

EGE UNIVERSITY
GRADUATE SCHOOL OF
NATURAL AND APPLIED SCIENCE

DEVELOPMENT AND VALIDATION OF
CHROMATOGRAPHIC AND VOLTAMMETRIC
METHODS FOR ANALYSIS OF
PHARMACEUTICALLY IMPORTANT COMPOUNDS

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Code of discipline:405.03.01
Presentation Date: 10.11.2017

Bornova-İZMİR
2017

K. Volkan ÖZDOKUR tarafından Doktora tezi olarak sunulan "*Development And Validation of Chromatographic And Voltammetric Methods For Analysis of Pharmaceutically Important Compounds*" (Farmasötik Önemi Olan Bileşiklerin Tayinine Yönelik Voltammetrik ve Kromatografik Yöntem Geliştirilmesi ve Validasyonu) başlıklı bu çalışma E.Ü. Lisansüstü Eğitim ve Öğretim Yönetmeliği ile E.Ü. Fen Bilimleri Enstitüsü Eğitim ve Öğretim Yönergesinin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve 10.11.2017 tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

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Kemal Volkan ÖZDOKUR

10/11/2017



ÖZET**FARMASÖTİK ÖNEMİ OLAN BİLEŞİKLERİN TAYİNİNE
YÖNELİK VOLTAMMETRİK VE KROMATOGRFİK YÖNTEM
GELİŞTİRİLMESİ VE VALİDASYONU**

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Kasım 2017, 103 sayfa

İlaç analizleri, halk sağlığı üzerinde hayati önemi olan kalite kontrol analizlerinde büyük bir yer tutmaktadır. Bu yüzden ilaç etken maddelerinin ve biyolojik maddelerin tayinine ilişkin basit, hızlı, seçimli, duyar ve doğru yöntem geliştirmeye yönelik birçok çalışma vardır. Özellikle, analitlerin ayrılması ve saptanmasının eş zamanlı gerçekleştiği kromatografik yöntemlerin kullanımı çok yaygındır. Bu yöntemler birçok molekül için çok seçimli ve duyar sonuçlar verse de bu yöntemlerin pahalı olmaları, kullanımlarının zor ve maliyetli olması gibi bazı sakıncaları vardır.

Günümüzde elektrokimyasal yöntemler nanoteknolojinin de kullanımıyla güncelliğini koruyan bir araştırma alanıdır. Elektrot yüzeylerinin nanoyapılarla zenginleştirilmesi, gıda maddelerinin kalite kontrolünde, çevresel, klinik ve farmasötiklerin analizinde birçok avantaj sağlamıştır. Nanomalzemelerle modifiye edilmiş sensörler, sinyalde önemli artış, aşırı potansiyelde azalma ve hızlı elektrot kinetiği nedeniyle analizlerde hızlı yanıt alma ve yüksek duyarlılık gibi üstünlükler sağlamaktadır. Ayrıca, elektrokimyasal sistemlerin ucuz oluşu ve kullanım kolaylığı gibi avantajları düşünüldüğünde, bu yöntemler birçok analizde kullanışlı bir alternatif olmaktadır.

Bu tez kapsamında biyolojik önemi olan moleküllerin kromatografik ve elektrokimyasal tayinlerine ilişkin yöntem geliştirilmiş ve valide edilmiştir. İlk olarak Parkinson hastalığının tedavisinde kullanılan Carbidopa, Droksidopa ve Levodopa için LC-MS/MS yöntemi geliştirilmiş ve bu üç molekülün bir arada tayini ilk defa HILIC ve C-18 kolonda gerçekleştirilmiştir. Geliştirilen yöntem,

ticari tablet ve kalite kontrol örnekleri üzerinde test edilmiş ve tatmin edici sonuçlar elde edilmiştir.

Öte yandan, UV detektörlü HPLC cihazı kullanılarak, Levodopa, Dopamin, ve metabolitleri homovanilik asit ve DOPAC için kromatografik yöntem geliştirilmiş ve bu yöntem de yapay beyin sıvısı ortamında valide edilmiştir.

Tez çalışmalarının elektrokimyasal kesiminde literatürde ilk defa ultrasonikasyonla eşleşmiş pulslü elektropolimerizasyon tekniğiyle polipirol karbon nanotüp kompozit filmler elde edilmiştir. Elde edilen bu filmin yapısı ve elektrokimyasal özellikleri; taramalı elektron mikroskobu (SEM), Fourier dönüşümlü kızılötesi spektroskopisi (FTIR), Elektrokimyasal empedans spektroskopisi ve döngüsel voltammetri tekniği kullanılarak aydınlatılmıştır. Ardından, carbidopa ve droksidopa'nın elde edilen bu elektrottaki (GCE/PPy-CNT) elektrokimyasal davranışları incelenmiştir. Ardından, ölçüm ortamının pH'ı, pirol derişimi, uygulanan puls sayısı, kullanılan karbon nanotüp miktarı optimize edilmiştir. Optimizasyon çalışmalarında döngüsel voltammetri, kalibrasyon çalışmalarında da duyarlılığı arttırmak için kare dalga voltammetrisi kullanılmıştır. Ancak, droksidopa ve carbidopa'nın yükseltgenme sinyalleri çakıştığından, çözüm olarak kare dalga voltammogramları kısmi en küçük kareler yöntemiyle modellenmiş ve yöntemin başarısı derişimi bilinen kalite kontrol örnekleriyle test edilmiştir. Benzer durum Levodopa ve Carbidopa için de gözlenmiş ve benzer bir yaklaşımla girişim engellenmiştir. Ayrıca yöntem ticari tabletlere uygulanmış ve tatmin verici sonuçlar elde edilmiştir.

Elde edilen son elektrodun son uygulaması çok bilinen anti-analjezik ilaç olan metamizol sodyum üzerinde yapılmıştır. PPy-CNT modifiye elektrot metamizolün yükseltgenmesinde katalitik etki göstermiştir. Geliştirilen yöntem ticari tabletlere uygulanmış ve yüksek gerikazanım değerleri bulunmuştur.

Anahtar sözcükler: Katekolaminler, Carbidopa, Droksidopa, Dopamin, sıvı kromatografi, elektrokimya

ABSTRACT**DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC
AND VOLTAMMETRIC METHODS FOR ANALYSIS OF
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PhD Thesis, Chemistry Department

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November, 2017, 103 pages

Drug analyses have played a vital role in quality control required by the great impact on public health. Therefore, a vast number of studies are dedicated to develop simple, fast, selective, sensitive and accurate method for the determination of active pharmaceutical and biological compounds. In particular, chromatographic methods are widely used in such analyses due to their separation and detection abilities at same time. This method provides selective and sensitive results for many analytes. However, some disadvantages such as; expensive instrumentation, labor usage and high working expenses are limits its usage

Electrochemical methods based on nanomaterial are currently an ongoing research field. Several advantages of nanomaterial as electrode modifiers have been demonstrated for the analysis of wide range of chemicals in food quality, clinical, pharmaceutical and environmental interest. Nanomaterial-modified sensors exhibit excellent sensitivity and fast response due to the signal enhancement provided by high surface area, low overvoltage, and rapid kinetics.

In this thesis, chromatographic and electrochemical methods for the determination of some biologically important compounds were developed and validated. Chromatographic studies have performed with liquid chromatography tandem mass spectrometry (LC-MS/MS) technique for the ternary analysis of the anti-Parkinson drugs Carbidopa, L-Dopa and Droxidopa for the first time. The method developed was tested by using commercial tablets and quality control samples and satisfying results have been obtained. In the second part, an HPLC-UV method was developed and validated for the simultaneous determination of L-Dopa, Dopamine, HVA and DOPAC in artificial cerebrospinal fluid.

In the electrochemical part of the thesis, a novel polypyrrole-carbon nanotube composite electrode was developed by using ultrasonic assisted pulsed electropolymerization technique. The film was characterized by Scanning Electron Microscopy (SEM), Fourier transfer infrared spectroscopy (FTIR), electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). Then after, the electrochemical behavior of carbidopa and droxidopa was investigated on GCE/PPy-CNT electrode. The influence of pH, pyrrole concentration, amount of CNT, applied pulse number was investigated by aid of cyclic voltammetry. Calibration studies have been conducted by using square wave voltammetry (SWV) for attaining higher sensitivity. Simultaneous analysis of carbidopa and L-Dopa at GCE/PPy-CNT electrode was hindered due to the overlapping peaks of the analytes. Thus, partial least square technique was used and the trade formulation of commercial tablets was analyzed. The obtained results were found in good agreement with label value. Similarly, simultaneous determination of carbidopa and droxidopa was performed with the electrode developed. The quality control samples with known concentration was analyzed and satisfactory recoveries was obtained.

The last application of the electrode has been carried out for the analysis of anti-analgesic drug in tablets. PPy-CNT/GCE electrode exhibited catalytic effect towards to metamizol sodium oxidation. Then, the method developed was applied to assay content analysis of commercial tablets and the results were found in good agreement with label value.

Keywords: Catecholamine, Carbidopa, Droxidopa, Dopamine, liquid chromatography, electrochemistry

ACKNOWLEDGEMENT

I would like to thank my supervisor Prof. Dr. F. Nil ERTAS for all her guidance, support, encouragement and motherly attitude over the PhD thesis.

I would like to express my gratitude to Prof. Dr. Suna TİMUR, Prof. Dr. Zekerya DURSUN and Assoc. Prof. Dr. Hasan ERTAŞ for their valuable suggestions during the PhD studies.

I am also grateful to İrem Çakar, Çağrı KOÇAK, Şükriye KARABİBEROĞLU and Deniz ELMACI and all of my laboratory colleagues for their kind help.

I am grateful to TUBITAK for financial support with a project number 114 Z 153 during my doctorate studies and I would like to acknowledge to ARGEFAR for supporting opportunity to use chromatographic systems.

I would like to thank my mother Nurhan ÖZDOKUR and my wife Seyhan ÖZDOKUR and the other member of my family for their encouragement, understanding and attitude throughout my study.

This thesis is dedicated to my father memories...



