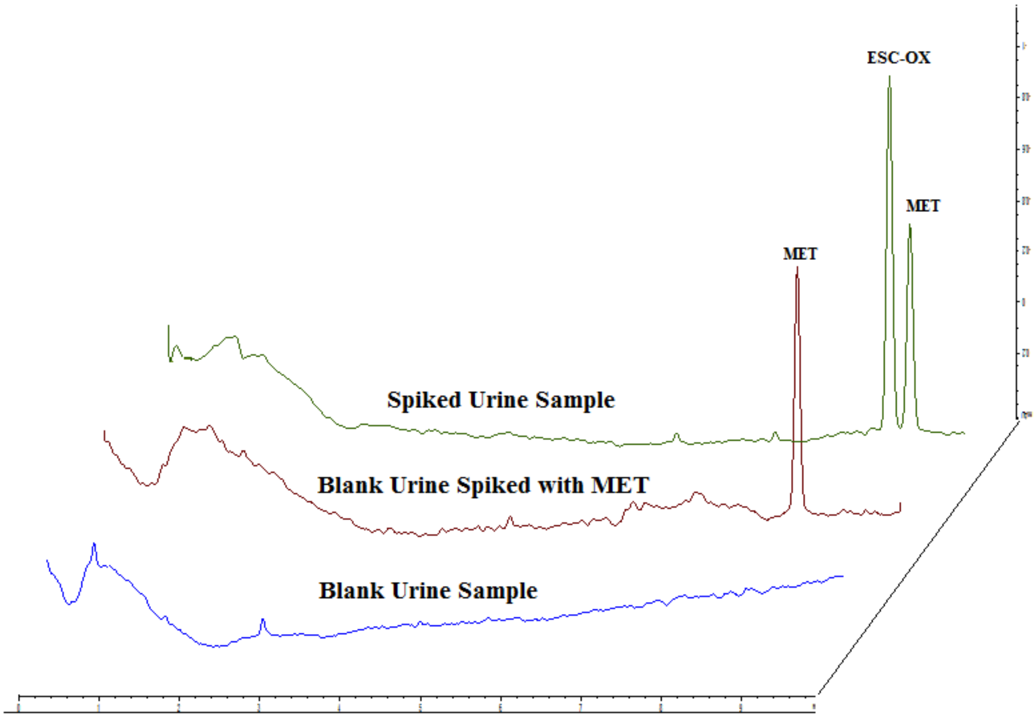


Figure 5.55. *The Electropherograms of the Blank Urine Sample, Blank Urine Spiked with MET and Spiked Urine Sample.*

5.4.8.5. Stability

The stability of standard solution and urine sample was examined and there was no change in the signal intensity for one month for standard and urine sample solutions when there were stored at +4°C and 24 hrs.

5.4.9. Analysis of human urine spiked with the ESC-OX solution

The main advantages of CE method for determination of drugs in biological samples are rapid pretreatment of sample such as LLE, use of inexpensive organic solvents, and other chemicals, small sample size requirements and low reagent consumption. Bioanalysis are usually a troublesome task since the matrix is often very complex. Urine was selected in this study instead of plasma because the collection of urine specimen is noninvasive procedure, also urine is easier to process than other biological fluids as it doesn’t contain of protein and another cellular constituent. Urine may contain salt, ions and organic compounds which are sources of high conductivity and lower effective separation due to peak-broadening. Therefore, LLE was performed to get a net electrophoretic sample profile, low detection limit and good reproducibility. The developed method was sensitive enough to obtain well-shaped peaks, without any interference at 6.62 ± 0.074 and 6.87 ± 0.09 min (mean ± SD, n=10), respectively, for ESC-OX and IS in spiked urine; the peaks were similar to those obtained from standards (Figure 5.55).

Shifting in migration time is accepted as normal in biological sample analysis, due to the presence of other charged ions in the sample. Statistical evaluation of the results is presented in Table 5.37- 5.39. Approximately 8.0% of ESC-OX is eliminated unchanged in urine (Søgaard et al., 2005, p.1400-1406). This CE method for ESC-OX spiked human urine has a LOD and LOQ of 0.00517 µg mL^{-1} and 0.01723 µg mL^{-1} respectively, which is lower than the percentage of ESC-OX eliminated in urine so this method can be used for determination of drug in urine and in pharmacokinetic studies.

5.4.10. Analysis of ESC-OX in pharmaceutical formulations

pKa of ESC is 9.5, so at pH 2.5 it becomes positively charged and transports to the cathode. At this pH, the silanol groups become more protonated and capillary wall is less charged leading to negligible EOF; the separation in this case depends solely on electrophoretic flow. Performing CE with reduced EOF has a number of benefits such as; more reproducible separation since a little change in buffer concentration or pH will cause a significant change in the EOF which result in change in migration time. At this pH most excipients are neutral, they will be stopped at the beginning of the capillary so they will not interfere with the separation of ESC-OX which offer superiority over HPLC analysis of pharmaceutical dosage form where the analyte peak can be masked by the co-elution of neutral excipient or flavoring compounds (Morzunova, 2005, p.158-170).

The current method was applied on two different pharmaceutical products; two products in tablet forms containing 20 mg or 10 mg active ingredient per tablet, and an oral solution containing 10 mg mL^{-1}. The peaks obtained from the all dosage forms were similar to those from ESC-OX standard and there was no interference from the matrix (Figure 5.56). The content of the tablets and oral solution was found to be within the limits specified by USP36. The results of the different dosage forms are shown in Table 5.43.

Table 5.43. *Assay Results of ESC-OX 20 mg, 10 mg Film Tablet and Oral solution (n=18).*

	Tablet (20 mg)	**Tablet (10 mg)**	**Oral Solution (10 mg mL^{-1})**
Mean (mg)	20.46	10.28	10.23
Minimum (mg)	20.06	10.08	10.14
Maximum (mg)	20.77	10.43	10.35
SD (mg)	0.2501	0.1256	0.07943
RSD %	1.22	1.22	0.78
SEM (mg)	0.1021	0.05129	0.03243
Bias%	-2.3	-2.8	-2.3
95% CI of Average	20.18 to 20.72	10.15 to 10.41	10.19 to 10.31
% Content Uniformity	102.3	102.8	102.3

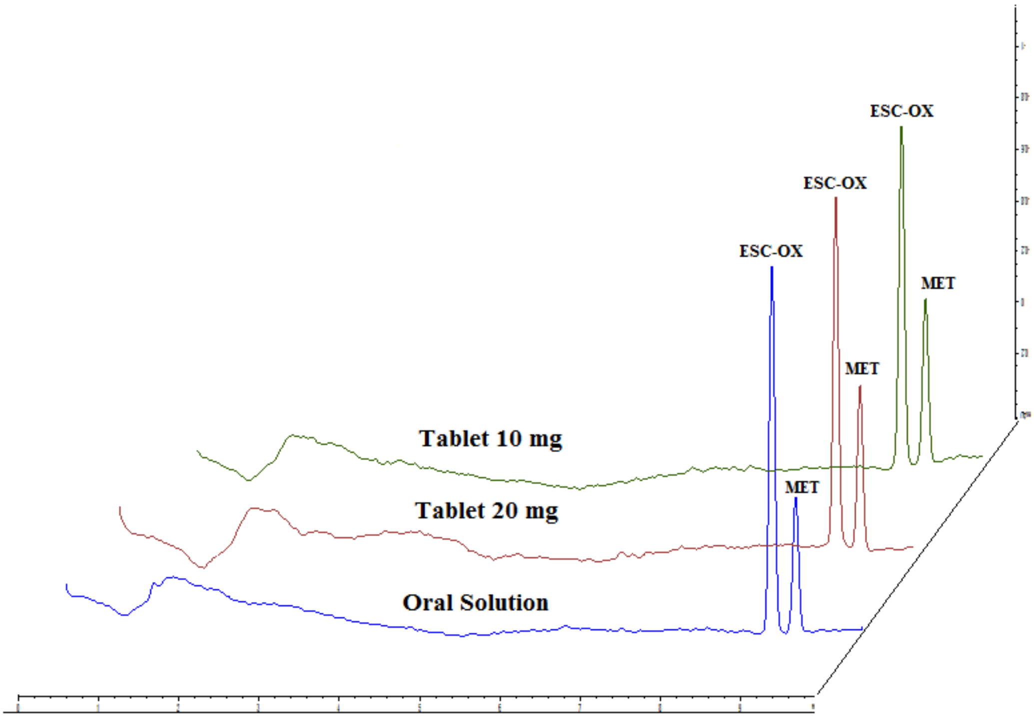


Figure 5.56. *The Electropherograms of the Pharmaceutical Preparations.*

5.4.11. General evaluation of CE method

The determination of ESC-OX in different pharmaceutical dosage forms and spiked urine sample has been accomplished by applying the developed CE method. The current method exhibited many merits such as rapid, economic, high sensitivity and reproducibility. Using of BGE, which is free from organic solvent and the simple extraction procedure for urine sample preparation makes the method a good analytical choose for determination of ESC-OX in its all available dosage forms and biological liquids for routine analysis instead of already present methods. The developed method shows excellent selectivity, since urine sample electropherograms display no interference from the endogenous substance. In spite of using spiked urine sample the developed method shows the possibility of monitoring this drug since the LOD and LOQ is lower than the amount excreted in the urine, which makes the method suitable for clinical, pharmacokinetic and forensic purposes.

5.5. Development and Optimization of the LC-ESI-MS/MS Method

In previous studies, determination of ESC-OX in urine samples was performed by using ESI-TOF-MS coupled with on-line extraction by using a silica capillary. In another study, a special bar was used for extraction of ESC-OX and its metabolites from urine (Johannesson and Bergquist, 2007, p. 1045–1048; Unceta et al, 2013 p. 448-453). The LOD was 10 pg mL^{-1} and 2 ng mL^{-1} in these studies, respectively. The developed method in this thesis provides estimation of the ESC-OX in urine by using simple LLE with low LOD.

LC-MS/MS has the ability to quantitively analyze and detect a small amount of analyte in biological fluids. LC-ESI-MS/MS was used because only 8% of the ESC-OX is eliminated in urine and by using the quadrupole analyzer even small amount of analyte can be analyzed with high sensitivity and low detection limits.

In this thesis, a simple LLE was used for urine pretreatment as it is considered economic and the most effective extraction procedure in comparison to SPE. Gradient elution was used to reduce the interference originating from the urine matrix, which is usually carrying hydrophilic inorganic salts that prevent proper ionization in ESI chamber (Xu et al, 2007, p. 342–355).

ESC-OX is a weak base salt and can be readily protonated. Thus, the positive mode was performed by ion spray ionization to produce molecular ions for triple quadrupole mass spectrometric detection. The instrumental and analytical conditions were optimized by using 7.8 ng mL^{-1} ESC-OX in 50% MeOH (*v/v*). Several instrumental modes were used (full scan, selective ion monitoring (SIM), multiple reaction monitoring (MRM), precursor ion scan) for sensitive analyte detection (Figure 5.57).

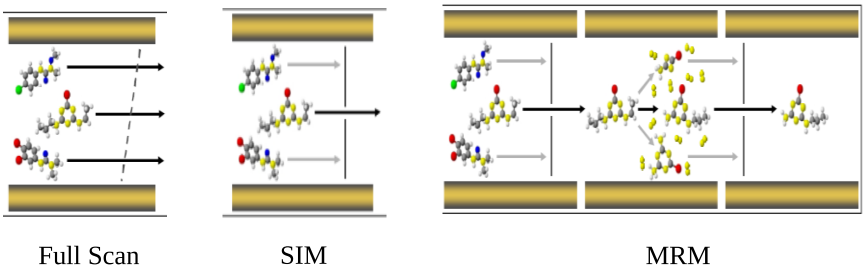
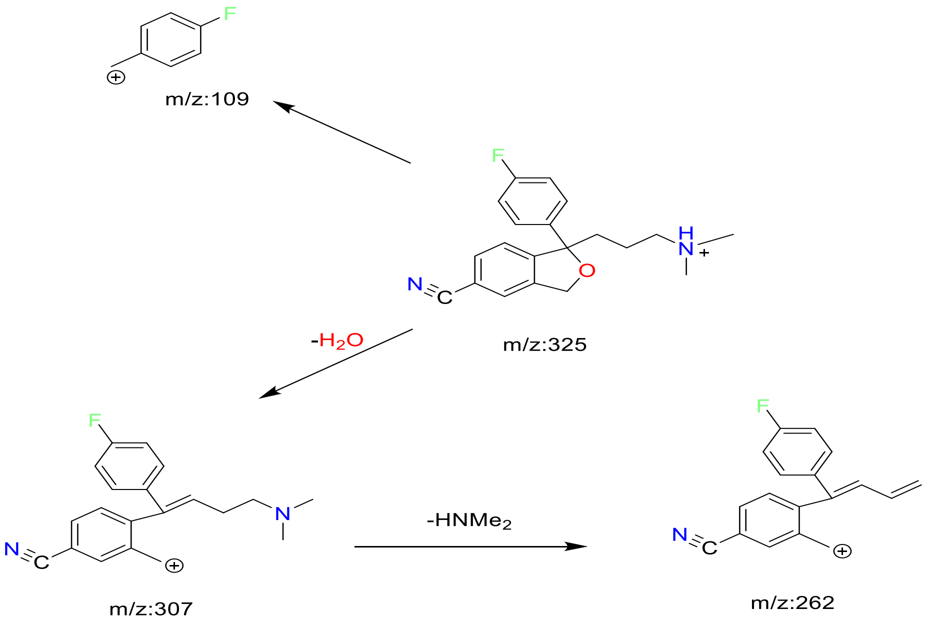


Figure 5.57. *Illustrative Image for Full Scan, SIM and MRM Modes tested ESC-OX Analysis.*
Reference: *Schreiber, 2010, p. 2.*

The quantitative analysis of ESC-OX was started by monitoring the full-scan chromatogram from 50 to 500 Da and scan-cycle time was 500 msec. The fragmentation voltage was 135 V and cell accelerator voltage 7 V. The most intense peak in the chromatogram was related to the $[M+H]^+$ with 325 m/z $[ESC+H]^+$. Then (SIM) was performed by changing the fragmentation voltage from 20, 40, 60, 80, 100, 120, 140 to 160 V. The most intense peak was found at 100 V. Thereafter, the product ions were selected by changing the collision frequency 0, 10, 20, 30 V in the MS^2 by fragmentation of 325 m/z parent ions. The most appropriate collision frequency was at 20 V. The most intensive product ion was 109.10 m/z that is why it is used for quantification as a quantifier and the second intensive product ion was 262.00 m/z it is used for qualification as a qualifier. To get signal of both ions, (MRM) was used. The expected fragmentation manner that was occurred for the parent ion molecule 325 m/z is illustrated in Scheme 5.2. The parent ion 325 m/z produced daughter ion 307 m/z by losing H_2O molecule (-18 Da), which was further loss side-chain amine (-45 Da). The product ion peak at 109 m/z can be related to the 4-fluoro-1-methylbenzene (Raman et.al., 2010, p. 895–901; Rao, Raju and Narsimha, 2010, p. 1729 – 1738).