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ABBREVIATIONS

ACN	: Acetonitrile
CV	: Cyclic Voltammetry
CNT	: Carbon nanotube
DA	: Dopamine
DP	: Differential Pulse
EIS	: Electrochemical Impedance Spectroscopy
GCE	: Glassy Carbon Electrode
HPLC	: High Performance Liquid Chromatography
L-Dopa	: Levodopa
LC–MS/MS	: Liquid Chromatography Tandem Mass Spectrometry
LSV	: Linear Sweep Voltammetry
PPy	: Polypyrrole
SEM	: Scanning electron microscopic
SWV	: Square Wave Voltammetry
TFA	: Trifluoro Acetic Acid

1. INTRODUCTION

1.1. Analysis of Pharmaceutically Important Compounds

Nowadays, pharmaceuticals play an important part in human health but, these compounds only serve their intent if they are administered in an appropriate amount and are free from impurities. The whole process starts with the innovation of a drug molecule that cures a particular disease (Siddiqui et al., 2017). The synthesis and characterization of the compound is followed by its analysis for safety reasons. The investigations on the experiments to prove that it is absorbed in the blood stream, distributed in the body, metabolized sufficiently but, the most importantly the compound should demonstrate non-toxicity. Therefore, toxicity and carcinogenicity tests are performed in vitro studies as well as the animal testing to check kinetics. Then, the clinical trials start to reveal the optimum dose and its potential interaction with other medicines. Finally, the drug has become commercial product for the patients. In each step, analytical methods are applied to monitor the drug interaction as well as the quality control purposes.

Therefore, pharmaceutical analyses require rapid, sensitive selective methods with high throughput for analyzing trace amounts in mixtures as well as the screening approaches (Lee and Kerns, 1999). Chromatographic methods are commonly used for the quantitative and qualitative analysis of drug substances in biological samples and up to date, liquid chromatography with tandem mass spectrometry is the method of choice in many laboratories (Mattrey et al., 2017).

In addition to the chromatographic methods, voltammetric techniques are also emerging as an economical alternative for pharmaceutical analysis offering high sensitivity, accuracy, and precision. A number of review articles have been presented in the field of electrochemical drug analysis by using solid electrodes (Özkan et al., 2003) and carbonaceous materials for electrode fabrication (Uslu and Özkan, 2007). Gupta and co-workers have reviewed the voltammetric techniques for drug analysis assessing the utility of several kinds of electrodes and surface-active agents as well for the determination (2011).

In a more recent paper, basic strategies for the enhancement of the sensory performance for drug assays by utilizing nanomaterials in the design of novel chemical and biochemical sensors have been reviewed (Rahi et al., 2016).

Pharmaceutically important compounds differ in structural and influential groups. Among them, antiparkinson drugs are popular nowadays. Parkinson's disease is a degenerative disorder of the CNS. The motor symptoms of Parkinson's disease result from the death of dopamine-generating cells in a region of the mid brain, called ***substantia nigra***. Early in the course of the disease, the most obvious symptoms are movement-related including shaking, rigidity, slowness of movement and difficulty with walking and gait.

Early motor symptoms of the disease can be managed by modern treatments effectively, which mainly use of L-Dopa and dopamine agonists (Ceravolo et al., 2009). The dopamine precursor ***levodopa*** (L-dopa) is employed for the treatment. For better therapeutic effect and lower toxicity, ***carbidopa*** is administered in association with levodopa in pharmaceutical formulation as an inhibitor on the decarboxylase activity. Therefore, the development of a method for the simultaneous determination of L-dopa and carbidopa is of great importance because of their coexistence in pharmaceutical preparations (Quintino et al., 2006, Tajik et al., 2013, Ardakani et al., 2013, Vilhena et al., 2014).

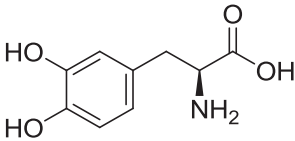
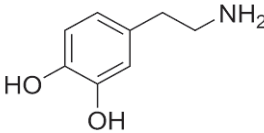
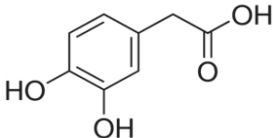
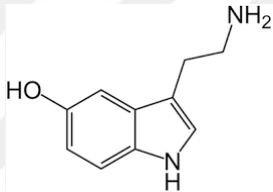
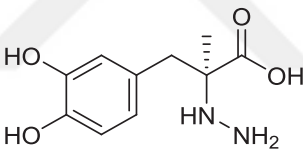
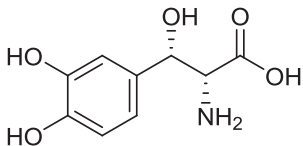
Droxidopa is another important psychoactive drug and synthetic amino acid precursor which acts as a prodrug to the neurotransmitters (Gupta et al., 2013). It is capable of crossing the protective blood-brain barrier and is metabolized by dopadecarboxylase enzyme. Next section involves detailed information on the catecholamines.

1.2. Catecholamines and its derivatives

A wide range of neural pathway originated catecholamines employ biogenic amines as neurotransmitters (Kobayashi et al., 2001). The neurotransmitters in cerebrospinal fluid can be an indicator of neuronal functioning in nearby diencephalon structures. Hence, it is very crucial to quantitate neurotransmitters and their metabolites level in the extracellular fluid for monitoring neurotransmission process (Deurwaerdère and Millan, 2017, Goldstein et al., 2014). Table 1.1 summarizes the chemical structure, properties of selected catecholamines.

Levodopa (L-3,4-dihydroxyphenylalanine, L-Dopa) is the precursor of catecholamines, which is produced and consumed as part of the normal biology of humans, some animals and plants. It is produced via biosynthesis from L-tyrosine.

Table 1.1 Chemical properties of selected catecholamines

Compound	Molecular Formula	MW (g mol ⁻¹)
Levodopa		197.19
Dopamine		153.18
DOPAC		168.15
Serotonine		176
Droxidopa		213.187
Carbidopa		226.229

L-Dopa can be manufactured and in its pure form is sold as a psychoactive drug with the trade names include Sinemet, Parcopa, Atamet, Stalevo, Madopar, and Prolopa. As a drug, it is used in the clinical treatment of Parkinson's disease and dopamine-responsive dystonia (Antonini et al., 2015).

Dopamine (3,4-dihydroxyphenethylamine, DA) is an important neurotransmitter or hormone of the catecholamine and phenethylamine families due to its a number of significant roles in the human brain and body. From the chemical point, it is an amine that is formed by loosing a carboxyl group from a molecule of L-Dopa.

DA is responsible to communications of nerve cells and plays a major role in reward-motivated behavior. Several distinct dopamine systems are occupied in the brain. Level and neuronal activity of dopamine increase by most type of reward and addictive drugs, respectively. Other dopamine systems are responsible in motor control and in releasing of other important hormones (Bjorklund and Dunnet, 2007). From the view of physiological importance, it is a challenge to monitor DA and its metabolites (Figure 1.1), because the control of DA level is vital in the treatment of Parkinson's disease.

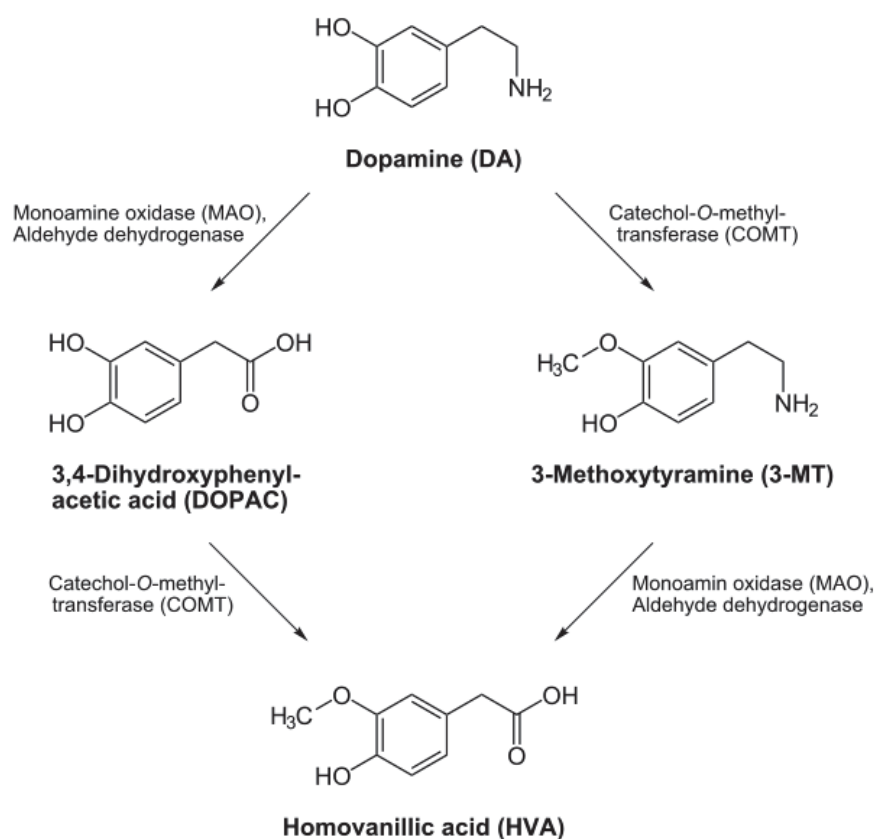


Figure 1.1 Metabolic pathway of dopamine

DOPAC (2-(3,4-Dihydroxyphenyl)acetic acid), is one of the metabolites of the DA. Concentrations of DOPAC in healthy adult human's antecubital venous plasma are about 30-50 times that of DA (Deurwaerdère and Millan, 2017).

Droxidopa, (L-DOPS) is a psycho-active drug and synthetic amino acid precursor for producing the norepinephrine (NP) and epinephrine (EP) neurotransmitters. While the L-DOPS can cross protective blood-brain barrier (BBB), NP and EP are not enable pass BBB (Movlaee et al., 2017).

L-DOPS was commercialized for the treatment of hypertension. It is metabolized by DOPA decarboxylase (DDC) as known as aromatic L-amino acid decarboxylase (AAAD). Increase at the levels of NE and EP in the PNS by L-DOPS, induces tachycardia or increased heart rate and hypertension or increased blood pressure, thereby enabling the body to maintain blood flow while standing.

Carbidopa is one of the most widely described anti Parkinson drug in order to inhibit peripheral metabolism of L-dopa. On the other hand, L-dopa utilized as an active compound is ineffective against Parkinson's disease, because of its conversion to dopamine by a decarboxylation process in extra cerebral tissue. Thus, only a small amount of L-Dopa can be transported across the cerebral tissue to the central nervous system. When carbidopa is along with used with L-dopa, as inhibitor for the decarboxylase activity, the amount of the transferred L-dopa is increased.