Scheme 5.2. *The Proposed Mechanism for the Fragmentation of the ESC.*

The ESI- parameters were optimized in order to enhance desolvation and focusing of ions. Improving of desolvation result in reduction in noise that may come from mobile phase and matrix. The parameters of optimization are listed in Table 5.44. The chromatograms of 109.10 m/z and 262.00 m/z, and MS/MS product ions spectra of 262.00 m/z and product ion 109.10 m/z are demonstrated in Figure 5.58.

Table 5.44. *Optimized MS/MS parameters for ESC-OX.*

Parameter	Value
Collision Gas	High purity nitrogen
Gas Temperature (°C)	300
Nebulizer gas(psi)	45
Capillary voltage (V)	3500
Gas Flow (L min^{-1})	5.0
Sheath Gas heater	250
Sheath Gas Flow (mL min^{-1})	11
Charging Voltage (V)	500

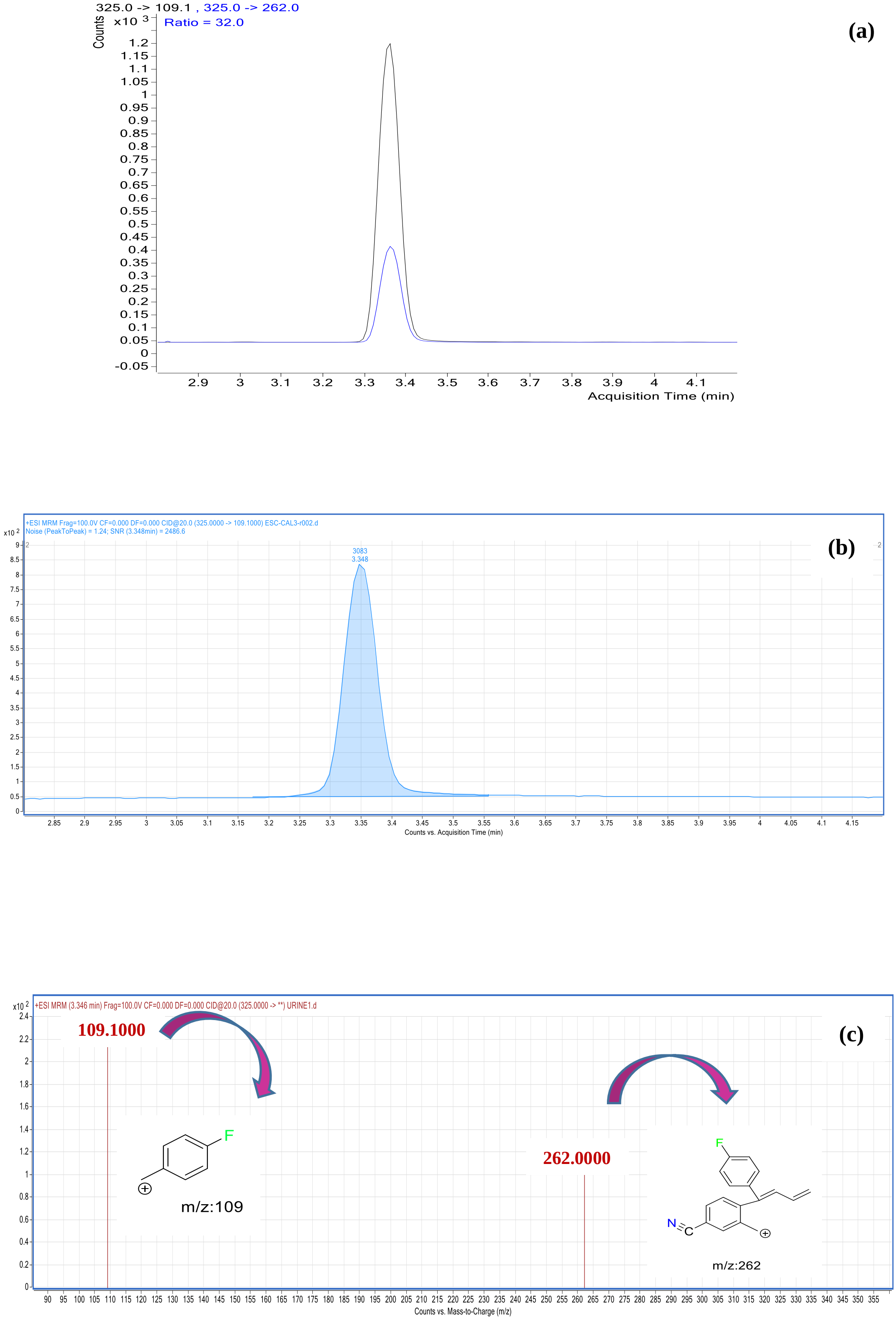


Figure 5.58. *The MRM Chromatogram of (a) 109.10 m/z and 262.00 m/z, (b) Chromatogram of 109.10 m/z (c) MS/MS spectra of daughter 262.00 m/z and product ion 109.10 m/z.*

The final selected conditions for the determination of ESC-OX by using LC-ESC-MS/MS are summarized in the table 5.45.

Table 5.45. *Final LC-ESI-MS/MS Conditions for Analysis of ESC-OX and its Impurities.*

Parameters	value
Mobile phase composition	A: 0.1 % F. A in ACN and B: 0.1 F. A in water
Column temperature	40 ℃
Flow rate	0.35 μL.min^{-1}
Injection volume	2μL

5.5.1. SST

The performance of the developed method was evaluated by using SST for the LC system. All parameters were calculated by Agilent Mass Hunter Workstation Software-Qualitative Analysis Program B.06.00. The parameters are summarized in Table 5.46. The *K'* was 27 which is more than the recommended value. However, it is preferred to avoid the interference from the biological matrix especially phospholipids.

Table 5.46. *SST for the Developed Method by Using Zorbax Eclipse RP C_{18}.*

Parameters	ESC-OX	Recommended value
N	24337	>2000
N/m	486740	
t_R	3.348	
T	1.1	< 2
As	0.89	$0.95 < A_S < 1.2$
K'	27	$1 < K' < 10$
RSD% of t_R	0.28	<1

5.5.2. Validation of LC-ESI-MS/MS method

5.5.2.1. Linearity and range

In this study, linearity was evaluated by using Weighted-Least Squares equation (1/x). Use of Weighted-Least Squares equation is recommended specially in analysis of pharmaceuticals in biological fluids because the variance may be not homogenous over the whole concentration range (Burrows and Watson, 2015, p. 1763–1774). The linearity of the proposed method was examined in the range 0.0078 to 391.370 ng mL^{-1} in the standard and urine samples. The calibration curve was constructed in the range of 0.079 to 196.450 ng mL^{-1}. IS was not used due to unavailability of isotopically labelled analogues. For this reason, the calibration curve was constructed in urine sample to eliminate the matrix effect. Three sets were prepared and calibration curve was calculated plotting peak area ratios against concentration. Data related to the method linearity is represented in Table 4.46. The inter-days' groups were examined with one-way ANOVA.

LOD was calculated from signal to noise ratio and multiplying by 3. The LOD of standard solution was 3.88 pg mL^{-1}. In case of urine, LOD was 10.66 pg mL^{-1}. The higher LOD in case of urine relative to standard solution could be due to the ion suppression effect from the endogenous urine matrix which may have not been eliminated completely during extraction process (Antignac et al, 2005, p.129-136). Another reason, is the matrix compounds that are not easily evaporated, reduce the solvent evaporation rate which result in increasing the analyte drop's surface tension and reduce the quantity of analyte that transformed into the gaseous phase (Kebarle and Tang, 1993, p. 972-986). In spite of using simple LLE the obtained LOD was acceptable.

LLOQ is most usually used parameter in LC-MS/MS for evaluation of the quantification capability of the method. It is the lowest quantity from the calibration curve that can be quantified with not more that ±20 % C.V of accuracy and precision (Shah et al, 2000, p.1551-1557). The statistical data are outlined in Table 5.47 and 5.48.

Table 5.47. *Statistical evaluation of Intra and Inter-Days Linearity Analysis of ESC-OX Standard Solution.*

	Intra-day (n=5)			**Inter-days (n=15)**
Range (ng mL^{-1})	0.079- 196.45			
Slope ± SE	335.9 ± 6.163	327.1 ± 1.325	333.4 ± 2.518	332.1 ± 1.554
Intercept ± SE	111.6 ± 27.60	113.9 ± 5.934	112.6 ± 11.28	112.7 ± 6.961
r^2	0.9990	1.000	0.9998	0.9999
95% CI of Slope	316.3 to 355.5	322.9 to 331.3	325.4 to 341.4	327.2 to 337.1
95% CI of Intercept	23.76 to 199.4	95.02 to 132.8	76.67 to 148.4	90.53 to 134.8
LLOQ (ng mL^{-1})	0.079	0.079	0.079	0.079
ANOVA	F (1.595, 6.378) = 0.6489, P = 0.5207			

Table 5.48. *Statistical Evaluation of Intra and Inter-Days Linearity Analysis of Urine Sample Spiked with ESC-OX Standard solution.*

	Intra- day (n=5)			**Inter-days (n=15)**
Range (ng mL^{-1})	0.079 - 196.450			
Slope ± SE	412.2 ± 5.583	399.6 ± 3.764	411.0 ± 4.929	413.9 ± 6.421
Intercept ± SE	692.2 ± 528.6	575.1 ± 356.4	670.6 ± 466.7	289.5 ± 28.76
r^2	0.9995	0.9997	0.9996	0.9993
95% CI of Slope	394.5 to 430.0	387.6 to 411.6	395.4 to 426.7	393.4 to 434.4
95% CI of Intercept	-990.5 to 2374	-558.9 to 1709	-814.6 to 2156	197.3 to 380.4
LLOQ (ng mL^{-1})	0.079	0.079	0.079	0.079
ANOVA	F (1.009, 4.035) = 2.847, P = 0.1663			

5.5.2.2. Accuracy

Accuracy evaluates the efficiency of the developed method and its applicability to real sample analysis. The accuracy of the method evaluated by percentage of recoveries of three different concentrations 0.079, 7.800 and 196.450 ng mL^{-1} in triplicate for three days. The accuracy study was carried out in standard solution and urine matrix. The result of accuracy study is represented in Table 5.49 and 5.50. The acceptable % of recovery for biological sample is (±20 %). The % of recovery which is above 100% is mainly due to presence of ion enhancement effect while the % of recovery below 100% indicates the presence of ion suppression effect.

The accuracy for the pharmaceutical products was performed by comparing the results of the sample analysis results with those of obtained in the HPLC method, as recommended in the ICH Q2(R1) guideline.

5.5.2.3. Precision

Repeatability (intra-day), intermediate precision (inter-days) were evaluated using three concentrations of the calibration curve 0.079, 7.800 and 196.450 ng mL^{-1}. The precision was evaluated for both standard solutions and in spiked urine samples. The RSD % for both intra-day and inter-day precision were below 15%. The result of precision studies is summarized in Table 5.51.

Table 5.49. *Results of Accuracy Studies for ESC-OX Standard Solution.*

		ESC-OX standard (n=18)		
Day 1 (n=6)	Added (ng mL^{-1})	0.079	7.800	196.450
	Found (mean ± SD)	0.077 ± 0.0014	7.79 ± 0.1119	193.79 ± 1.3937
	Recovery%	97.89	99.86	98.65
	SD	1.89	1.43	0.65
	SEM	0.77	0.59	0.27
	RSD%	1.93	1.44	0.66
Day 2 (n=6)	Added (ng mL^{-1})	0.079	7.8	196.45
	Found (mean ± SD)	0.076 ± 0.0013	7.83 ± 0.1273	198.63 ± 1.5555
	Recovery%	96.20	100.32	100.94
	SD	1.83	1.63	0.79
	SEM	0.75	0.67	0.32
	RSD%	1.90	1.63	0.78
Day 3 (n=6)	Added (ng mL^{-1})	0.079	7.8	196.45
	Found (mean ± SD)	0.076 ± 0.0013	7.96 ± 0.0966	196.24 ± 1.6286
	Recovery%	96.82	101.99	100.21
	SD	1.77	1.24	0.83
	SEM	0.72	0.51	0.83
	RSD%	1.83	1.21	0.34
Inter-days (n=18)	Added (ng mL^{-1})	0.079	7.800	196.450
	Found (mean ± SD)	0.076 ± 0.0013	7.857 ± 0.1119	192.22 ± 1.5259
	Recovery%	96.19	100.73	97.70
	SD	1.83	1.43	0.76
	SEM	0.748	0.586	0.309
	RSD%	1.91	1.43	0.77

Table 5.50. *Results of Accuracy Studies for ESC-OX in Spiked Human Urine Sample.*

		Spiked urine (n=9)		
Day 1 (n=3)	Added (ng mL^{-1})	0.079	7.800	196.450
	Found (mean ± SD)	0.064 ± 0.003	7.51 ± 0.133	186.01 ± 3.4056
	Recovery%	81.96	96.35	94.67
	SD	4.38	1.71	1.73
	SEM	0.731	0.285	0.29
	RSD%	5.35	1.78	1.83
Day 2 (n=3)	Added (ng mL^{-1})	0.079	7.800	196.45
	Found (mean ± SD)	0.067 ± 0.006	7.44 ± 0.36	180.34 ± 6.27
	Recovery%	84.77	95.35	91.80
	SD	7.83	4.58	3.19
	SEM	1.31	0.76	0.53
	RSD%	9.24	4.80	3.47
Day 3 (n=3)	Added (ng mL^{-1})	0.079	7.8	196.45
	Found (mean ± SD)	0.064 ± 0.007	6.94 ± 0.084	187.58 ± 3.33
	Recovery%	80.93	89.04	95.48
	SD	9.02	1.08	1.70
	SEM	1.50	0.18	0.28
	RSD%	11.14	1.21	1.78
Inter-days (n=18)	Added (ng mL^{-1})	0.079	7.800	196.450
	Found (mean ± SD)	0.065 ± 0.006	7.30 ± 0.192	184.63 ± 4.33
	Recovery%	82.56	93.58	93.98
	SD	7.078	2.46	2.21
	SEM	1.18	0.41	0.37
	RSD%	8.58	2.60	2.36

Table 5.51. *Statistical Evaluation of Precision Studies for ESC-OX Standard solution and Spiked Urine samples.*

Medium	**Parameters**	**Lower Concentration (n=18)**	**Medium Concentration (n=18)**	**High Concentration (n=18)**
ESC-OX Standard Solution	Average (ng mL^{-1})	138.6	2758	61830
	Lower limit CI	138.1	2732	61479
	Upper limit CI	139.1	2784	62181
	SD	1.053	51.97	705.7
	SEM	0.2481	12.25	166.3
	RSD%	0.76	1.88	1.14
	Parameters	**Lower Concentration (n=9)**	**Medium Concentration (n=9)**	**High Concentration (n=9)**
ESC-OX Spiked Urine Sample	Average (ng mL^{-1})	311.2	3306	76721
	Lower limit CI	309.5	3201	75088
	Upper limit CI	312.8	3412	78354
	SD	2.14	137.2	2125
	SEM	0.71	45.75	708.3
	RSD%	0.69	0.041	0.027

5.5.2.4. Selectivity

The chromatogram of standard ESC-OX solution and spiked urine sample were compared with the blank urine sample chromatogram (Figure 5.59) as well as the chromatogram of the blank oral solution and tablet matrix were compared with the chromatograms of different pharmaceutical dosage forms (Figure 5.60). The analysis of the blank urine sample chromatogram and pharmaceutical dosage forms indicated that there were no interfering peaks at the retention time of ESC-OX. Subsequently, the selectivity and specificity of the developed method were confirmed.