It is very crucial to control the content of drug assay in pharmaceutical preparations, for achieving a improved medicinal effect and lower toxicity. In particular, concentration of catecholamines plays key role on CNS disorders. Therefore, many studies are dedicated to develop a simple, inexpensive, fast, sensitive and accurate method for the simultaneous determination of drugs and their metabolites. Next headline includes the analytical methodologies developed for determination of Carbidopa, Droxidopa and L-Dopa, Dopamine and DOPAC in different matrixes.

1.3. Analytical Methods for Quantation of Catecholamines

Numerous methods such as; spectrophotometry , gas chromatography (GC) with mass detection, high performance liquid chromatography (HPLC) with various detection system ,chemiluminescence , amperometric and voltammetric determination, potentiometry , radioimmunoassay and flowinjection analysis (FIA) have been reported for the determination of catecholamines in various biological samples and pharmaceutical preparations (Bugamelli et al., 2011). Table 1.2 summarizes the electrochemical methods developed for catecholamine derivatives.

Table 1.2 Literature Survey for Electrochemical Determination Methods of Catecholamines

Catecholamine	Method	Electrode/Detector	Linear Range (M)	LOD (M)	Reference
DOPAC	Amperometry	Tyrosinase-chitosan modified GCE	0.006 – 200	0.003	Liu et al., 2005
Carbidopa	DPV	GCE	0.5 - 5.4	0.38	Quantino et al., 2006,
Levodopa	CV		0.2 - 750	0.042	
Dopamine	DPV	Gold nanoparticles modified ITO electrode	0.001 – 500	0.0005	Goyal et al., 2007,
Serotonin	DPV	Graphene modified electrode	0.01 – 250	0.003	Kim et al., 2010
Dopamine	DPV		4 - 100	2.64	
Levodopa	DPV	GCE (Multivariate Calibration)	110 – 1300	5.12	Zapata-Urzua et al., 2010
Carbidopa			31 – 470	2.16	
Benserazide			31 – 620	2.77	
Dopamine	DPV	Iron(II) phthalocyanine modified MWCNT CPE	0.5 – 8.85	0.205	Patrascu et al., 2011
Carbidopa	DPV	EBNBH and CNT modified CPE	35 – 700	0.094	Ardakani et al., 2011
Carbidopa	SWV	MBIDZBr modified CNT PE	0.1 – 420	0.06	Beitollah et al., 2012
Carbidopa	SWV	Chloranil and CNT modified CPE	0.09 - 400	0.071	Rastakhiz et al., 2012
Droxidopa	SWV	ZnO nanoparticles and BmimPF ₆ modified CPE		0.15	Gupta et al., 2013
Droxidopa	SWV	CNT and 5AEB modifye CPE	0.12 – 225	0.05	Tajjik et al., 2013
Carbidopa	DPV	Graphene modified GCE	1 – 16	1.8	Wang et al., 2013
Levodopa	DPV	IL–TiO ₂ nanoparticles modified CPE	0.10– 900.0	0.041	Ardakani et al., 2013
Carbidopa			20.0–900.0	0.38	
Levodopa	DPV	Co(OH) ₂ nanoparticles and MWCNT modified carbon IL electrode	0.25–225	0.12	Babaei et al., 2013
Serotonin			0.05–75	0.023	
Levodopa	SWV	Graphene oxide (RGO), gold nanoparticles and 2-(3,4-dihydroxy phenyl) benzothiazole (DHB) modified glassy carbon electrode	0.05–1200.0	0.018	Benvidi et al., 2015
Dopamine	DPV	Cu np PPy modified GCE	0.001-0.1	0.00085	Ulubay and Dursun, 2010
Uric acid			0.001-10	0.00080	

Due to the electroactive nature of catecholamines, voltammetric methods are very promising (Gupta et al., 2011). However, most of the sample matrixes are very complex and electrochemical methods are so receptive to interferences. This limitation of the method can be overcome by using chemometric approach (Zapata-Urzuu et al., 2010).

Alternatively, in most cases chromatographic methods are promising due to their high selectivity and sensitivity. Some of these methods developed for catecholamines are listed in Table 1.3.

Table 1.3 Chromatographic Methods developed for Catecholamine determination

Catecholamine	Method	Detector	Linear Range (μM)	LOD (μM)	Ref.
Carbidopa	HPLC	UV detection	1.3 – 512	0.8	Issa et al., 2011
Carbidopa	HPLC	Electrochemical detection	0.082 – 16.4	0.016	Bugamelli et al., 2011
Carbidopa Levodopa 3-o-methyldopa	LC-MS/MS	ESI-MS/MS	65 – 800 75 – 800 20 – 400	65 75 20	Vilhena et al, 2014

According to literature survey, binary and ternary mixtures of catecholamines have been analysed simultaneously and very low detection limits were achieved. However, operating these devices is expensive and time consuming. Furthermore, most of chromatographic methods require sample preparation and preconcentration step prior to analysis which needed many chemical reagents, solvents and labour processes.

It is important to note that; combination of separation abilities of liquid chromatography and detection ability of electrochemical methods are superior alternative for simultaneous determination of catecholamines.

1.4. Metamizole Sodium

In this thesis, in addition to the selected catecholamines, metamizole sodium (Met) was also studied. Metamizol sodium or dipyrone is a pyrazolone derivative (sodium salt of the 1-phenyl-2,3-dimethyl- 4-methyl aminomethane sulfonate-5-pyrazolone), and water soluble white crystalline powder which shows analgesic and antipyretic effects like paracetamol (Figure 1.2).

Met is widely used as analgesic, antipyretic, and antispasmodic drug in many pharmaceutical preparations such as analgin, novalgin, and other commercial trademarks since 1922. This compound showed short maximal systemic adsorption time after hydrolysis in the gastric juice (Maier, 1999). Selected physicochemical parameters of metamizol sodium are listed in Table 1.4.

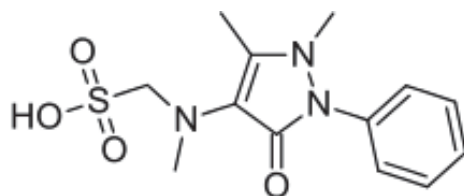


Figure 1.2 Chemical Structure of Metamizole

Table 1.4 Physicochemical properties of Metamizole Sodium

Parameter	Value
Molecular Formula	C ₁₃ H ₁₆ N ₃ NaO ₄ S
Molecular weight	333.339
Water Solubility	5.8 mg/mL
log P	1.07
log S	-1.8
pKa	-1.2

Although Met has many advantages, its use in some countries is restricted because of the risk of adverse reactions and can cause occasional or rare reactions as transitory disturbances and inflammation of the renal tissue, mainly in patients with renal disease history or in cases of overdose (Pavlic et al., 2004). Therefore, the sensitive, accurate and precise measurement of this molecule in pharmaceutical preparation is very crucial for the public health. Numerous analytical methods have been developed for the determination of Metamizol sodium such as aqueous and nonaqueous titration, coulometry, polarography, potentiometry, fluorimetry, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (Domínguez-Ramírez et al., 2012; Dinç et al., 2001). Among these methods electroanalytical methods are promising, due to their simplicity cheap instrumentation and reagents, and greener applications. Therefore, this part of the thesis was dedicated to develop a sensor for the determination of metamizol sodium by using polypyrrole modified GCE.

1.5 The aim of the Thesis

Nowadays, analytical chemistry mostly focused on food, environmental and drug analyses. Due to development drug industry analyses of drug assay in pharmaceuticals and biological fluids became very popular. Among the chromatographic methods liquid chromatography with MSMS detection is promising however, the price of analytical device and usage expenses are relatively much higher than other methods. Electrochemical methods provides cheaper, easy alternative for the analysis of pharmaceutical assays.

In this thesis, it was aimed to develop an electrochemical method for simultaneous determination of carbidopa, levodopa and droxidopa in pharmaceutical formulations and tablets. Appropriate modifiers for the electrode surfaces have been searched in terms of selectivity and sensitivity.

Today, nanomaterial integrated sensor technologies are in continuous progress due to their widespread applications in clinical, pharmaceutical and environmental analysis. Composite materials developed by coupling the conducting polymers with carbonaceous nanomaterials, carbon nanotubes in particular, have displayed a synergetic effect with greater application potentials in many fields. However, the structural consistency of the composite suffers from the poor dispersion of CNTs in the coating solution and sonication is the method of choice for preventing agglomeration by accelerating the mass transfer.

Present study describes a new synthesis route for the preparation of polypyrrole/CNT composite film via combining the ultrasonication and electrochemical pulsed deposition for the first time. The effectiveness of nanostructured composite materials based on polymeric films, carbon nanotubes and metallic nanoparticles has been explored. have been determined by chromatographic methods.

Chromatographic studies have been carried out in two sections; In addition to the dopamine, Carbidopa and Droxidopa determined by LC-MS/MS system, L-Dopa and two metabolites namely; DOPAC and HVA have been also determined by HPLC-UV system. Following the optimization studies, the method validation was performed and LC-MS/MS method has been used for verification of data set.

1.6. Theoretical Background of the Analytical Methods

1.6.1. Liquid Chromatography

The International Union of Pure and Applied Chemistry (IUPAC) published the definition for chromatography as follows: “*Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction. A mobile phase is described as a fluid which percolates through or along the stationary bed in a definite direction*” (IUPAC, 2012).

It may be a liquid, a gas or a supercritical fluid, while the stationary phase may be a solid, a gel or a liquid. If a liquid, it may be distributed on a solid, which may or may not contribute to the separation process. Thus, four components can be accounted for a chromatographic system, namely: device for sample introduction, mobile phase, stationary phase and detector. These four components can be differed for various types of chromatographic techniques related to the chemical nature of sample (Harvey, 2000).

Liquid chromatography is a separation technique for trace amount of nonvolatile, very wide range of organic compounds, such as; drug assays, their metabolites, peptides, proteins, pesticides etc. This technique, used to separate the components in a mixture, to identify each component, and to quantify each component in analytical chemistry. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.

Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column. Flow scheme was shown in the Figure 1.3. Although many detectors are available for various applications in LC system, such as; Ultra Violet (UV), Diode Array (DAD), Florescence (FD), Reflective Index (RI), mass spectrometers (MS) are superior detectors in terms of selectivity and sensitivity. These detectors works for most compounds across a wide polarity and molecular weight range. It can be tuned to be selective to reduce interference, enhance detection limits and reduce sample preparation.