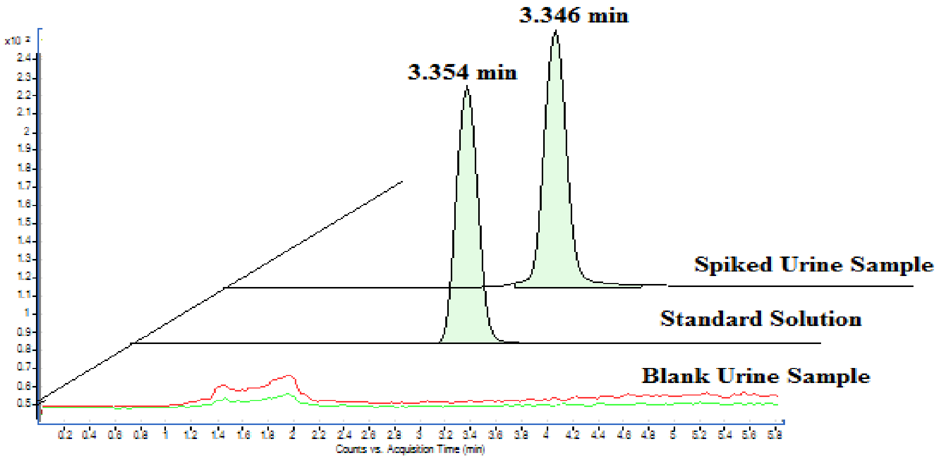


Figure 5.59. *The MRM Chromatogram of the Blank Urine Sample and ESC-OX Standard solution, Urine Sample Spiked with ESC-OX.*

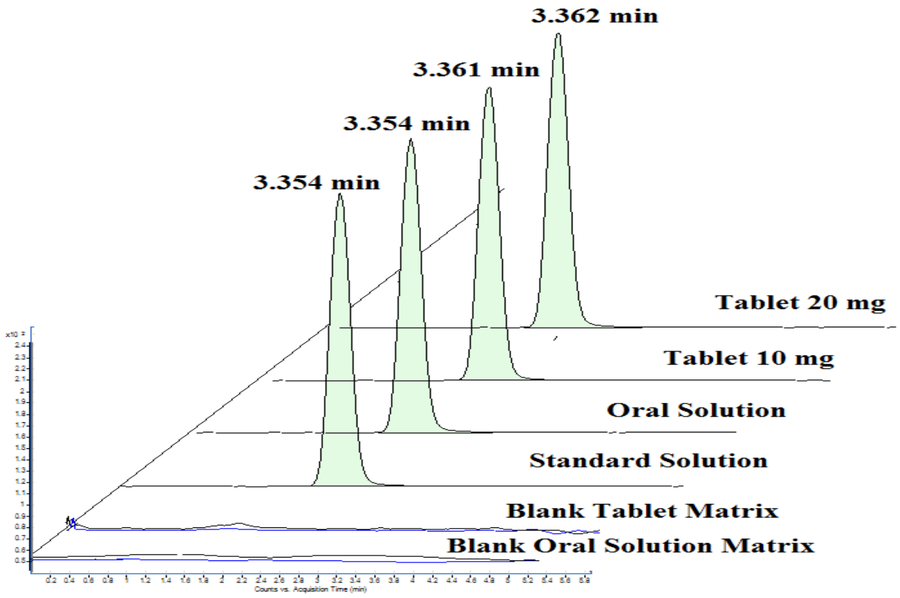


Figure 5.60. *The MRM Chromatogram of the Blank Oral Solution Matrix, Blank Tablet Matrix, ESC-OX Standard solution and ESC-OX Dosage Forms.*

5.5.2.5. Stability

The stability of standard solution and urine sample was examined and there was no change in the signal intensity for one month for standard and urine sample solutions when there were stored at +4℃ and 24 hrs.

5.5.3. Analysis of the human urine spiked with the ESC-OX solution

The results illustrated above in Table 5.45- 5.49, demonstrate the validity and utility of the developed method for analysis of ESC-OX in urine sample. The percentage of recovery from low concentration was 81.96 % which is within range of 80-120% recoveries for the bioanalysis (EMA, 2011; FDA, 2001). However, this percentage can be improved by double or triple extraction step.

5.5.4. Analysis of pharmaceutical dosage forms

The developed method was also applied for determination of ESC-OX in dosage forms; tablet from two company one tablet contains 20 mg and the other contain 10 mg, in addition, oral solution of 10 mg mL $^{-1}$. The results are represented in Table 5.52. The chromatograms related to the different dosage forms are demonstrated in Figure 5.61.

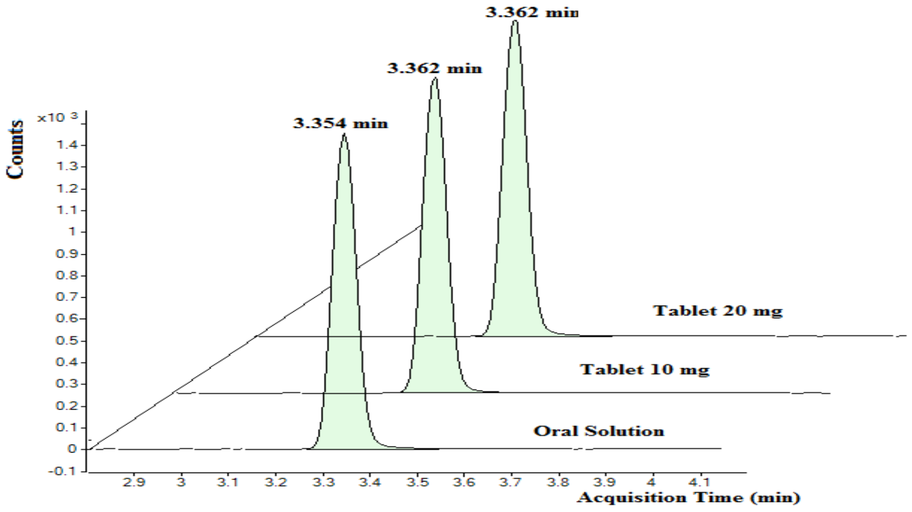


Figure 5.61. *The MRM Chromatogram of the Three-Dosage Forms.*

Table 5.52. *Assay Results of ESC-OX 20 mg, 10 mg Film Tablet and Oral solution (n=18).*

	Tablet (20 mg)	**Tablet (10 mg)**	**Oral Solution (10 mg mL^{-1})**
Mean (mg)	20.38	10.23	10.33
Minimum (mg)	20.16	10.17	10.13
Maximum (mg)	20.66	10.37	10.46
SD (mg)	0.1887	0.07329	0.1312
RSD %	0.93	0.72	1.27
SEM (mg)	0.07704	0.02992	0.05354
Bias%	-1.9	-2.3	-3.3
95% CI of Average	20.18 to 20.58	10.15 to 10.30	10.19 to 10.46
% Content Uniformity	101.9	102.3	103.3

5.5.5. General evaluation of LC-ESI-MS/MS method

A sensitive LC-ESI-MS/MS was established and validated for determination of ESC-OX in spiked human urine and in different pharmaceutical dosage forms. By developing of this acceptable precise method, the requirement of clinical, toxicological and quality control laboratories could be satisfied. In comparing the LOD with the previously published studies, the current method showed that LOD achieved by using simple LLE instead of time consuming and expensive extraction technique was 10 pg mL^{-1}, which considered convenient for monitoring of ESC-OX for pharmacodynamics and clinical purposes. One additional advantage is the shortest analysis time (3.36 min) in comparison to the previous literature 8.6 min (Unceta et al., 2013, p. 448- 453) and 25 min for all process of clean up and detection (Johannesson and Bergquist, 2007, p. 1045–1048). More promising improvement in the precision of this method could be accessed by using isotopically labelled analogues as IS. The results were encouraging for the possibility to use the proposed method for detection of the ESC-OX in other biological fluids such as plasma.

5.6. Comparison of the HPLC, DPV, CE, and LC-ESI-MS/MS Methods

The harmonization of the developed methods for quantification of ESC-OX in three pharmaceutical dosage forms were tested by using one-way ANOVA. The statistical data refers that all of the developed methods are in accordance with each other for the determination of ESC-OX in the different pharmaceutical dosage forms; that means any method can be used for analysis of the ESC-OX in its different dosage forms. The statistical data of one-way ANOVA is outlined in Table 5.53 and related graphics are given in Figure 4.62.

Table 5.53. *Assay Results of ESC-OX 20 mg Film Tablet by Using DPV, CE and LC-ESI-MS/MS.*

ESC-OX 20 mg Tablet (n=6)				
	DPV	**CE**	**LC-ESI-MS/MS**	**HPLC***
Mean (mg)	20.39	20.46	20.38	20.12
RSD %	1.51	1.22	0.93	0.23
Bias%	-1.95	-2.3	-1.9	-0.60
ANOVA	F (3, 20) = 2.673, P = 0.0750			
ESC-OX 10 mg Tablet (n=6)				
Mean (mg)	10.17	10.28	10.23	10.29
RSD %	0.99	1.22	0.72	0.14
Bias%	-1.7	-2.8	-2.3	-3.1
ANOVA	F (3, 20) = 2.144, P = 0.1266			
ESC-OX Oral Solution 10 mg mL^{-1} (n=6)				
Mean (mg)	10.28	10.23	10.33	10.25
RSD %	1.19	0.78	1.27	0.66
Bias%	-2.8	-2.3	-3.3	-2.9
ANOVA	F (3, 20) = 1.020, P = 0.4047			

* By Using Kinetex® Phenyl-Hexyl Column.

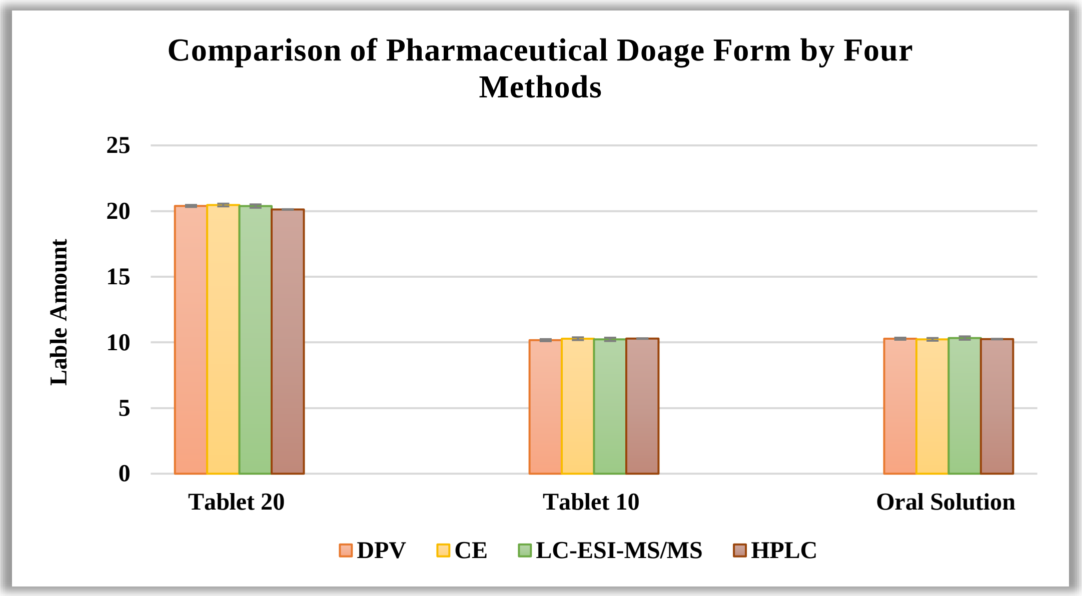


Figure 5.62. *Comparison Study for Evaluation of Dosage Forms by Using DPV, CE, LC-ESI-MS/MS and HPLC.*

In order to compare the performance of the established methods for the determination of the ESC-OX, the validation parameters were compared. The result of comparison is listed in table 5.54.

Table 5.54. *Comparison of Validation Data of the Developed Methods for ESC-OX Standard Solution.*

Parameters	**DPV**	**CE**	**LC-ESI-MS/MS**	**HPLC**$^{\blacklozenge}$
Time of Analysis (min)	5	6.59 ± 0.88	3.360 ± 0.20	6.712 ± 0.031
Linearity Range (µg mL^{-1})	4.14- 29.00	0.0264 -2.750	7.9 ×10^{-5}- 0.19645	0.236- 70.70
Correlation Coefficient	0.9984	0.9999	0.9999	0.9999
LOD (µg mL^{-1})	1.16*	0.00385	3.88×10^{-6}	0.006
LOQ (µg mL^{-1})	3.87*	0.0128	7.9×10^{-5}	0.017
Inter-day Precision (RDS%) at Low Concentration	0.70	1.76	0.76	0.76
Selectivity	Selective	Selective	Selective	Selective

*Calculation Based on the SD of the Response and the Slope, $^{\blacklozenge}$By using Kinetex® Phenyl-Hexyl Column.

The comparison between the performance of the three (DPV, CE and LC-ESI-MS/MS) methods in the determination of ESC-OX in the human urine was carried out by comparing the validation results. The chosen parameters of the three developed methods for the detection of the ESC-OX in the human urine sample were compared and listed in Table 5.55.

Table 5.55. *Comparison of Validation Data of the Developed Methods for Spiked Urine Samples.*

Parameters	**DPV**	**CE**	**LC-ESI-MS/MS**
Time of Analysis (min)	5	6.51 ± 0.07	3.348 ± 0.28
Linearity Range (µg mL^{-1})	4.14 - 29.00	0.0269 - 4.351	7.9×10^{-5}-0.19645
Correlation Coefficient	0.9979	0.9987	0.9993
LOD (µg mL^{-1})	1.31*	0.00517	10.66×10^{-6}
LOQ (µg mL^{-1})	3.97*	0.01723	7.9×10^{-5}
Inter-day Precision (RDS%) at Low Concentration	0.67	2.73	2.55
%Recovery at Low Concentration (Mean)	104.27	87.22	82.56
Selectivity	Selective	Selective	Selective

*Calculation Based on the SD of the Response and the Slope.

Comparison of the analysis time shows that LC-ESI-MS/MS required only 3.348 min for analysis which is the shortest analysis time over the HPLC, CE and DPV method. The percentage of recoveries and precision for all developed methods were within the limit of the bioanalysis regulations (EMA, 2011; FDA, 2001) as well as the RSD % for ESC-OX standard solution was within the permitted range stated in ICH regulations.

In the case of DPV technique, the percentage of recovery for urine samples was 104.27%; this may be due to presence of some interfering compounds in the urine samples that are reduced at the same cathodic reduction potential of analyte. The electrochemical method has moderate selectivity in comparison to the CE and LC-MS/MS because it does not allow an effective separation. The DPV technique provides the simplest pretreatment preparation steps before analysis in regard of other chromatographic methods.

In the analysis of ESC-OX standard solution the three chromatographic methods exhibit similar correlation coefficient for the linearity range (0.9999) which is close to the unity in comparison with the DPV technique (0.9984). In case of urine samples, LC-ESI-MS/MS shows superiority over DPV and CE method in term of LOD, linearity range and correlation coefficient.

The developed HPLC method has an advantage of not only detection of ESC-OX but also its impurities in short analysis time with good separation power. As well as, the usage of sensitive UV-visible detector provides a detection of ESC-OX and its impurities with low LOD which is the most preferred characteristics for the detection of low concentration of impurities. All these pros make the developed HPLC method a good alternative for the already existed methods.

Electrochemical method provides the most economic method but LC-ESI-MS/MS the most expensive and may be unavailable in the ordinary laboratories. The obtained data from the CE method showed that this method is suitable for bioanalysis and pharmaceutical analysis. CE provided good separation efficiency with low cost, and it is available in mostly developed laboratories. HPLC is considered as the most widely accessible and applicable instrument in the quality control and industrial laboratories. HPLC is one of the most used methods stated in official pharmacopeias for the analysis of pharmaceutical preparations.

Overall summary indicated that all developed methods were precise and accurate from the analytical point of view. The analyst can select the method according to the intention of analytical study required for the ESC-OX and accessible instrument in their laboratories.

6. CONCLUSION AND RECOMMENDATION

The main aim of using pharmaceuticals is helping to elevate the health standard by reducing the prevalence of disease in the society. For the drugs to be therapeutically effective they must be produce the intention from their use and free from the other compounds that may affect the human being health. For this reason, the pharmaceutical analysis is in continuous progress to develop a new method of drug evaluation that can be optimized with the laboratory's requirements. The main aim of this thesis is providing incorporated analytical data about ESC-OX.

The primary intension of this thesis was fulfilled by the development of an HPLC method for the detection of the most presence impurities in the pharmaceutical formulation and raw material. The established method is simple and fast in comparison to the official method USP 36. By the developing of this method, the safety and purity of the ESC-OX was verified. The developed method was validated according to the ICH guidelines and the statistical data was encouraging for the utilization of this method in the quality control and industrial laboratories. Simultaneously, the developed method was applied by using columns with divers stationary phases (core-shell and fully porous particle) and the performance of the various columns were evaluated. The comparison indicated that the core-shell columns performance is preferable over the fully porous particle columns. In addition, different chemical stationary phases were used and the phenyl-hexyl column provided the best separation with reasonable resolution for all compound. Accordingly, the second objective of the thesis was achieved.

The other two objectives of this thesis were the development of divers analytical methods to detect and quantify the ESC-OX in the human urine sample and different pharmaceutical dosage forms. The difference between the developed method for determination of ESC-OX in pharmaceutical dosage forms was evaluated by using one-way ANOVA, the data displays that there is no difference in the quantification of the ESC-OX in dosage forms by all developed method.

In case of urine analysis, simple LLE technique was used for preparation of the urine samples. The percentage of recovery for low concentration in case of LC-ESI-MS/MS and CE was at the lower limit (82.56-87.22%, respectively) stated in the official regularities for the bioanalysis (80-120%). Using of SPE techniques for urine preparation is recommended to get higher percentage of recoveries.

LC-ESI-MS/MS exhibit prevalence in term of LOD over CE and DPV methods. Also, CE has superiority over DPV in LOD. The obtained information from the DPV and CV about the redox reaction of ESC-OX can be used to explore the physiological behavior of drug in the body. Each of the developed method in this thesis has its advantages. The pharmaceutical analyst can select the appropriate technique according to the purpose of analysis and available laboratory facilities.

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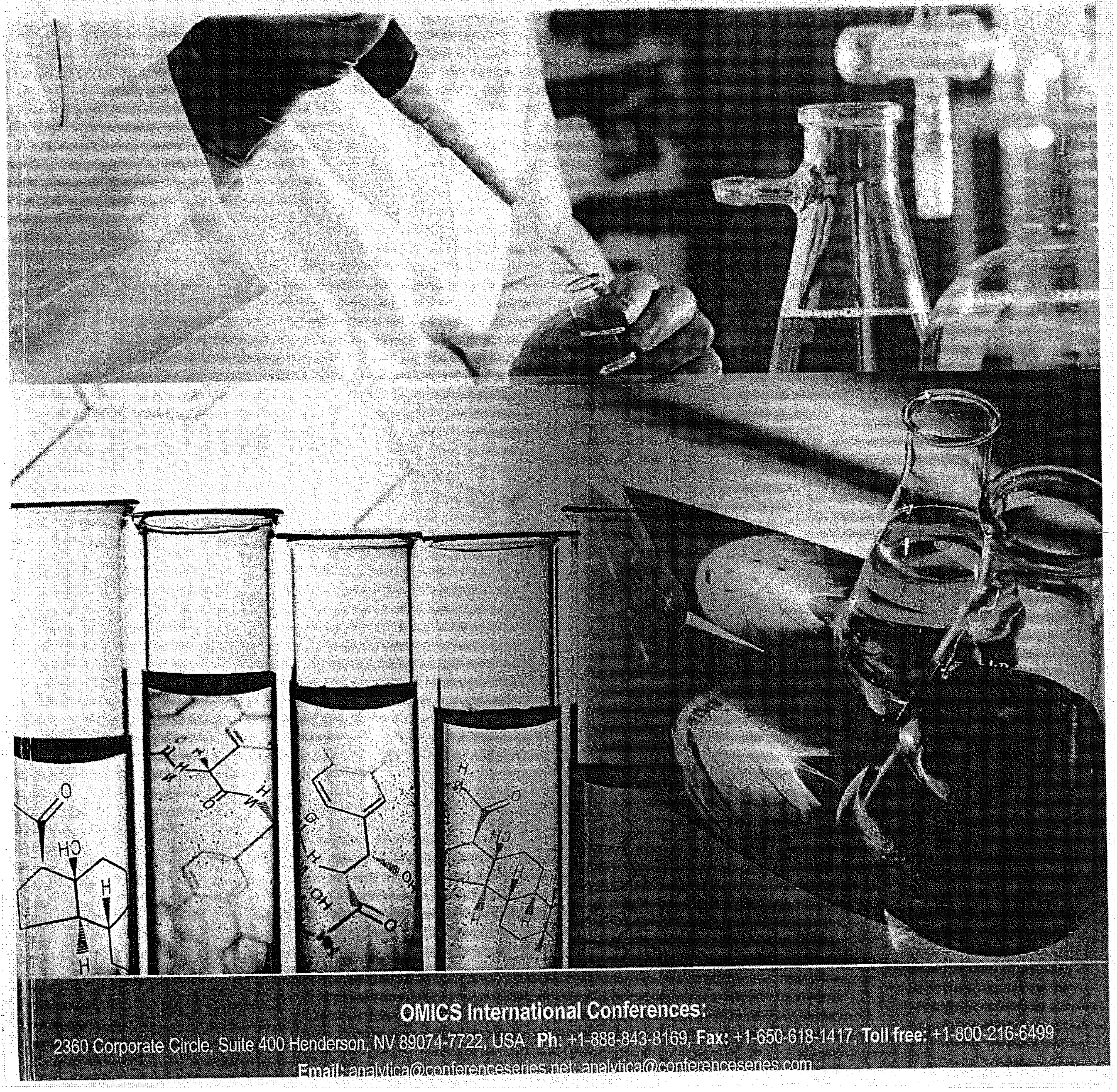

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Validated determination of escitalopram by capillary zone electrophoresis in pharmaceutical preparations

Arın Gül Dal and **Wafa Badulla**
Anadolu University, Turkey

Escitalopram is an oral serotonin re-uptake inhibitor. In this study, a simple, fast and sensitive method was developed for the determination of escitalopram by capillary electrophoresis in pharmaceutical preparations. Metoprolol was used as IS. Separation was achieved by a fused silica capillary with 40 cm effective (48.5 cm total, 75 μm i.d.) length. The run buffer was composed of 15 mM phosphate buffer (pH 2.5). The applied potential was 25 kV and the samples were injected at 50 mbar pressure for 10 s. The migration times under these optimum conditions were 6.51±0.07 and 6.73±0.08 minutes for escitalopram and IS, respectively. The method was validated for linearity, precision, accuracy, specificity and sensitivity. The LOQ was calculated as 3.57×10-7 M for escitalopram. The method was successfully applied to tablets, film coated tablets and oral drops of escitalopram.

Biography

Arın Gül Dal has completed her PhD in 2009 from Anadolu University, Institute of Health Sciences. She is now working at Anadolu University, Faculty of Pharmacy as an Assistant Professor. She has papers on separation methods like capillary electrophoresis and liquid chromatography in international periodicals and meetings.

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ABSTRACT BOOK

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P14 ELECTROCHEMICAL BEHAVIOUR and DETERMINATION of ESCITALOPRAM OXALATE on HANGING MERCURY DROP ELECTRODE

Wafa Badulla[1], *Zeki Atkoşar*[2], *Göksel Arlı*[2]

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Escitalopram is an orally administered highly selective serotonin-reuptake inhibitor, developed for the treatment of depression and anxiety disorders [1]. The main objectives of this work were to investigate the electrochemical behavior of Escitalopram and its determination possibilities in bulk and pharmaceutical formulations at hanging mercury electrode using differential pulse voltammetry (DPV). This technique allow analyst to save time and decrease cost compared with already published methods. A BAS 100B electrochemical analyzer, a CGME polarographic stand with a hanging mercury drop electrode as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl electrode as the reference electrode was used in the analyses. The solution in the analytical cell was composed of 0.3 M KCl, 0.004 M Britton-Robinson buffer at pH 6.5 and 5% methanol in water. The electrochemical behavior was examined by cyclic voltammetry and the overall electrode process was found to be mainly diffusion-controlled and quasi-reversible. The optimum reduction potential was determined to be - 0.6 V. Different parameters were tested to optimize the differential pulse voltammetric conditions for determination of Escitalopram both in bulk and dosage forms. The dependence of current intensity and potential on pH, concentration of electrolyte as well as various instrumental parameters were investigated. The developed method was validated according to the ICH Q2(R) 1 guideline. The method was linear over the range of 4.143µg/mL– 29µg/mL with a regression coefficient of 0.9984. The LOD and LOQ were found to be 1.17µg/mL and 3.54µg/mL, respectively. The recovery was between 99.91 - 102.52% for all cases. The proposed procedure was successfully applied for determination of Escitalopram in bulk and three different pharmaceutical formulations.

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ABSTRACT BOOK

P46

LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF ESCITALOPRAM IN HUMAN URINE

Wafa Badulla[1], **Erol Şener**[2], **Zeki Atkoşar**[2], **Göksel Arli**[2]

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Escitalopram oxalate (ESC) is an orally administered highly selective serotonin-reuptake inhibitor, developed for the treatment of depression and anxiety disorders [1]. ESC is metabolized to S-demethylcitalopram (S-DCT) and S-didemethylcitalopram (S-DDCT). In humans, unchanged ESC is the predominant compound in plasma. Following oral administration of ESC, the fraction of drug recovered in the urine as ESC and S-demethylcitalopram (S-DCT) is about 8% and 10%, respectively [2]. The main objectives of this work were to determine the drug in pharmaceutical dosage form and spiked ESC in human urine consequently, a new method of a liquid chromatography/tandem mass spectrometry (LC-MS/MS) was developed. The method involved liquid–liquid extraction of the analyte from human urine after alkalization with ammonium hydroxide then extraction with hexane. The absolute recovery of spiked ESC from urine was more than 70%.The chromatographic separation was achieved within 3.36 min by using mobile phase consisted of a binary gradient elution profile comprising 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Zorbax Eclipse C18, 50 mm x 2.1mm analytical column; the flow-rate was 0.35 ml/min. The mass transitions were selected according to their stabilities and intensities. As precursor ion, the protonated molecular ion [M+H] was observed at 325.0 m/z. The transition of 325.0 109.0 m/z was selected as quantifier and 325.0 262.0 m/z as qualifier. The developed method was validated according to the ICH Q2(R) 1 guideline. The method was linear over the range of 0.1-500 ng/ml with 1/x weighted regression coefficient of 0.99878.The LLOQ by using CV% was found to be 0.1ng/mL. The proposed procedure was successfully applied for determination of ESC in urine and different pharmaceutical dosage forms.

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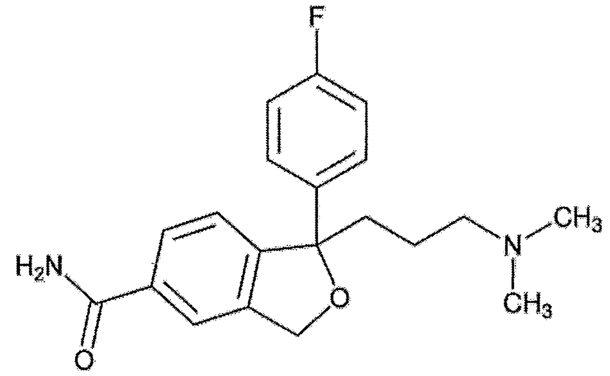
Certificate

CITALOPRAM RELATED COMPOUND A

(1-(3-Dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carboxamide)

USP Catalog No.:	1134244
USP Lot No.:	R015E0



CAS No.:	64372-56-1
Molecular Formula:	$C_{20}H_{23}FN_2O_2$
Molecular Weight:	342.41

LABEL TEXT

For use with specified USP compendial tests.
Not for use as a drug. See SDS prior to use at
www.usp.org/sds.