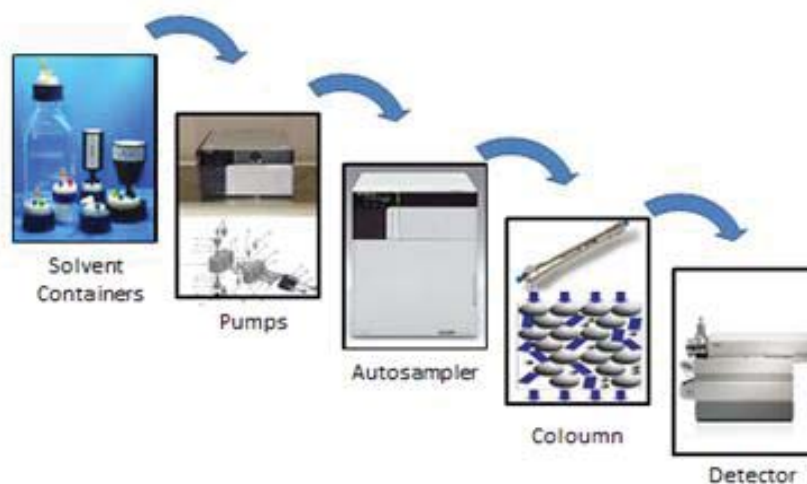


Figure 1.3 Schematic illustration of LC system

MS has linear dynamic ranges between 3 and 5 orders of magnitude and can provide information during quantitation in terms of molecular weight and fragmentation pattern. As such, MS is quickly becoming the detector of choice for routine analysis. The advantage of the mass spectrometer is stated that it can provide that absolute identification along with molecular weight of the analyte. Furthermore, the mass spectrometry can be used for identification with a small amount of analyte about in picogram (pg) amounts with high accuracy and precision (Mattrey, 2017).

Although many types of MS detectors are available three quad MS/MS (or tandem MS) system are very practical for routine analyses. As can be followed from Figure 1.4, the analyte molecules are separated and ionized by ESI system then precursor ions are detected in Q1. Then after the charged ions split into small fragments in Q2 or namely, collision cell and the fragment ions are detected in Q3.

Ionization Systems for Tandem Mass Spectroscopy: Since HPLC system utilizes a liquid mobile phase, containing significant amounts of water and organic solvents in various ratios, the compatibility of this liquid effluent was the major problem in early development stages.

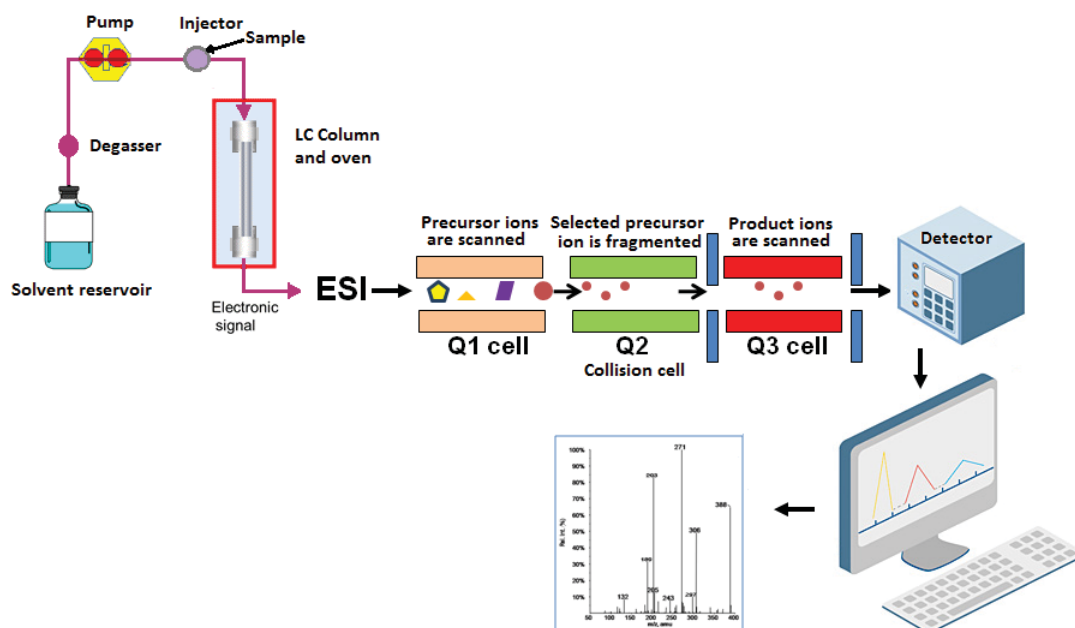


Figure 1.4 Flow Chart for ESI-MSMS system.

On the other hand, high vacuum is required for the ion transport from ion source of the mass spectrometer to the detector. The mean free path of an ion at atmospheric pressure is around 10 nm and hence, the ion cannot reach the detector. Considering that mean free path is inversely proportional to the pressure, it is reduced to 10^{-6} torr to increase this path to around 10 m allowing the ions to reach the mass spectrometer.

Furthermore, the analyte molecules have to be ionized prior to mass analysis due to the mass spectrometers detect the mass to charge ratios (m/z). Thus, an interface technology is required to satisfy two demands. First is to separate the undesired mobile phase from the analyte passing into the mass spectrometer while other ions are ionizing. For this purpose there are three common techniques developed for ionization process;

- Electrospray ionization (ESI)
- Atmospheric pressure chemical ionization (APCI)
- Atmospheric pressure photoionization (APPI)

Present thesis utilizes the ESI and therefore, detailed information is given. There are two electrodes in ESI source being the ES capillary and the counter electrode as an atmospheric pressure aperture plate of the mass spectrometer. A potential difference as high as 3 kV is applied between the electrodes. This electric field applied forces the polarized ions migrate toward the liquid at the capillary tip.

Thus, the liquid surface is drawn out of the capillary forming a **Taylor cone**. When the excess of ions accumulated at the surface of the liquid, coulombic forces become sufficient to overcome the surface tension of the liquid. The droplets about the size of 1 μm are emitted from the capillary and shrink by solvent evaporation (Figure 1.5). Then, very small charged droplets produce gas-phase ions by a very soft ionization technique. The skimmer electrodes are used to sample these ions to be analysed in the MS analyser.

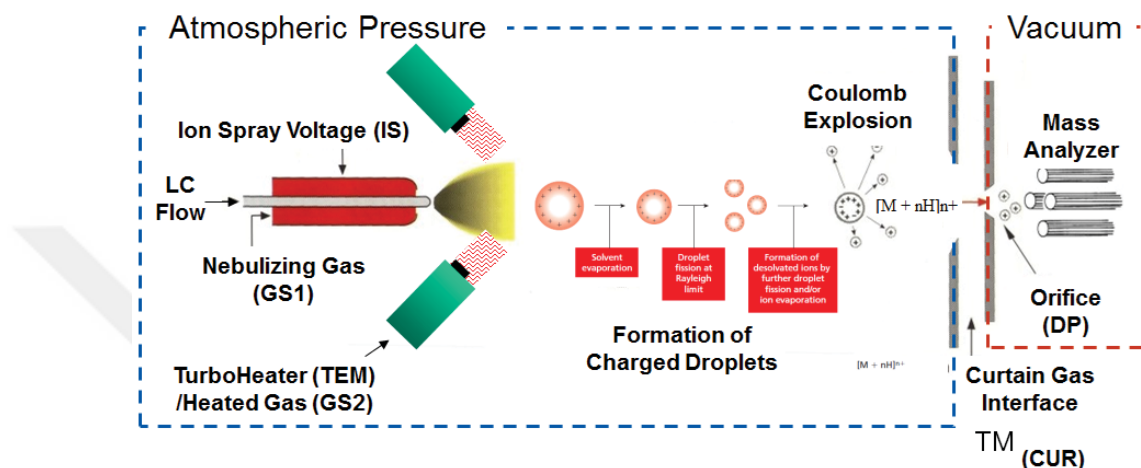


Figure 1.5 Schematic illustration of ESI interface for liquid chromatography mass spectroscopy

During this process, the deposits on electrodes and skimmers might have a damaging effect and therefore, a gas curtain is applied to blow away the neutrals and large particles away from the MS system. Spraying at an angle improves the sensitivity and ruggedness of the interfaces and a protonated molecular ion MH^+ can be observed alone in a electrospray spectrum. However, ammonium acetate buffer might lead formation of an adduct MNH_4^+ as the predominant ion.

1.6.2. Fundamentals of Voltammetry

Voltammetry represents a group of electroanalytical techniques in which the current flowing through an electrochemical cell is measured while a varied potential is applied to the electrodes. In electro-analytical chemistry generally the focus is on the interfacial processes where the analyte at the solution-electrode interface undergoes redox reaction. The working electrode used is so small that the amount of analyte consumed can be ignored (Wang, 2006)

The working electrode may be constructed from a wide variety of conducting materials including noble metals such as gold or platinum, a liquid metal; mercury and various forms of carbon materials. Other electrodes in the cell are reference and counter electrode providing desired potential and current measurement. Commonly used reference electrodes are the Ag/AgCl electrode or saturated calomel electrode. The cell solution displays a resistance to the flow of current which can be reduced by adding a high concentration of a supporting electrolyte (Tural et al., 2003).

There are a number of controlled potential techniques developed for the particular analysis. Cyclic voltammetry provides information about the redox behavior while differential pulse and square wave voltammetries able us to determine inorganic and organic substances in ppb ranges. Therefore, the development of modern techniques has contributed to the elucidation of electrode processes of compounds of biological interest.

Electrochemical methods are based on nanostructural materials are very popular research field. Several advantages of nanomaterial as the electrode modifiers have been demonstrated for the analysis of wide range of chemicals in biological, pharmaceutical environmental and food analysis. Drug analyses have played a vital role in quality control required by the great impact on public health. Therefore, a huge number of studies have been dedicated to develop sensitive methods for the determination of pharmaceutical and biological compounds. Electrochemical detection of neurotransmitters along with their metabolites is drawing attention due to their high sensitivity speed and low cost (Liu et al. 2005).

However, the major drawback of the technique is that electroanalytical methods are prone to interferences of other electroactive organic substances and surface active ingredients those deteriorate the voltammetric signal. In this case an appropriate separation method is required prior to the analysis for maintaining selectivity. The overlapped peaks can be resolved by applying statistical methods.

1.7 Multivariate Calibration

The basic of multivariate calibration is to establish a regression model relating the measured analytical signals to certain properties of samples (Martens and Naes, 1991). The quantitative model is, then, applied to predict the same properties of samples not involved in the calibration set from their responses.

Many methods can be used for multivariate calibration such as; Multiple Linear Regression (MLR), Principal Component Regression (PCR), Partial Least Squares (PLS), Artificial Neural Network (ANN), Support Vector Machine (SVM). However, the quality of the model determines the reliability and accuracy of the predicted results and consequently the quality of the models must be improved by optimization studies.

Generally, the PLS is the method of choice since it is very practical and versatile technique. This technique is simply a combination of principal component analysis (PCA) and multi-linear regression (MLR). Consequently, the data can be handled with strong co-linearity and noise and even if the number of variables exceeds the number of available samples (Brereton, 2003).

1.8. Method Validation in Analytical Chemistry

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. The validation data can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice occurs (Eurochem Guide, 2014).

Analytical methods have to be validated according to regulations and quality standards that have an impact on analytical laboratories. The validation parameters can be differed in many applications according to the regulation may use the word “validation” directly, or suggest validation with statements such as: “test methods should be appropriate for their intended use”.

While the aim of these thesis to develop a new chromatographic and voltammetric methods for drug analysis, EMA regulation will be emphasized and the validation parameters will be discussed.

Drug concentrations in biological matrices (such as serum, plasma, blood, urine, and saliva) are an important step for development of medicinal product. The obtained data may be critical for new active compound and generics as well as variations to authorised drug products. Toxicokinetic and clinic trails along with the bioequivalence results, support critical decisions for the safety and efficacy of a drug assay or product. It is therefore paramount that the applied bioanalytical methods used are well characterised, fully validated and documented to a satisfactory standard in order to yield reliable results.

The main characteristics of a bioanalytical method that are essential to ensure the acceptability of the performance and the reliability of analytical results are: selectivity, lower limit of quantification, the response function and calibration range (calibration curve performance), accuracy, precision, matrix effects, stability of the analyte(s) in the biological matrix and stability of the analyte(s) and of the internal standard in the stock and working solutions and in extracts under the entire period of storage and processing conditions.

Acceptance criteria wider than those defined in this guideline may be used in special situations. This should be prospectively defined based on the intended use of the method.

Selectivity: The analytical method should be able to differentiate the analyte(s) of interest and IS from endogenous components in the matrix or other components in the sample. Selectivity should be proved using at least 6 individual sources of the appropriate blank matrix, which are individually analysed and evaluated for interference.

Normally, absence of interfering components is accepted where the response is less than 20% of the lower limit of quantification for the analyte and 5% for the internal standard.

Carry Over: Carry-over from the previous injection should be assessed during the validation. Injections of blank samples after a high concentration standard should not exceed 20% of the lower limit of quantification (LLOQ; see below) and 5% for the internal standard.

Limit of Detection and Quantification: Limit of Detection often used as, detection limit, is defined as “smallest amount of analyte that can be determined by analytical method in predetermined confidence interval”. The IUPAC definition for detection limit is “the smallest concentration or absolute amount of analyte that has a signal significantly larger than the signal arising from a reagent blank. From the mathematical point, detection limit can be estimated by using signal at the detection limit $((S_A)_{DL})$ and expressed as follows, where S_{reag} designates the signal arising from reagent blank and ∇_{reag} its standard deviation, z is the factor accounting for the desired confidence level.

$$(S_A)_{DL} = S_{reag} + z \nabla_{reag} \quad \text{Eq.1}$$

Generally, the z values are accepted as 3 (corresponding significance level $\Rightarrow \uparrow \downarrow \leftarrow \rightarrow$ for estimation of LOD and for quantitative analysis z values is set to 10. These values are selected due to compromise type 1 and type 2 errors. Type 1 error occurs where the probability curves for sample at LOD levels, and reagent blank overlaps as shown grey area in the Figure 1.6.

On the other hand, if the z value set to greater than 3 type 2 error occurs. However, in many cases, regulation of method validation differs the calculation criteria of detection limits. EMA regulations offers the analyte signal of the LOQ sample should be at least 5 times the signal of a blank sample for LOQ values. Furthermore the analytical devices are able to calculate signal to noise ratio which can allow us to directly estimate LOD and LOQ values.

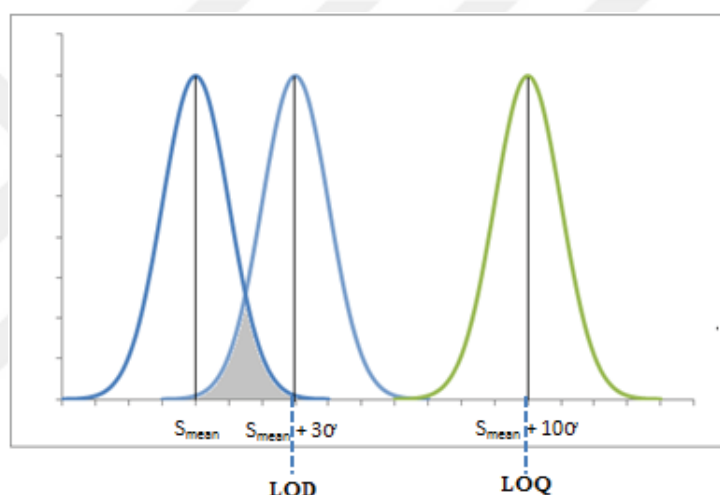


Figure 1.6 The normal distribution for reagent blank and sample at LOD and LOQ levels.

Linearity: According to EMA Guideline it was described as the concentration range directly proportional to the detector signal. Linear range starts with LLOQ up to the upper limit of quantification (ULOQ). At least 6 concentration levels must be contained in linear range and difference between calculated and nominal concentrations should be within $\pm 15\%$, except for the LLOQ for which it should be within $\pm 20\%$.

Accuracy: It is defined as the closeness of the determined value by the developed method to the actual or nominal concentration of the analyte. The appropriate portion of sample are spiked with standard solution of the analyte known as the quality control samples (QC samples) and then, analyzed by the method to reveal the difference in the determined and the nominal value. The recovery is given in percentages for low, medium and high QC samples

Precision: This term describes the closeness of repeated measurements to each other and it is expressed as the coefficient of variation (CV). Precision is calculated low, medium and high QC samples, within a single run and between different runs.



2. EXPERIMENTAL

2.1 Reagents and Apparatus

LC-MS grade acetonitrile (ACN), water and methanol (Met) was purchased from Merck and used for preparing the standard solutions and mobile phase. S-Carbidopa, Droxidopa, Levodopa, Serotonin HCl, Dopamine, DOPAC, Homovanilic Acid, Metamizole Sodium, Trifluoro acetic acid and Formic acid are of analytical grade and used without further purification. 1000 mg.L⁻¹ stock solutions of all analytes were prepared and stored at -20 °C. The standard solutions were prepared freshly by diluting the stock solutions prior to study.

Pyrrole was purchased from Alfaeaser and double distilled under argon atmosphere before each experiment. Multiwall carbon nanotube (MW-CNT) was purchased Sigma Aldrich and used without any purification. LiClO₄ was used as supporting electrolyte for PPy-CNT film fabrication. Britton Robinson buffer system containing 0.04 M H₃PO₄, CH₃COOH and H₃BO₃ was used for maintaining pH of the solution in the range of 2.0 – 12.0.

2.2. Instrumental Conditions

LC-MS/MS Conditions: Preliminary studies have been performed by using Thermo Scientific TSQ Quantum Access MAX Triple Quadrupole LC-MS system. Further studies were carried out with Shimadzu Prominence ultra-fast liquid chromatography system (Kyoto, Japan) equipped with AB Sciex 4000 Turbo Ion Spray tandem mass spectrometric system. The columns used were Phenomenex C18 Luna octadecylsilane 3 column (4.6 mm × 150 mm, 3 µm) and Atlantis HILIC column (2.1 mm × 150 mm, 5 µm). The LC conditions were the same for both C18 and HILIC columns. The mobile phase consisting of 0.5% (v/v) formic acid in water (60%) and acetonitrile (40%) was prepared from LC-MS grade reagents. Experiments have been carried out by using isocratic flow with a rate of 1.0 mL min⁻¹. The column oven was kept constant at 25 °C during the analysis. Auto sampler was set to 4°C and the injection volume was 10 µL.

The studies comprise the studies on developing LC gradient programme for achieving well resolved peaks and short analysis time. The gradient programme was used as given in Table 2.1 where the mobile phase (MP) A is % 0.5 F.A. aqueous solution and B is % 0.1 F.A. solution prepared in ACN.

Table 2.1 The LC gradient programme

Time (min)	MP A Ratio (%)	MP B Ratio (%)
0.00	90	10
2.00	20	80
6.00	60	40
8.00	90	10
10.0	90	10

HPLC-UV Conditions:

The columns used were Phenomenex C18 Luna octadecylsilane 3 column (4.6 mm × 150 mm, 3 µm) and Atlantis HILIC column (2.1 mm × 150 mm, 5 µm) and Zorbax C-8 (4.6 mm × 150 mm, 5 µm) The mobile phase consisting of 0.05% (v/v) TFA in water and acetonitrile was prepared from HPLC grade reagents. Experiments have been carried out by using gradient flow with a rate of 1.0 mL min⁻¹. The gradient programme was as follows; 0 to 1.50 min 10% ACN, 1.50 to 9.0 min ACN ratio was increased to 30% and 9.0 to 11.50 min ACN increased to 50% finally 11.50 to 15.0 min ACN was decreased 10 %ACN. The analytes detected at 274 nm. The autosampler kept at 10 oC and injection was 20 µL.

Electrochemical Conditions: Electrochemical measurements were carried out by using Autolab PGST 204 and EIS measurement Autolab PGSTAT 128N voltammetric analyzer, equipped with conventional three electrode system consisting of Ag/AgCl (sat. KCl) as the reference electrode, Pt wire (99.99 %) as the counter electrode and GCE (BASi, 3 mm diameter, geometric surface area 0.0707 cm²) as the working electrode.