USP REFERENCE STANDARD

CITALOPRAM RELATED COMPOUND A 25 mg

(1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carboxamide)

Do not dry. Keep container tightly closed. Protect from light.

USP, 12601 Twinbrook Pkwy, Rockville, MD, +1-301-881-0666
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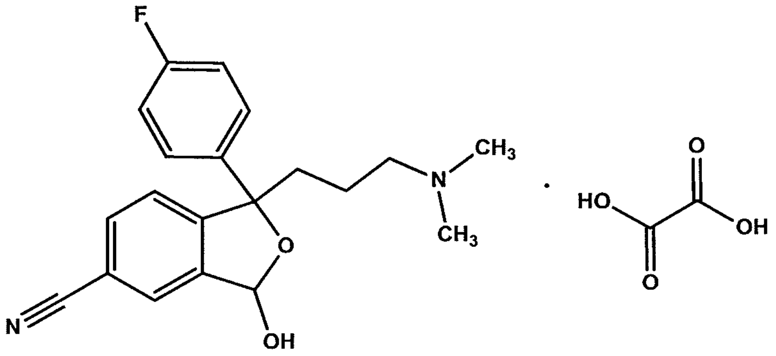

Molecular Formula
$C_{22}H_{23}FN_2O_6$

Molecular Weight
430.43

CAS Number
n/f

LABEL TEXT

For use with specified USP compendial tests.
Not for use as a drug. MSDS available on
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REFERENCE STANDARD

CITALOPRAM RELATED COMPOUND B 25 mg
(1-(3-Dimethylaminopropyl)-1-(4-fluorophenyl)-3-hydroxy-1,3-dihydroisobenzofuran-5-carbonitrile oxalate)

Do not dry. Keep container tightly closed. Protect from light.

USP, 12601 Twinbrook Pkwy, Rockville, MD, +1-301-881-0666
CAT. NO. 1134255 Material mfd. in Denmark

LOT: G0L552

Alice Lin
Quality Assurance

25-Mar-13

Calculation Value

Unless otherwise stated on the Reference Standard label, a value of 100.0% should be used in the compendial applications for which the use of this Reference Standard is intended. Please refer to the specific Reference Standard label for further information.

Expiration

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Instructions for Use

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Non-Monograph Use

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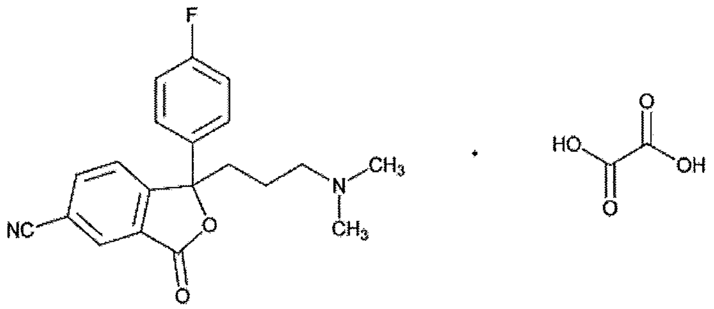
Certificate

CITALOPRAM RELATED COMPOUND C

(3-(3-Dimethylaminopropyl)-3-(4-fluorophenyl)-6-cyano-1(3H)-isobenzofuranone oxalate)

USP Catalog No.: 1134266
USP Lot No.: R030U0

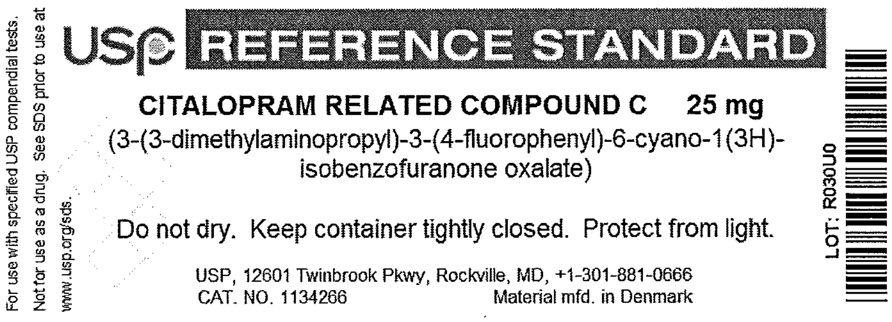


CAS No.: N/A

Molecular Formula: $C_{20}H_{19}FN_2O_2\cdot C_2H_2O_4$

Molecular Weight: 428.42

LABEL TEXT



For use with specified USP compendial tests.
Not for use as a drug. See SDS prior to use at
www.usp.org/sds.

USP REFERENCE STANDARD

CITALOPRAM RELATED COMPOUND C 25 mg
(3-(3-dimethylaminopropyl)-3-(4-fluorophenyl)-6-cyano-1(3H)-isobenzofuranone oxalate)

Do not dry. Keep container tightly closed. Protect from light.

USP, 12601 Twinbrook Pkwy, Rockville, MD, +1-301-881-0666
CAT. NO. 1134266 Material mfd. in Denmark

LOT: R030U0

Teri L. Toth
Quality Assurance

Calculation Value
If a value is not provided on the label or accompanying documentation and the Reference Standard has a quantitative USP compendial application, a value of 100.0% is used. The purity value is not applicable for qualitative uses. Please refer to the specific Reference Standard label for further information.

Expiration
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Instructions for Use
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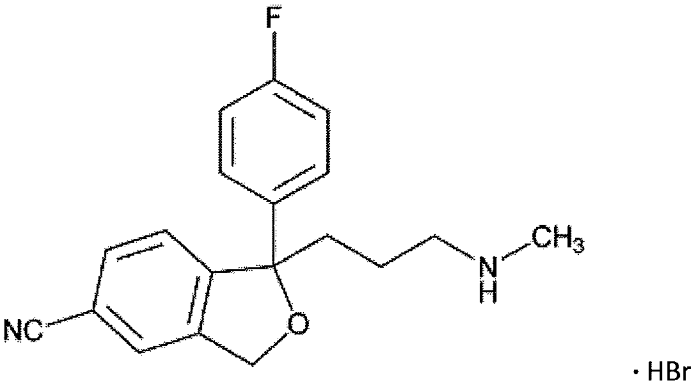
U.S. Pharmacopeial Convention

Certificate

CITALOPRAM RELATED COMPOUND D

(*1-(4-Fluorophenyl)-1-(3-methylaminopropyl)-1,3-dihydroisobenzofuran-5-carbonitrile hydrobromide*)

USP Catalog No.:	1134277
USP Lot No.:	R01270



CAS No.: N/A

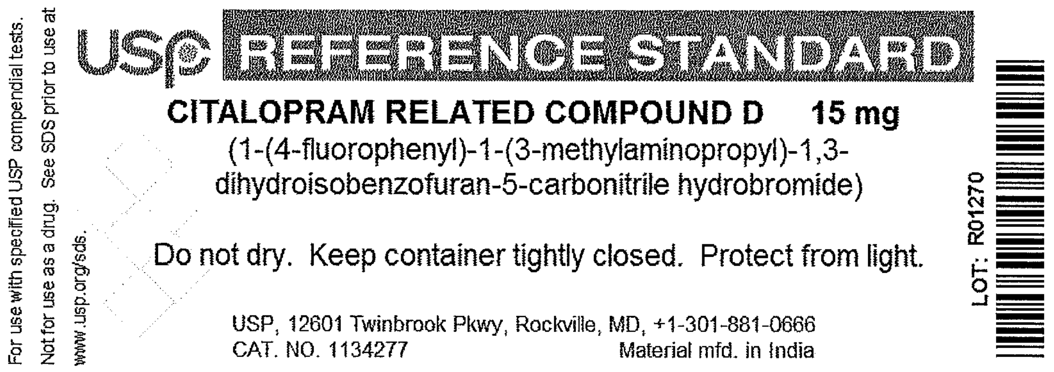
Molecular Formula: $C_{19}H_{19}FN_2O \cdot HBr$

Molecular Weight: 391.28

Certificate Date: 14Jan2015

U.S. Pharmacopeial Convention

LABEL TEXT



USP REFERENCE STANDARD

CITALOPRAM RELATED COMPOUND D 15 mg

(1-(4-fluorophenyl)-1-(3-methylaminopropyl)-1,3-dihydroisobenzofuran-5-carbonitrile hydrobromide)

Do not dry. Keep container tightly closed. Protect from light.

USP, 12601 Twinbrook Pkwy, Rockville, MD, +1-301-881-0666
CAT. NO. 1134277 Material mfd. in India

For use with specified USP compendial tests.
Not for use as a drug. See SDS prior to use at
www.usp.org/sds.

LOT: R01270

Teri L. Toth

Quality Assurance

Calculation Value

If a value is not provided on the label or accompanying documentation and the Reference Standard has a quantitative USP compendial application, a value of 100.0% is used. The purity value is not applicable for qualitative uses. Please refer to the specific Reference Standard label for further information.

Expiration

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Instructions for Use

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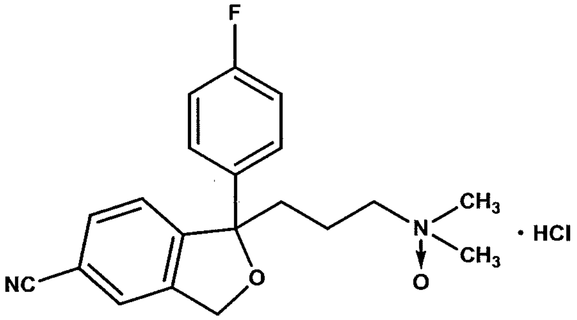
U.S. Pharmacopeia
The Standard of Quality℠

USP Certificate

Citalopram Related Compound E

LOT G0M155



Molecular Formula

$C_{20}H_{21}FN_2O_2 \cdot HCl$

Molecular Weight

376.85

CAS Number

62498-71-9

LABEL TEXT

For use with specified USP compendial tests.
Not for use as a drug. MSDS available on
www.USP.org.

REFERENCE STANDARD

CITALOPRAM RELATED COMPOUND E 25 mg

(1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile-N-oxide hydrochloride)

Do not dry. Keep container tightly closed. Protect from light.

USP, 12601 Twinbrook Pkwy, Rockville, MD, +1-301-881-0666
CAT. NO. 1134288 Material mfd. in Denmark

LOT: G0M155

Teri L. Toth
Quality Assurance

22-May-13

Calculation Value

Unless otherwise stated on the Reference Standard label, a value of 100.0% should be used in the compendial applications for which the use of this Reference Standard is intended. Please refer to the specific Reference Standard label for further information.

Expiration

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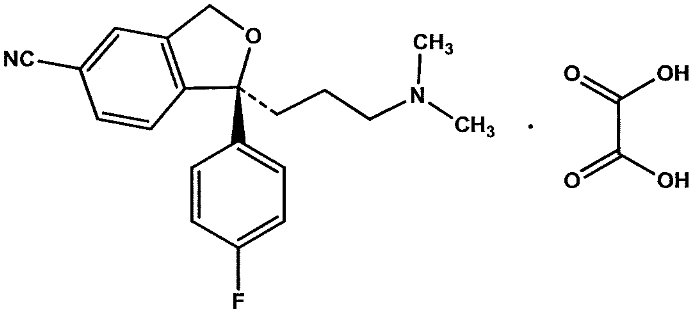
USP Certificate

Escitalopram Oxalate

LOT G0M272



Molecular Formula

$C_{20}H_{21}FN_2O \cdot C_2H_2O_4$

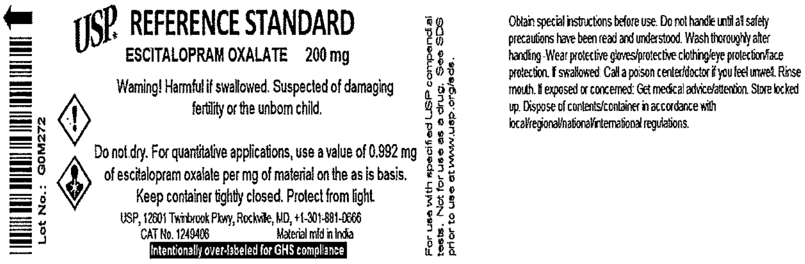
Molecular Weight

414.43

CAS Number

219861-08-2

LABEL TEXT



USP® REFERENCE STANDARD
ESCITALOPRAM OXALATE 200 mg

Warning! Harmful if swallowed. Suspected of damaging fertility or the unborn child.

Do not dry. For quantitative applications, use a value of 0.992 mg of escitalopram oxalate per mg of material on the as is basis. Keep container tightly closed. Protect from light.

USP, 12601 Twinbrook Pkwy, Rockville, MD, +1-301-881-0666
CAT No. 1249406 Material mfd in India
Intentionally over-labeled for GHS compliance

Lot No.: G0M272

For use with specified USP compendial tests. Not for use as a drug. See SDS prior to use at www.usp.org/sds.

Obtain special instructions before use. Do not handle until all safety precautions have been read and understood. Wash thoroughly after handling. Wear protective gloves/protective clothing/eye protection/face protection. If swallowed: Call a poison center/doctor if you feel unwell. Rinse mouth. If exposed or concerned: Get medical advice/attention. Store locked up. Dispose of contents/container in accordance with local/regional/national/international regulations.