Electrode Modification: PPy-CNT electrode was prepared via pulsed deposition technique under the aid of ultrasonication. In a typical experiment, 10 mg of MW-CNT was weighed and dispersed in 100 mL of N-N-dimethylformamide (DMF) and 1.06 g of LiClO₄ was added into this solution. Required amount of pyrrole was transferred into 10.0 mL aliquot of CNT solution and electropolymerization of the PPy-CNT film was performed by applying potential pattern (5s, -0.25V; 5s, 2.2V) in an ultrasonic bath as given in Figure 1.1.

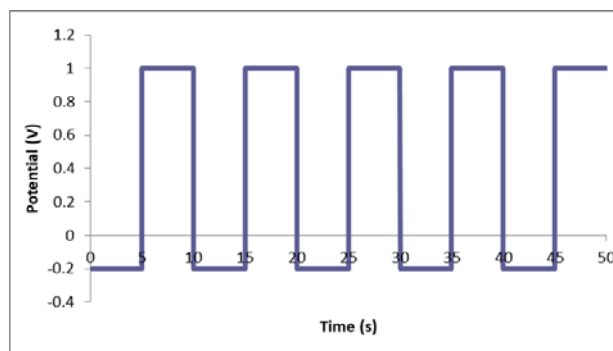


Figure 2.1 Applied Pulsed Deposition Pattern

Characterization Studies: Close images of the modified electrodes have been obtained by using SEM (FEI Quanta 250 SEM), XPS (ThermoScientific) and Perkin Elmer Fourier Transform Infrared Spectrometer (FTIR) was used to monitor characteristic peaks of Polypyrrole and carbon nanotube films.

Sample Preparation: In sample application step, a Sinemet tablet prescribed for the treatment of Parkinson's disease was used. The tablet which contains 25 mg of Carbidopa and 250 mg of Levodopa was obtained from local pharmacy and used without any treatment. The weighed tablet was dissolved in mobile phase consisting of 0.5% (v/v) formic acid in water (60%) and acetonitrile (40%) with the aid of sonication. Then, the sample was filtered through 0.22 μm Teflon syringe membrane filter prior to the injection to the LC-MS/MS system for subsequent quantification.

In metamizol determination in real samples, a novalgine tablet containing 500 mg metamizole was dissolved in PBS solution and filtered through 0.45 μm Teflon syringe membrane filter prior to analysis.

Artificial Cerebrospinal Fluid Preparation: Artificial Cerebrospinal Fluid (ACF) was prepared according to former study published by Stell et al. (Stell, 2003). The content of the ACF is 126 mM NaCl, 2.5 mM KCl, 2.0 mM CaCl_2 , 2.0 mM MgCl_2 , 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3 and 25 mM D-Glucose. The mixture was kept in +4 $^\circ\text{C}$.



3. RESULTS and DISCUSSION

3.1 Chromatographic Studies

Chromatographic studies include both quality control analysis of in dosage forms and clinical analysis. Former studies conducted with LC-MS/MS system for the determination of Carbidopa, Droxidopa and Levodopa assay in dosage forms and applied for the real tablet analysis.

The latter studies include the multi-component HPLC-UV analysis of Levodopa, Dopamine along with their metabolites namely; Homovanilic Acid (HVA) and 3,4-Dihydroxyphenylacetic acid (DOPAC) for clinical purposes verified with recovery studies conducted in synthetic brain fluid. The details of these studies have been given under related headings.

3.1.1. LC-MS/MS Studies

Preliminary studies have been dedicated to introduce the standard solution of analytes prepared in 500 $\mu\text{g L}^{-1}$ concentrations in a carrier stream consisting of 0.5% formic acid and acetonitrile at a flow rate of 0.50 mL /min. Table 3.1 summarizes the molecular and product ions.

Table 3.1 The molecular and product ions of the targeted analytes

Compound	Levodopa	Droxidopa	Carbidopa
Precursor	198.0	214.4	227.1
Product ions	152.1, 107.1	152.1, 135.1	181.1, 123.1

Optimization studies have been accomplished by using self-optimization of the system given all the parameters in a suitable range. The parameters related to the analytes (declustering, entrance and collision cell exit potential along with collision energy) and other parameters (collisional activated dissociation gas, curtain, nebulizing and drying gas pressures, ion spray voltage, temperature and interphase heater energy) have been optimized by flow injection analysis. Table 3.2 lists the optimal parameters determined for the targeted analytes.

Table 3.2 optimal mass spectrometer parameters for the analysis of targeted analytes

Compound-related parameters			
Compound	L-Dopa	Droxidopa	Carbidopa
Declustering potential (V)	40	50	50
Entrance Potential (V)	8	8	9
Collision Energy (arb)	20	30	20
Collision Exit Potential (V)	22	22	22
Non-compound-related parameters			
Collisional Activated Dissociation Gas Pressure (psi)			8
Curtain Gas Pressure (psi)			35
Nebulizing Gas Pressure (psi)			30
Drying Gas Pressures (psi)			30
Ion spray voltage (V)			2500
Temperature (°C)			550
Interphase Heater Energy (arb)			0.6

Then, the column studies have been carried out at 25°C by using aqueous mobile phase consisting of 0.5% formic acid solutions and 0.1% formic acid solution prepared in ACN under gradient flow at a rate of 500 μ L min⁻¹.

Considering the polarity of the analytes, Atlantis HILIC (4.6 id x 150mm, 5 μ m) and Inertsil ODS-2 column (4.6 id x 150mm, 5 μ m) have been used for the chromatographic separation. However, no satisfactory resolution was obtained for the analytes at different gradient elution programs.

It is well known that the pH of the mobile phase can have a strong effect on the resolution, the formic acid concentration was changed to adjust the pH but, the chromatograms given in Figure 3.1 have revealed poor resolution.

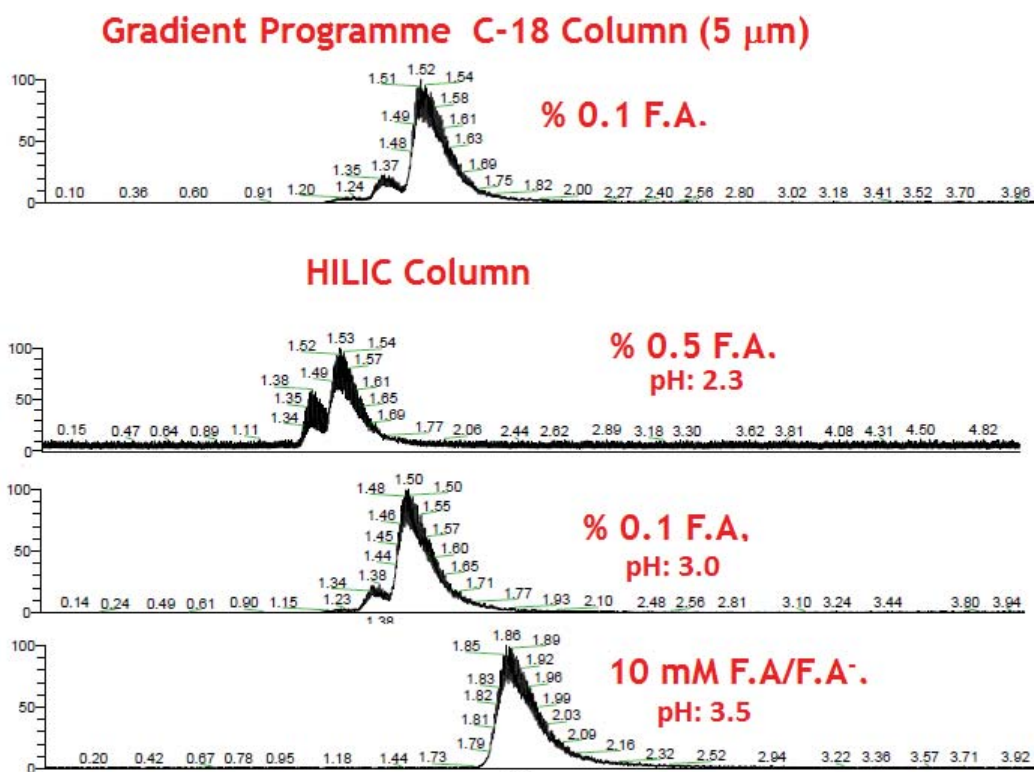


Figure 3.1 Total ion chromatograms of L-Dopa, Carbidopa and Droxidopa standards by using two different columns and different percentages of formic acid as the mobile phase.

Then, the effect of the particle size of the column on the resolution has been studied and Luna Octadecylsilane 3 column with a 3 μ m particle size has been used. Figure 3.2 shows the effect of the pH of mobile phase on the separation on this column.

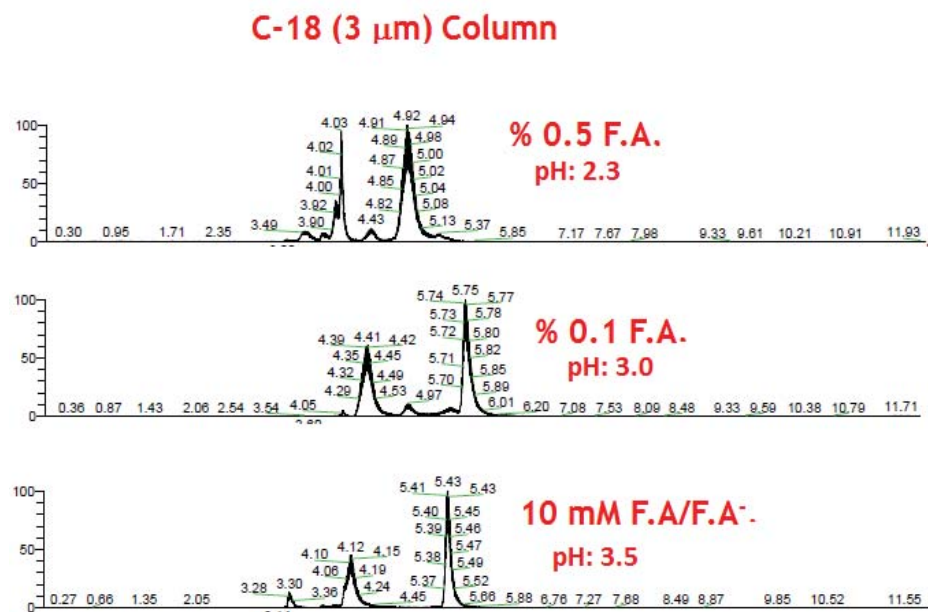


Figure 3.2 Total ion chromatograms obtained with Luna ODS-2 (3 μ m) at different pHs

As can be deduced from Figure 3.2, 0.5% formic acid solution with pH 2.3 has displayed the best resolution for the analytes. In addition, further improvement in resolution by means of peak shape was observed at isocratic flow 1.0 mL min^{-1} . Figure 3.3 shows the best resolved peak formation for the three analytes at 50 ng mL^{-1} concentration.

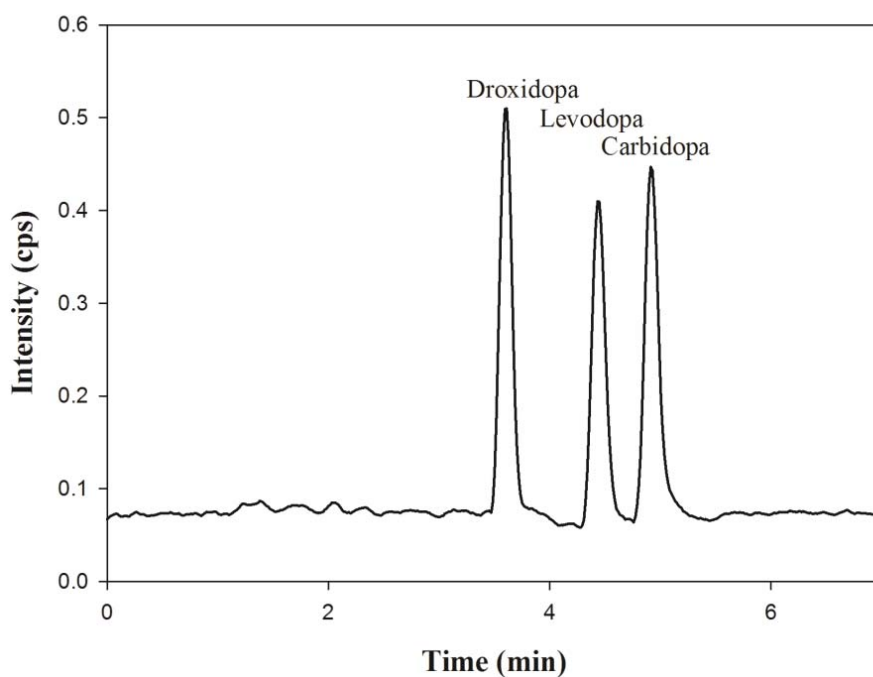


Figure 3.3 The chromatograms obtained with LC-MS/MS system for 50 ng mL^{-1} standard mixtures of Carbidopa, Droxidopa and Levodopa with C-18 column

After achieving satisfactory chromatographic separation by using C-18 column and optimization of mass parameters, analytical merits of the method were studied. In comparison, the performance of the HILIC column was tested at same chromatographic conditions. The method developed was exhibited linear response 100 fold concentration range. Figures between 3.4 and 3.9 showed calibration curves for all analytes obtained with both C-18 and HILIC columns.

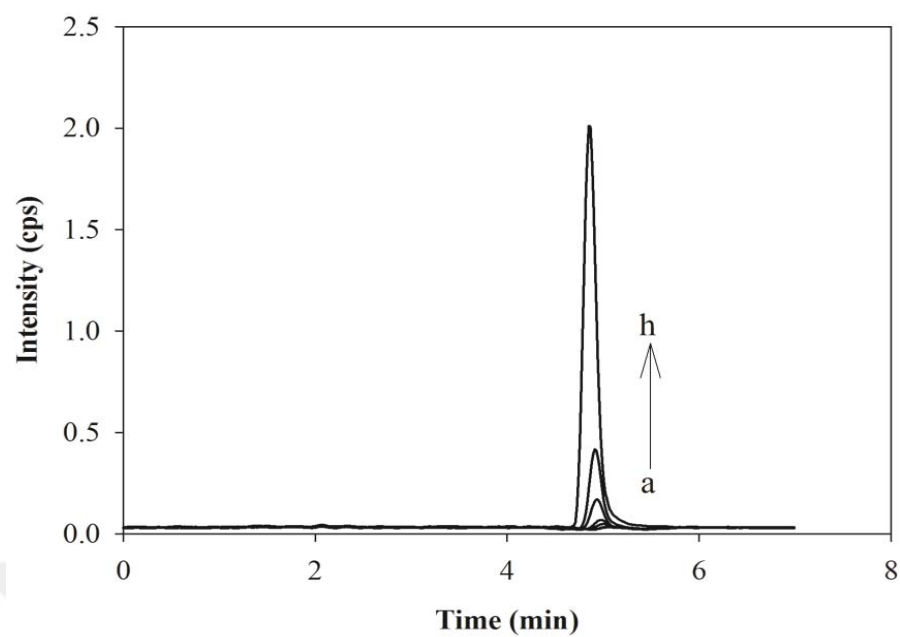


Figure 3.4 MRM chromatograms for Carbidopa in concentration of a) 2.0, b) 5.0, c) 10, d) 20, e) 50, f) 100, g) 200 and h) 500 ng mL⁻¹ obtained by using C-18 column.

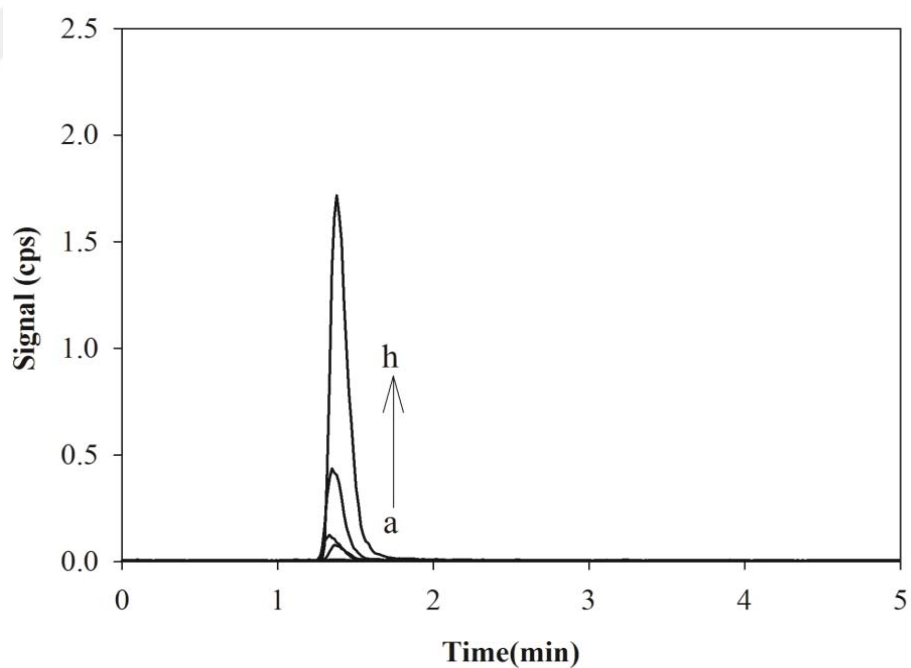


Figure 3.5 MRM chromatograms for Carbidopa in concentration of a) 2.0, b) 5.0, c) 10, d) 20, e) 50, f) 100, g) 200 and h) 500 ng mL⁻¹ obtained by using HILIC column.

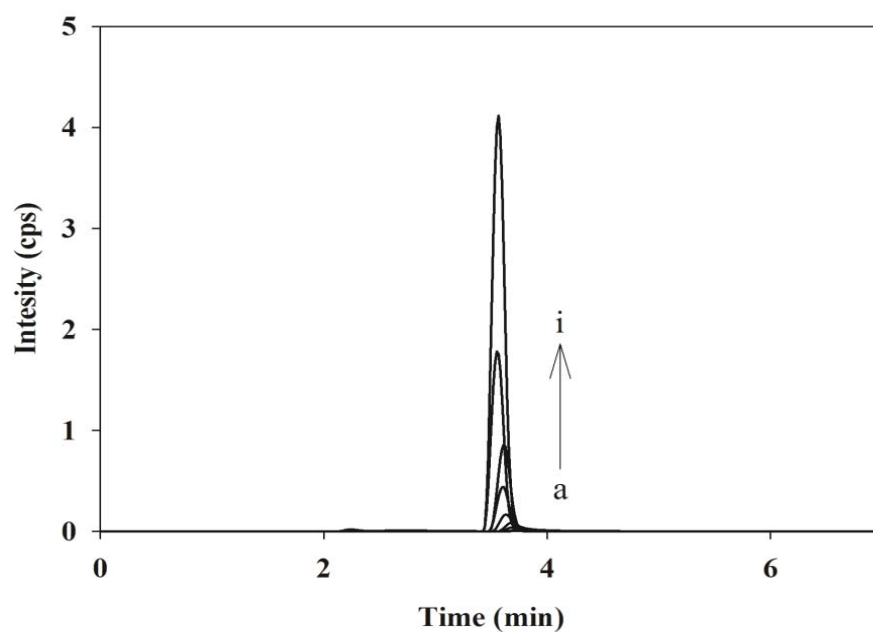


Figure 3.6 MRM chromatograms for Droxidopa in concentration of a) 1.0, b) 2.0, c) 5.0, d) 10, e) 20, f) 50, g) 100, h) 200 and i) 500 ng mL⁻¹ obtained by using C-18 column.

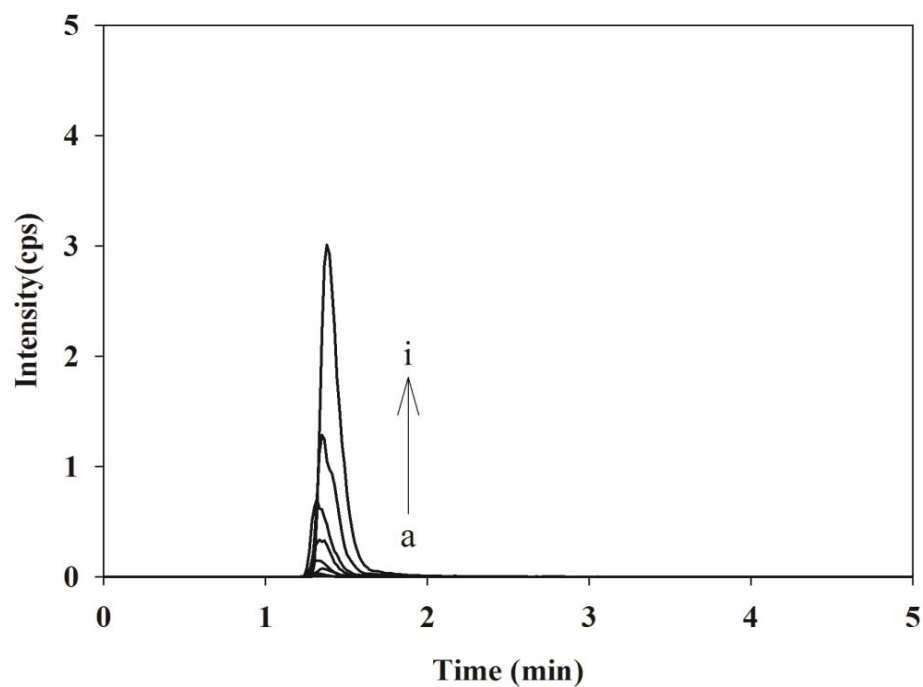


Figure 3.7 MRM chromatograms for Droxidopa in concentration of a) 1.0, b) 2.0, c) 5.0, d) 10, e) 20, f) 50, g) 100, h) 200 and i) 500 ng mL⁻¹ obtained by using HILIC column.