Teri L. Toth

Quality Assurance

Calculation Value

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Instructions for Use

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Non-Monograph Use

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LEGAL NOTICE

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Certificate of Analysis

PHASE: Kinetex 2.6 µm Phenyl-Hexyl

BATCH NO.: 5602-0143

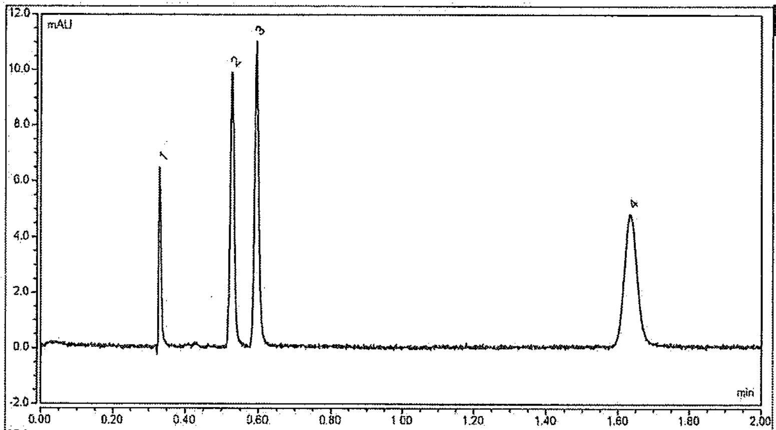
SILICA ANALYSIS

PARAMETER:	SPECIFICATION:	BATCH RESULT:
Pore Diameter (A)	82 - 102	99
Particle Size (µm)	2.4 - 2.6	2.4
Particle Size Distribution: dp90/10	<= 1.2	1.1

BONDED PHASE ANALYSIS

PARAMETER:	SPECIFICATION:	BATCH RESULT:
Surface Coverage (µmole/m2)	2.9 - 3.9	3.5

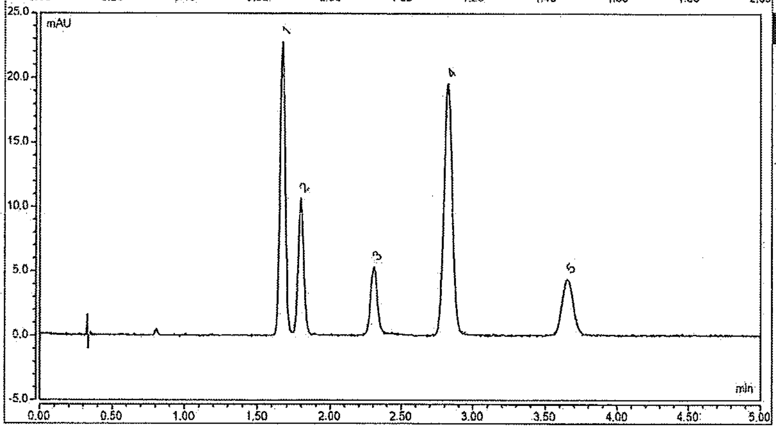
CHROMATOGRAPHIC RESULTS



PHENOMENEX QC MIX 870P

CONDITION:
Column dimensions: 50 X 4.6 mm
Mobile Phase: 20 mM K2HPO4 pH7.0/ methanol (20:80)
Flow Rate: 1.5 mL/min
Injection Volume: 0.2 µL
Temperature: 40 °C
Detector: UV@254 nm
Sample:
1. uracil
2. toluene
3. ethyl benzene
4. clomipramine

PARAMETER:	SPECIFICATION:	BATCH RESULT:
a2,3	1.32 - 1.35	1.34
k'3	0.68 - 0.80	0.80
Asym4	<= 1.50	1.10



Methoxybenzenes

CONDITIONS
Column Dimensions: 50 X 4.6 mm
Mobile Phase: Methanol : Water (40:60)
Flow Rate: 1.5 mL/min
Injection Volume: 2 µL
Temperature: 40 °C
Detector: UV@254 nm
Sample:
1. 1,2-dimethoxybenzene
2. 1,2,4-trimethoxybenzene
3. 1,4-dimethoxybenzene
4. 1,3-dimethoxybenzene
5. 1,3,5-trimethoxybenzene

PARAMETER:	SPECIFICATION:	BATCH RESULT:
a1,2	1.08 - 1.12	1.10
a3,4	1.24 - 1.27	1.26
k'5	8.30 - 10.40	10.14

QUALITY ASSURANCE

Approved By: ____________

Date Approved: July 25, 2016

FR68370616_W

Certificate of Analysis

PHASE: Kinetex 2.6µm C18

BATCH NO.: 5569-0214

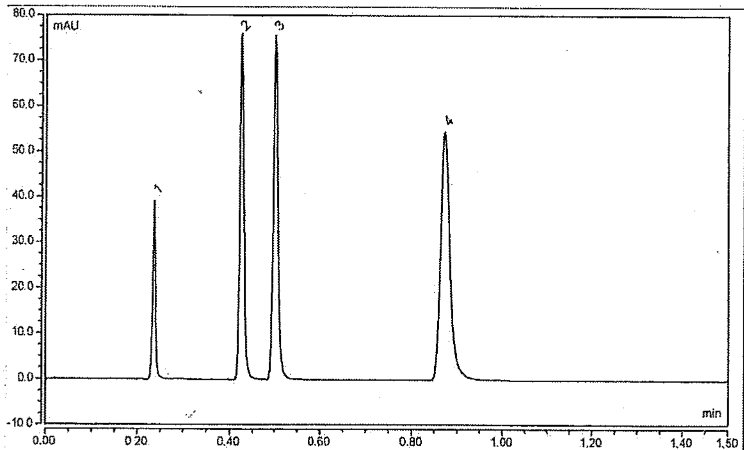
SILICA ANALYSIS

PARAMETER:	SPECIFICATION	BATCH RESULT
Pore Diameter(Å)	82 - 102	101
Particle Size (µm)	2.4 - 2.6	2.4
Particle Size Distribution: dp90/10	<= 1.2	1.2

BONDED PHASE ANALYSIS

PARAMETER	SPECIFICATION	BATCH RESULT
Surface Coverage(µmole/m2)	2.7 - 3.4	3.2

CHROMATOGRAPHIC RESULTS

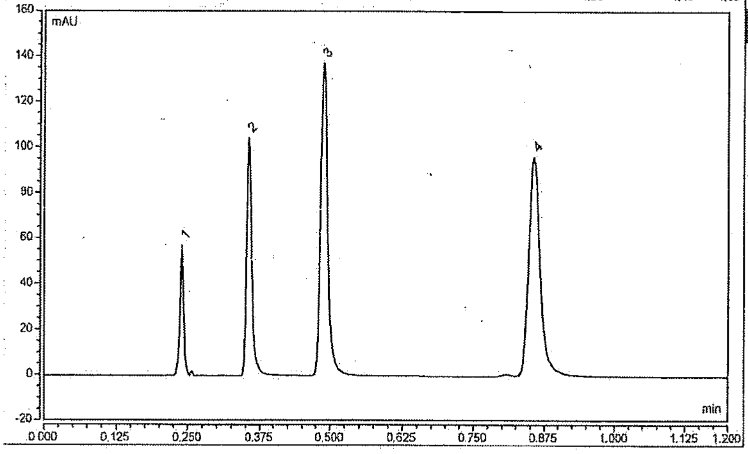


PHENOMENEX QC MIX 870

CONDITION:

Column dimensions: 50 x 4.6 mm
Mobile Phase: 20 mM KH2PO4 pH7.0/ MeOH (20:80)
Flow Rate: 2.0 mL/min
Injection Volume: 1µL
Temperature: 40 °C
Detector: UV @ 254 nm
Sample:
1. uracil
2. toluene
3. ethyl benzene
4. amitriptyline

PARAMETER:	SPECIFICATION:	BATCH RESULT:
Asym4	<= 1.65	1.33
a2,3	1.35 - 1.43	1.40



BASIC DRUGS

CONDITIONS

Column Dimensions: 50 x 4.6 mm
Mobile Phase: 20 mM Na2HPO4 pH9.75/MeOH/ACN (35:55:10)
Flow Rate: 2.0 mL/min
Injection Volume: 1 µL
Temperature: 40 °C
Detector: UV @ 230 nm
Sample:
1. thiourea
2. pindolol
3. metoprolol
4. propranolol

PARAMETER:	SPECIFICATION:	BATCH RESULT:
Asym4	<= 1.45	1.32
a2,3	2.04 - 2.20	2.15
Rs2,3	>= 6.5	7.3

QUALITY ASSURANCE

Approved By:

Date Approved: May 13, 2016

Effective 04/22/15, the specification for a2,3 has been updated from 1.95-2.15 to 2.04-2.20

If you have any questions about the above change, please contact the Phenomenex Technical team at http://www.phenomenex.com/Home/TechnicalSupport

FR66370714_W

QUALITY MANAGEMENT SYSTEM
CERTIFIED BY DNV
ISO 9001:2008

Certificate of Analysis

PHASE: **Kinetex 2.6 µm F5**

BATCH NO.: **5723-016**

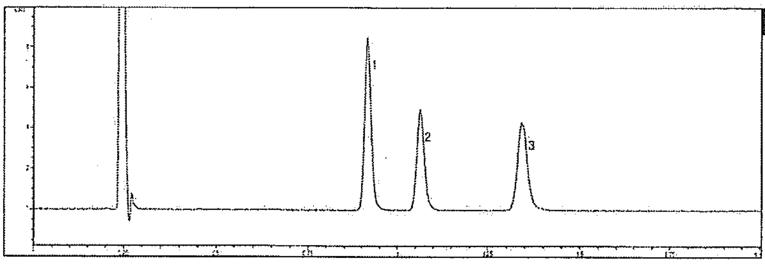
SILICA ANALYSIS

PARAMETER:	SPECIFICATION:	BATCH RESULT:
pore diameter (Å)	82 - 102	99
particle size (µm)	2.4 - 2.6	2.4
particle size distribution: dp90/10	≤ 1.2	1.1

BONDED PHASE ANALYSIS

PARAMETER:	SPECIFICATION:	BATCH RESULT:
surface coverage (µmole/m^2)	2.1 - 2.4	2.3

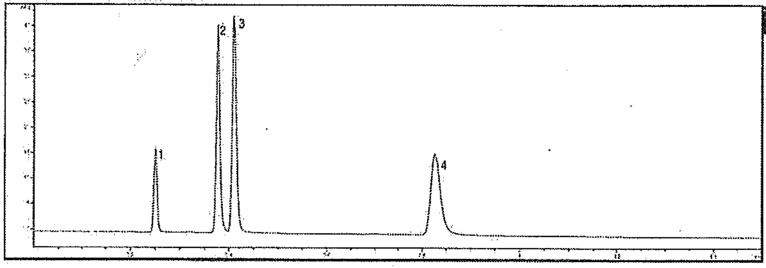
CHROMATOGRAPHIC RESULTS



POSITIONAL ISOMERS

CONDITIONS:
Mobile Phase: A-0.1% Formic acid in water
B-0.1% Formic acid in acetonitrile
60:40 A:B
Flow Rate: 2.0 mL/min
Temperature: 25 °C
Detector: UV @ 254 nm
Sample: 1. 2,6-Dichlorophenol
2. 2,5-Dichlorophenol
3. 3,5-Dichlorophenol

PARAMETER:	SPECIFICATION:	BATCH RESULT:
K'_3	4.20 - 4.70	4.48
$R_{1,2}$	≥ 3.7	4.2
$R_{2,3}$	≥ 5.7	6.4



PHEN MIX 870P

CONDITIONS:
Mobile Phase: A-20 mM potassium phosphate pH 7
B-MeOH
20:80 A:B
Flow Rate: 2.0 mL/min
Temperature: 40 °C
Detector: UV @ 254 nm
Sample: 1. uracil
2. toluene
3. ethyl benzene
4. clomipramine

PARAMETER:	SPECIFICATION:	BATCH RESULT:
K'_3	0.60 - 0.68	0.63
K'_4	2.00 - 2.40	2.29
$\alpha_{2,3}$	1.25 - 1.29	1.26
Sym_4	≥ 0.60	0.71

QUALITY ASSURANCE

Approved By:

Date Approved: June 4, 2015

FR6837O714_W

www.phenomenex.com/mysupport

QUALITY MANAGEMENT SYSTEM
CERTIFIED BY DNV
ISO 9001:2008

Certificate of Analysis

PHASE: Luna 3µm Phenyl-Hexyl

BATCH NO.: 5295-0037

SILICA ANALYSIS

PARAMETER:	SPECIFICATION:	BATCH RESULTS:
Particle Size (µm)	2.90 - 3.30	3.01
Particle Size Distribution: dp90/10	1.40 - 2.00	1.55
Pore Diameter (Å)	90 - 110	101
Surface Area (m2/g)	370 - 430	402
Metal Content (ppm)	< 30.0	4.2

BONDED PHASE ANALYSIS

PARAMETER:	SPECIFICATION:	BATCH RESULTS:
Total Carbon (%)	16.50 - 18.70	18.08
Surface Coverage (µmole/m2)	3.80 - 4.80	4.23

CHROMATOGRAPHIC RESULTS

Inertness

CONDITIONS:

Column Dimension: 150 x 4.6 mm
Mobile Phase: CH3CN:H2O (50:50)
Flow Rate: 1 mL/min
Injection Volume: 5µL
Temperature: ambient
Detector: UV @ 254 nm
Sample: 1. Pyridine
2. Phenol
3. Toluene

PARAMETER:	SPECIFICATION:	BATCH RESULTS:
Asym 1/2	1.00 - 1.50	1.30

Metal Test

CONDITIONS:

Column Dimension: 150 x 4.6 mm
Mobile Phase: CH3CN:H20 (50:50)
Flow Rate: 1 mL/min
Injection Volume: 5µL
Temperature: ambient
Detector: UV @ 230 nm
Sample: 1. 2,7 dihydroxynaphthalene
2. 2,3 dihydroxynaphthalene

PARAMETER:	SPECIFICATION:	BATCH RESULTS:
Asym 2/1	1.05 - 1.55	1.15

Hydrophobic Index

CONDITIONS:

Column Dimension: 150 x 4.6 mm
Mobile Phase: CH3CN:H20 (80:20)
Flow Rate: 1 mL/min
Injection Volume: 5µL
Temperature: ambient
Detector: UV @ 254 nm
Sample: 1. Butyl benzene
2. Amyl benzene

PARAMETER:	SPECIFICATION:	BATCH RESULTS:
a1,2	1.11 - 1.31	1.21

Basic Drugs

CONDITIONS:

Column Dimension: 150 x 4.6 mm
Mobile Phase: A=20mM Na2HPO4,pH9.75
B=CH3OH, C=CH3CN
A:B:C = (35:55:10)
Flow Rate: 1 mL/min
Injection Volume: 5µL
Temperature: 35°C
Detector: UV @ 230 nm
Sample: 1. Thiourea
2. Pindolol
3. Metoprolol
4. Propanolol

PARAMETER:	SPECIFICATION:	BATCH RESULTS:
Asym3	< 1.45	1.19
a1,2	1.65 - 1.85	1.81
a2,3	2.30 - 2.50	2.46

QUALITY ASSURANCE

Approved By: ____________________

Date Approved: August 10, 2016

FR63370616_W

www.phenomenex.com/mysupport

QUALITY
MANAGEMENT SYSTEM
CERTIFIED BY DNV GL
= 9001:2008 =

Certificate of Analysis

PHASE: Luna 3µm PFP(2)
BATCH NO.: 5536-056

SILICA ANALYSIS

SILICA SURFACE CHARACTERISTICS

PARAMETER:	SPECIFICATION:	BATCH RESULT:
surface area (m^2/g)	370 - 430	416
pore diameter (Å)	90 - 110	96
pore volume (cm^3/g)	0.95 - 1.05	1.00

PARTICLE SIZE ANALYSIS

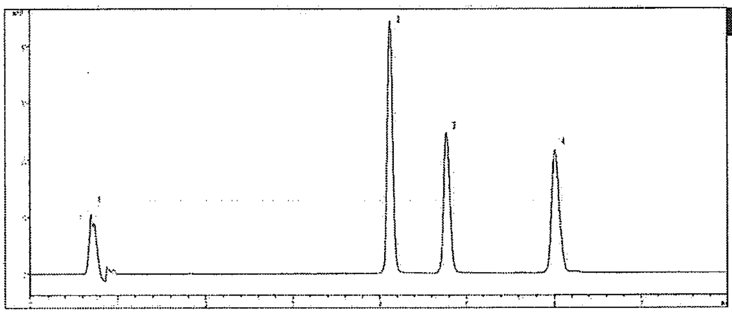
PARAMETER:	SPECIFICATION:	BATCH RESULT:
particle size (µm)	2.90 - 3.50	3.05
dp 90/10	≤ 2.0	1.5

BONDED PHASE ANALYSIS

ELEMENTAL ANALYSIS

PARAMETER:	SPECIFICATION:	BATCH RESULT:
% carbon	10.50 - 12.50	12.16

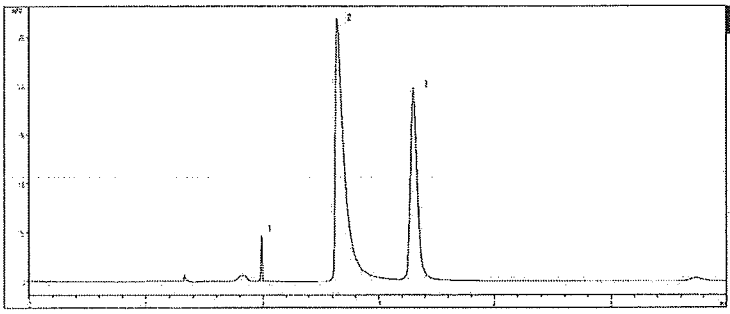
CHROMATOGRAPHIC RESULTS



PHENOLIC POSITIONAL ISOMERS

CONDITIONS:
Column Dimensions: 100 x 4.6 mm
Mobile Phase: A=0.1%formic acid in H_2O
B=0.1%formic acid in CH_3CN
Flow Rate: 1.4 mL/min.
Gradient: 40% - 50% B for 10 min; re-equib. at 40% B for 5 min
Column Temperature: ambient
Injection Volume: 10 µL
Detection: 254 nm
Samples: 1. Thiourea
2. 2,5-dichlorophenol
3. 2,6-dichlorophenol
4. 3,5-dichlorophenol

PARAMETER:	SPECIFICATION:	BATCH RESULT:
R_1	> 3.5	5.5
R_2	> 6.0	8.8
k'_4	6.20 - 7.40	7.21



GEOMETRIC ISOMERS

CONDITIONS:
Column Dimensions: 100 x 4.6 mm
Mobile Phase: A=CH_3CN
B=20mM Potassium Phosphate, pH 2.5
A:B=3:97
Flow Rate: 1.0 mL/min.
Column Temperature: ambient
Injection Volume: 2 µL
Detection: 210 nm
Samples: 1. Uracil
2. Maleic acid
3. Fumaric acid

PARAMETER:	SPECIFICATION:	BATCH RESULT:
k'_2	1.60 - 2.40	1.77
$\alpha_{2,3}$	1.10 - 1.50	1.39

QUALITY ASSURANCE

Approved By: EKim
Quality Assurance

Date Approved: April 17, 2015

Effective 09/10/14, the specification for Pore Volume has been tightened from 0.90-1.10 to 0.95-1.05
Effective 04/16/15, the specification for %C was changed from 10.00-12.00 to 10.50-12.50

FR68370714_W

QUALITY MANAGEMENT SYSTEM
CERTIFIED BY DNV
ISO 9001:2008

phenomenex
...breaking with tradition™

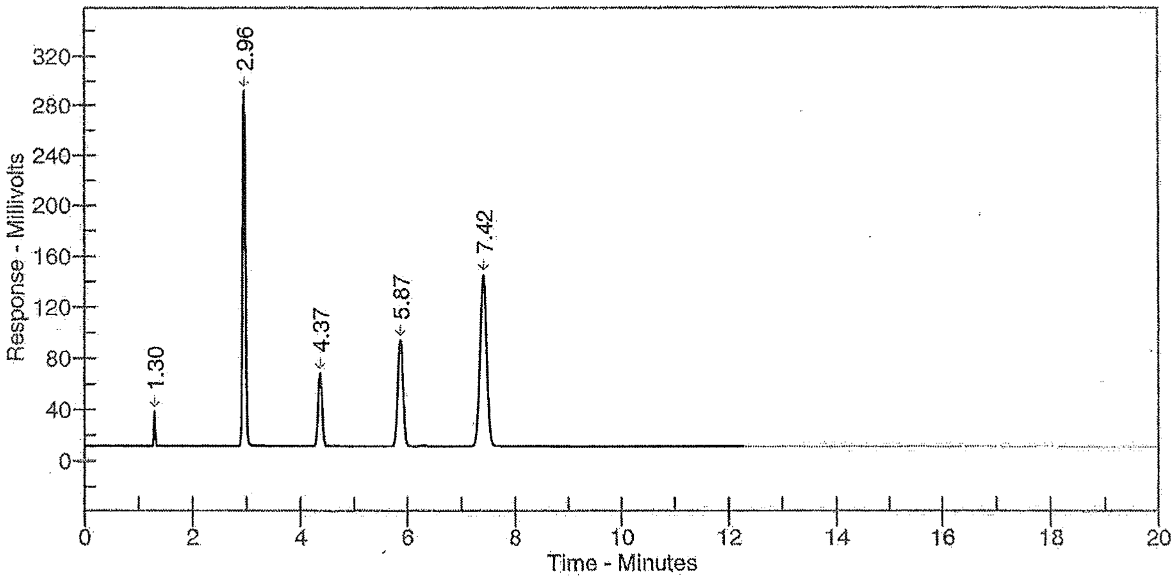
Certificate of Quality Assurance

Part No.: 00F-4375-E0
Description: Synergi 4u Hydro-RP 80A

Serial No.: 408417-18
Column Size: 150 X 4.6 mm

Measured Parameters Based on the NAPHTHALENE Peak

Efficiency: 107480 plates/meter
Peak Asym.: 0.95
k Factor: 4.719
Ret. Time: 7.42 minutes
Batch No.: 5375-27



Test Analytes

Peak	Analyte	Time	Area	k Factor	Width	Eff.	Asym.
1	Uracil	1.30	55096	0.000	0.030	10262	1.35
2	Acetophenone	2.96	1004676	1.282	0.055	15852	0.92
3	Benzene	4.37	290888	2.373	0.079	16688	1.07
4	Toluene	5.87	566455	3.530	0.107	16465	1.07
5	Naphthalene	7.42	1181520	4.719	0.137	16122	0.95

Testing Condition

Mobile Phase:	65% MECN/H2O	**Temperature:**	AMBIENT
Flow Rate:	1 ml	**Injection:**	1 µl
Detection:	254 NM	**Back Pressure:**	1030 psi

Checked By:	LK	**Instrument:**	QC42
		Tested:	Wednesday, August 15, 2007
Approved By:		**Printed:**	Wednesday, August 15, 2007

Column Performance Report

Cat.No. 5020-01771
Serial No. 7FI82421

Acquired : 07/06/11 14:14:43
User : TAHARA

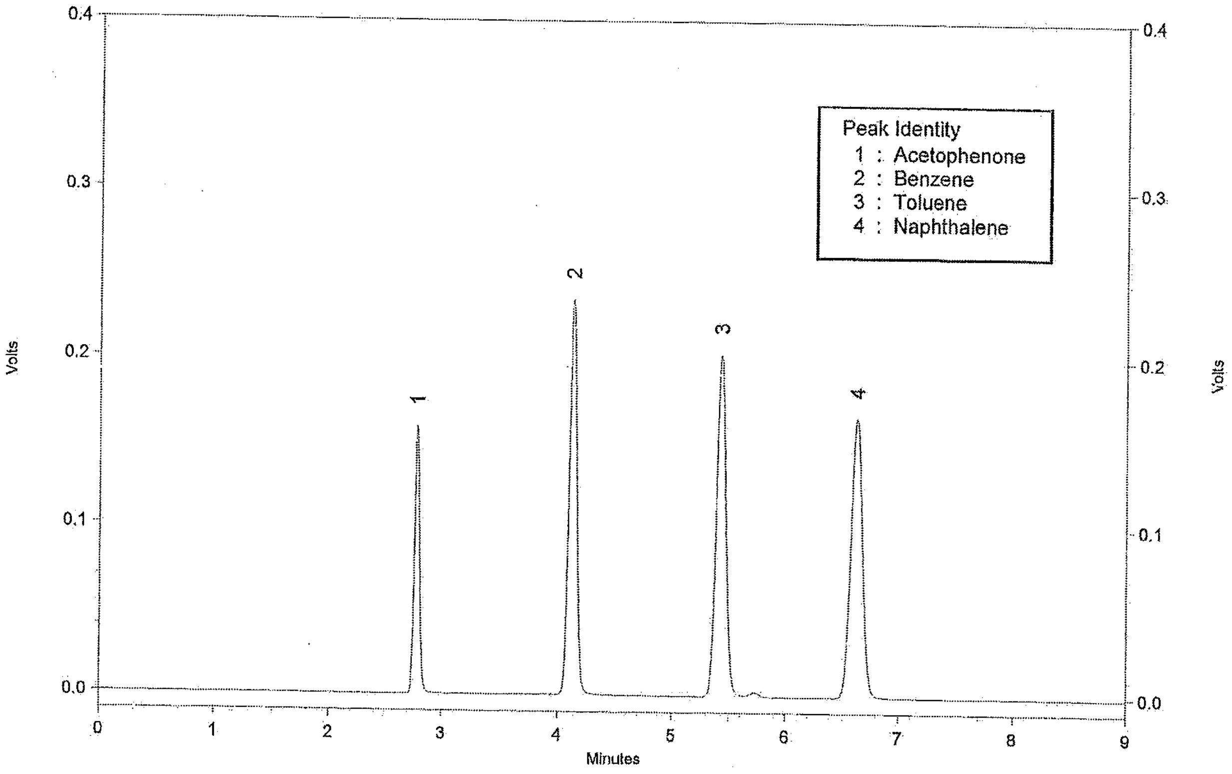
Channel A Method

Column Specification

Column Packing	Inertsil	ODS-3	3	[um]
Material Lot.No.	TQ3-2519			
Column Dimension	4.6 I.D.	x	150	[mm]
Guarantee	N =	19,000		

Test Condition

Mobile Phase	:	CH3CN / H20 =	65 / 35
Flow Rate	:	1.0	[ml/min]
Pressure	:	8.9	[MPa]
Column Temp.	:	40	[deg]
Detector UV	:	254	[nm]
Sample Size	:	1.0	[ul]



Channel A Results

Peak No.	Name	Time	W1/2	N=5.54(t/W1/2)^2	Asy=W0.05/2f
4	Naphthalene	6.63	0.110	19953	1.04

GL Sciences Inc.

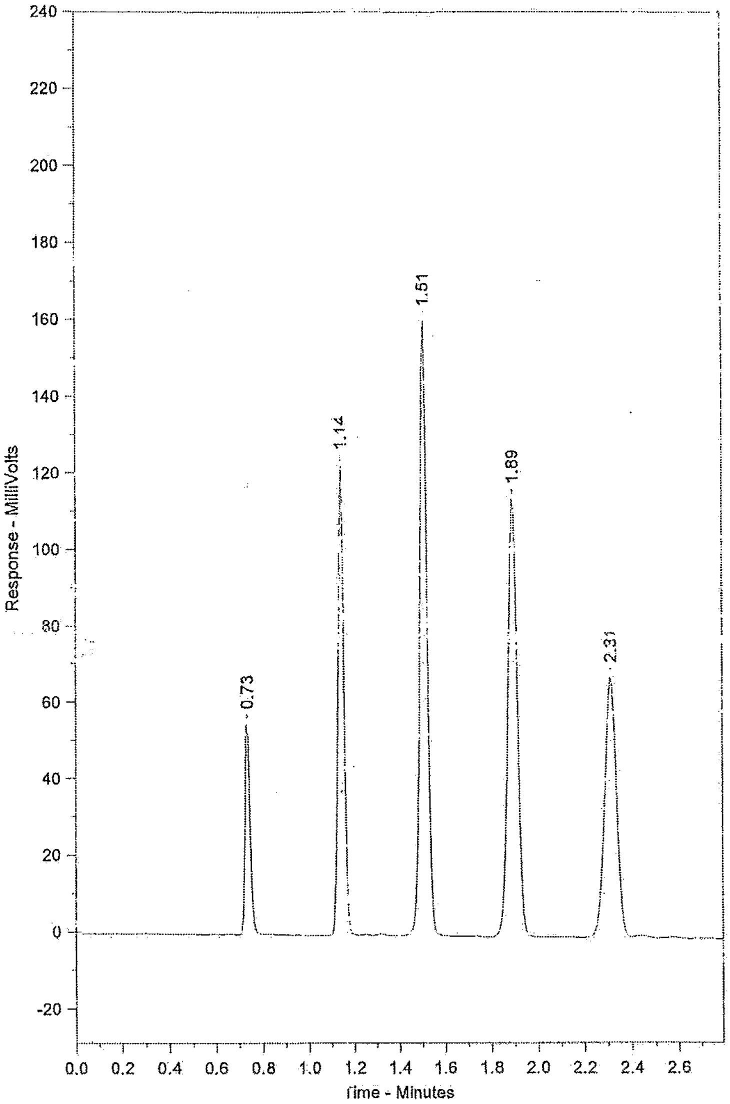
Thermo SCIENTIFIC

Certificate of Analysis

Column: FLUOPHASE RP
Lot Number: 8410
Length (mm): 100
I.D. (mm): 4.6
Detection: UV @ 254nm

Part Number: 82505-104630
Particle Size (um): 5
Temperature: Ambient
Injection Volume: 5 uL
Eluent: 60/40 ACN/HOH

Serial Number: 1072442E
Pore Size (A): 100
Pressure (psi): 800
Flow: 1.25 mL/min
QC Approval:



Peak No	Peak Name	Ret Time (min)	Capacity Factor	Selectivity
1	Theophylline	0.73	0.00	0.00
2	p-Nitroaniline	1.14	0.56	ERR
3	Methyl Benzoate	1.51	1.05	1.89
4	Phenetole	1.89	1.58	1.50
5	o-Xylene	2.31	2.15	1.36

Efficiency o-Xylene (n/m) = 100058
Asymmetry (10%) o-Xylene = 0.965

f:\CPWIN\SBDATA2\1072442E.0000.RAW

2

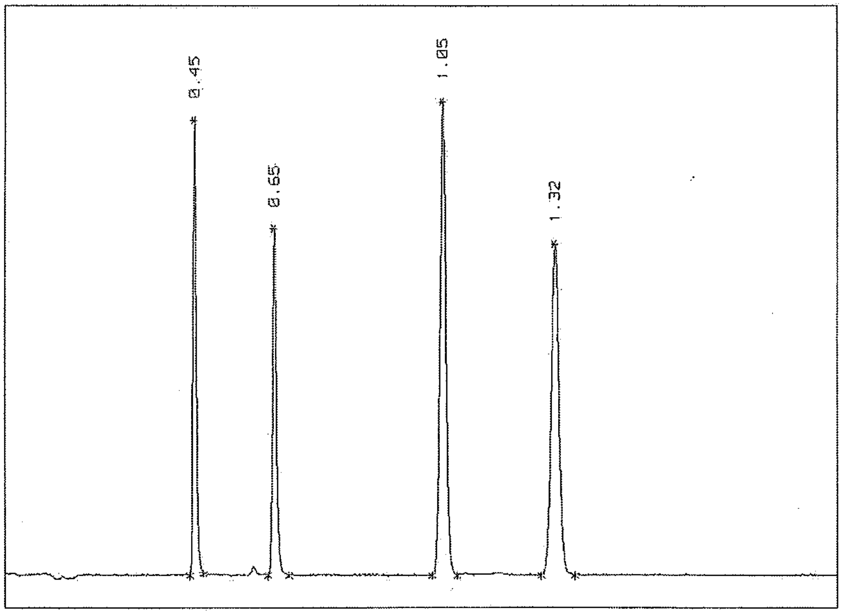
SUPELCO™
Analytical
595 North Harrison Road
Bellefonte, PA 16823-0048 USA
Telephone 800-247-6628 • 814-359-3441
Fax 800-447-3044 • 814-359-3044
email: supelco@sial.com
sigma-aldrich.com/supelco

SERIAL NO.: USBN001316
COLUMN: Ascentis Express F5 10cm x 4.6mm
PART NO.: 53590-U
PACKING LOT NO.: S11006

TEST CONDITIONS:
MOBILE PHASE = 60/40 ACETONITRILE/WATER
PRESSURE = 200 bar
FLOW = 1.8 mL/min
LINEAR VELOCITY = 0.370 cm/sec
TEMPERATURE = AMBIENT

PERFORMANCE RESULTS FOR NAPHTHALENE (K' = 1.91)

PARAMETER	SPECIFICATION	COLUMN USBN001316
THEORETICAL PLATES	MIN: 22000	28827
USP TAILING FACTOR	MAX: 1.25	1.09
SELECTIVITY	RANGE: 1.40 - 1.60	1.46



QC Sample:

Uracil (30 µg/ml)
Phenol (500 µg/ml)
4-Cl-Nitrobenzene (120 µg/ml)
Naphthalene (200 µg/ml)

29/01/2015 : Yikamalar sonrası p
145 bar (k. HPLC)
150 bar (〃)

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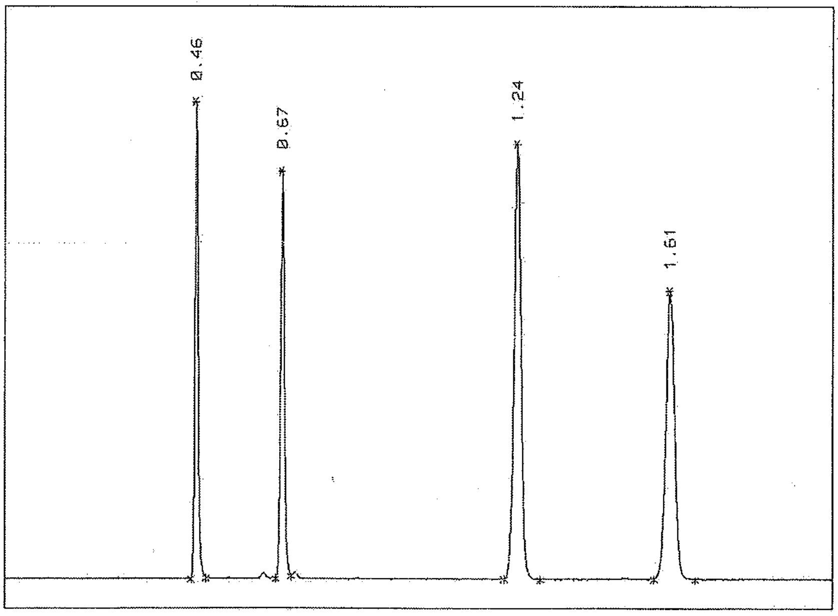
Fax 800-447-3044 • 814-359-3044
email: supelco@sial.com
sigma-aldrich.com/supelco

SERIAL NO.: USRT001358
COLUMN: Ascentis Express Phenyl-Hexyl 10cm x 4.6mm
PART NO.: 53352-U
PACKING LOT NO.: S11045

TEST CONDITIONS:
MOBILE PHASE = 60/40 ACETONITRILE/WATER
PRESSURE = 200 bar
FLOW = 1.8 mL/min
LINEAR VELOCITY = 0.360 cm/sec
TEMPERATURE = AMBIENT

PERFORMANCE RESULTS FOR NAPHTHALENE (K' = 2.46)

PARAMETER	SPECIFICATION	COLUMN USRT001358
THEORETICAL PLATES	MIN: 22000	28277
USP TAILING FACTOR	MAX: 1.25	1.05
SELECTIVITY	RANGE: 1.40 - 1.60	1.48



QC Sample:
Uracil (30 µg/ml)
Phenol (500 µg/ml)
4-Cl-Nitrobenzene (120 µg/ml)
Naphthalene (200 µg/ml)

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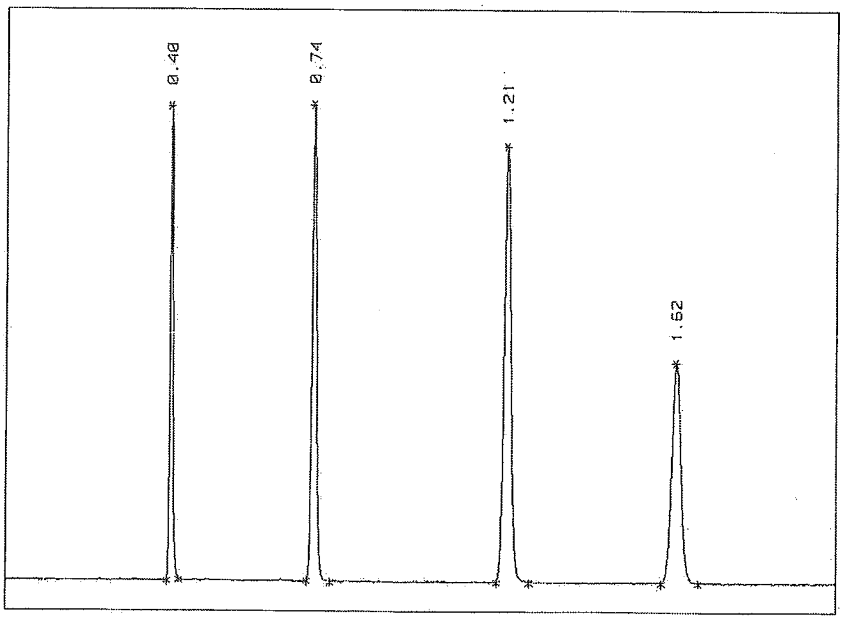
sigma-aldrich.com/supelco

SERIAL NO.: USPY005363
COLUMN: Ascentis Express C18 10cm x 4.6mm
PART NO.: 53827-U
PACKING LOT NO.: S12008

TEST CONDITIONS:
MOBILE PHASE = 60/40 ACETONITRILE/WATER
PRESSURE = 230 bar
FLOW = 1.8 mL/min
LINEAR VELOCITY = 0.420 cm/sec
TEMPERATURE = AMBIENT

PERFORMANCE RESULTS FOR TOLUENE (K' = 3.07)

PARAMETER	SPECIFICATION	COLUMN USPY005363
THEORETICAL PLATES	MIN: 22000	26580
USP TAILING FACTOR	MAX: 1.25	1.07
SELECTIVITY	RANGE: 1.37 - 1.57	1.50

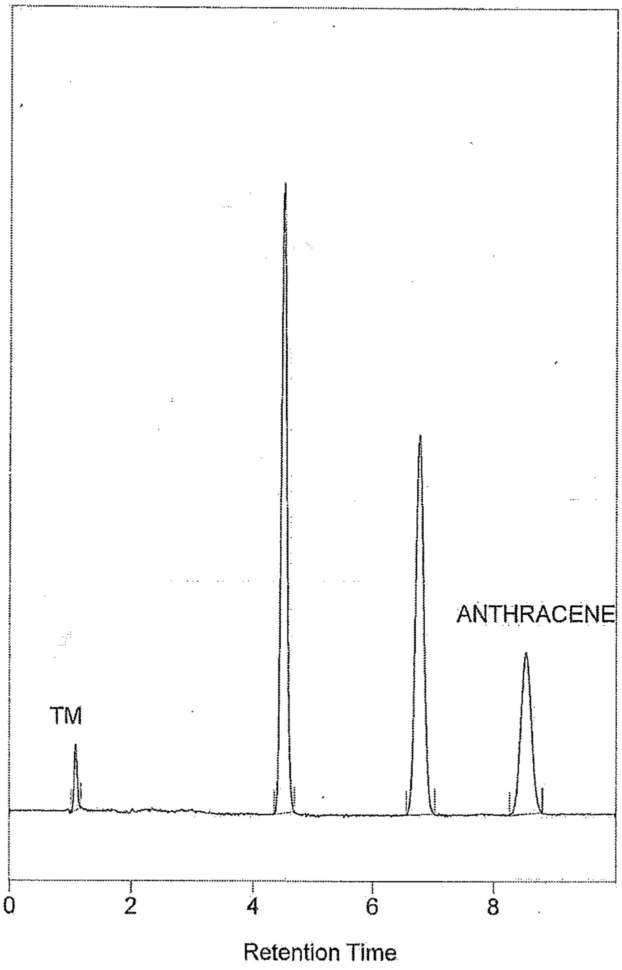


QC Sample:

Uracil (20 µg/ml)
Acetophenone (30 µg/ml)
4-Cl-Nitrobenzene (80 µg/ml)
Toluene (2.40 mg/ml)

Teknokroma®
Professionally Friendly

Part number: TR-013111 **Serial number:** N36715 **Date:** 26/10/2004
NUCLEOSIL 100 C18 3 µm 10x0.46



Chromatographic Conditions

Eluyent:	ACETONITRILE/WATER 65:35
Proportion:	65:35
Flow:	0.9 ml/min
Pressure:	76 bars
Injection volume:	10 µl
Temperature:	AMB.
Detection:	UV254

Assay solution composition

URACIL (DT)	0.002 mg/ml
NAPHTHALENE	0.100 mg/ml
ACENAPHTHENE	0.200 mg/ml
ANTHRACENE	0.004 mg/ml

Remarks

Results

Component	t_R	K'	N/m	h	As
ANTHRACENE	8.52	6.80	116427	2.86	1.04

Aproved:

CURRICULUM VITAE (CV)

Name-Surname : Wafa Farooq Suleman Badulla

Languages : Arabic (Fluent, mother language)
English (Professional, TESOL-certified)
Turkish (Functional, TÖMER-certified)
Urdu (Indian language, spoken-only)

Place and Date of Birth : Aden, Yemen / 18th Nov. 1976.

E-Mail : aden.wf.77@gmail.com.

Education and Professional Experience:

- 2017, Anadolu University, Health Sciences Institute, Department of Analytical Chemistry.
- 2001, Aden University, Faculty of Pharmacy, B.A.
- 2002-uptodate, Research Assistant and Lab Demonstrator, Aden University, Faculty of Pharmacy.
- 2002-2003, Pharmacist, Privet Pharmacy.
- 2014-2015, Pharmacist, Government poly clinic pharmacy.

Conference Papers Delivered:

- Soyseven, M., Badulla, W., Atkosar, Z., Arli, G., Can, N.Ö. Development and validation of a method consisting of spectrophotometric, flow injection and voltammetric determinations for the analysis of montelukast sodium in dosage form, 74th International Pharmaceutical Federation Congress (FIP-2014-Bangkok, Thailand, 31 August - 4 September (2014).
- Badulla, W., Atkosar, Z., Arli, G. Electrochemical behavior and determination of escitalopram oxalate on hanging mercury drop electrode, International Multidisciplinary Symposium on Drug Research & Development'15 (DRD-2015-Turkey, October 15-17 (2015).
- Badulla, W., Şener, E., Atkosar, Z., Arli, G, liquid chromatography–tandem mass spectrometry method for the determination of escitalopram in human urine, International Gazi Pharma Symposium Series (GPSS 2015), Gazi University Faculty of Pharmacy, Antalya-Turkey, November 12-15 (2015).
- Dal, A. G., Badulla, W. Validated determination of escitalopram by capillary zone electrophoresis in pharmaceutical preparations, 6th International Conference and Exhibition on Analytical and bioanalytical techniques (OMICS 2015), Spain, Valencia, 01-03 (2015).

Publications:

- Badulla, W., Arli, G. (2017). Comparative study for direct evaluation of montelukast sodium in tablet dosage form by multiple analytical methodologies. Rev. Roum. Chim., 2017, 62(2), 173-179.

Academic awards:

- 2005, Postgraduate certificate in teaching way in higher education awarded by Aden University.
- 2001, Scientific Achievement Certificate for obtaining the first highest score of BA degree in the academic year 2000/2001, awarded by the president of Yemen Republic.

Skills, knowledge and other activities

- Computer skills; Microsoft Office Word, Excel and PowerPoint.
- I have an experimental animal using certificate from Eskisehir Osman Gazi University (2014).
- Member of Yemen Pharmacists Syndicate.
- Reading, Handicraft, Knitting and Rose cultivation.