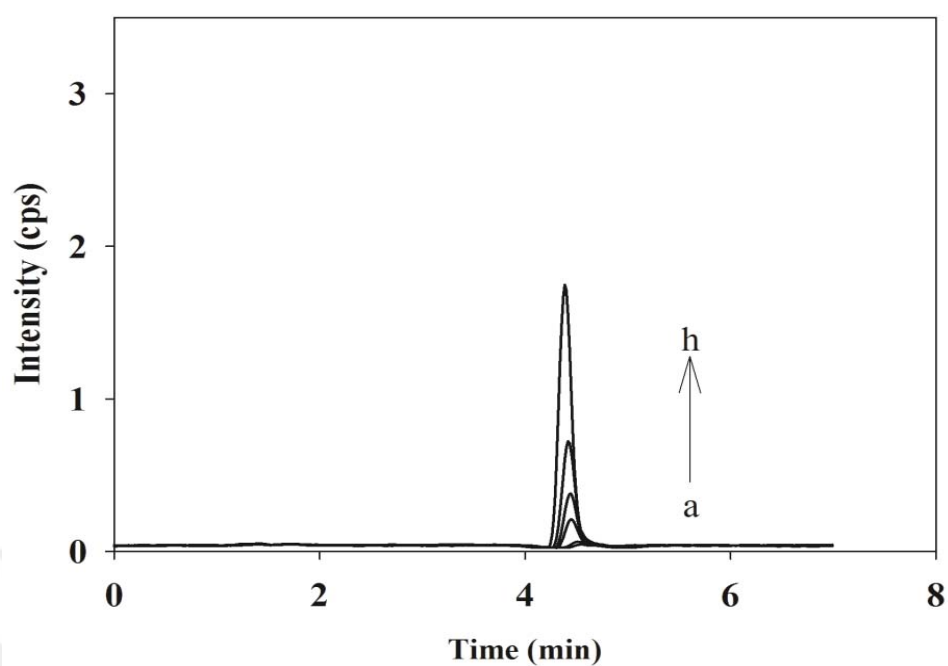


Figure 3.8 MRM chromatograms for Levodopa in concentration of a) 2.0, b) 5.0, c) 10, d) 20, e) 50, f) 100, g) 200 and h) 500 ng mL⁻¹ obtained by using C-18 column.

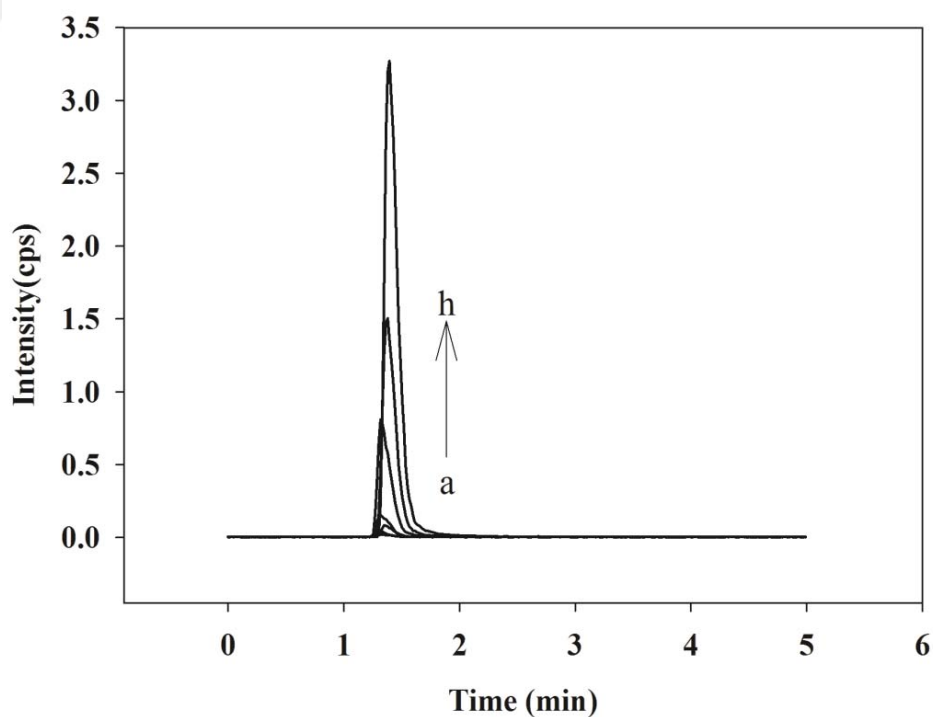


Figure 3.9 MRM chromatograms for Levodopa in concentration of a) 2.0, b) 5.0, c) 10, d) 20, e) 50, f) 100, g) 200 and h) 500 ng mL⁻¹ obtained by using HILIC column.

The other analytical parameters of the method were abstracted in Table 3.3, precision of the method was found to be quite high for 20 ng/mL concentration level however, relative standard deviation were calculated as nearly 5% at higher concentration level (200 ng/mL) for all analytes. LOQ of the method were estimated by decreasing the concentration level and LOD was calculated from LOQ value by relationship ($\text{LOD} = \text{LOQ}/3.3$) between LOQ and LOD.

Table 3.3 Analytical merits of LC-MS/MS method developed for the analytes by using C18 and HILIC column

Analytical characteristics	Carbidopa		Droxidopa		Levodopa	
	C18	HILIC	C18	HILIC	C18	HILIC
Linear Range (ng mL^{-1})	5.0 - 500	5.0 - 500	2.0 - 500	2.0 - 500	5.0 - 500	5.0 - 500
Equation	0.63C	0.39C - 0.16	0.07C + 0.11	0.05C	0.03C + 0.19	0.55C
R ²	0.9926	0.9983	0.9993	0.9979	0.9996	0.9959
LOD (ng mL^{-1})	1.6	1.6	0.7	0.7	1.6	1.6
LOQ (ng mL^{-1})	5.0	5.0	2.0	2.0	5.0	5.0
RSD (%) at 20 ng mL^{-1}	17.7	15.2	3.0	2.7	6.0	4.8
RSD (%) at 200 ng mL^{-1}	3.0	6.5	3.4	2.7	2.4	1.5

The accuracy of the method was tested with quality control (QC) samples and then, method applied to commercial tablet analysis. Table 3.4 displays the recovery values of the QC samples and results of commercial tablet analysis.

Table 3.4 Recoveries of spiked quality control samples and commercial tablet sample

Sample type	Analyte	Nominal Value ($\mu\text{g mL}^{-1}$)	Found ($\mu\text{g mL}^{-1}$)	Recovery (%)
Quality Control-1	Carbidopa	0.0200	0.0207	103.3
	Droxidopa	0.0200	0.0210	105.0
	Levodopa	0.0200	0.0203	101.8
Quality Control-2	Carbidopa	0.200	0.2040	101.8
	Droxidopa	0.200	0.2000	100.0
	Levodopa	0.200	0.2037	101.0
Tablet Sample	Carbidopa	25.0	25.8	103.0
	Levodopa	250	265.0	106.0

Overall results have revealed that the method developed provides a simple, selective and sensitive for the analysis of Carbidopa, L-Dopa and Droxidopa. The performance of the method developed has been compared to the other developed methods encountered in the literature and given in the Table 3.5. According to these data, the method developed provides very sensitive tool for the targeted analytes in a wide linear range.

Table 3.5 Comparison of the LC-MS/MS method developed for the targeted analytes

	Analyte	Technical Details	Linear Range (μM)	LOD (μM)	Ref.
Voltammetric Methods	Carbidopa L-Dopa	DP voltammetry at a GCE	0.500 - 5.40 0.200 - 750	0.38 0.042	Quintino et al. 2006
	Carbidopa L-Dopa	DP voltammetry at a hydroquinone and CNT modified CPE	10.0 - 600 35.0 - 700	7.2 0.094	Ardakani et al. 2011
	Carbidopa	SW voltammetry at a chloranyl and CNT modified CPE	0.090 - 400	0.071	Rasthakiz et al. 2012
	Carbidopa	SW voltammetry at a ionic liquid modified CNT paste electrode	0.100 - 420	0.06	Beitollah et al. 2012
	Droxidopa	SW voltammetry at a CNT modified CPE	0.120 - 225	0.05	Taijik et al. 2013
	Droxidopa	SW voltammetry with a ZnO and ionic liquid modified CPE	5.00 - 310	0.15	Gupta et al. 2013
	L-Dopa Carbidopa	DPV Graphene modified GCE	1.0 - 14 μM 3.0 - 16 μM	0.8 1.8	Wang et al. 2013
Chromatographic Methods	L-Dopa	LC-MS/MS	0.25 - 5.0	0.25	Pan et al. 2010
	Carbidopa	HPLC-UV	1.300 - 512	0.80	Issa et al. 2011
	Carbidopa	HPLC-ECD	0.082 - 16.4	0.016	Bugamelli et al. 2011
	Carbidopa Droxidopa L-Dopa	LC-MS/MS at a HILIC column	0.021 - 2.100 0.008 - 0.800 0.021 - 2.100	0.007 0.003 0.007	This study
	Carbidopa Droxidopa L-Dopa	LC-MS/MS at a C18 column	0.008 - 0.800 0.004 - 0.400 0.021 - 2.100	0.003 0.002 0.007	This study

3.1.2. HPLC-UV Studies

In this part of the thesis, simultaneous analysis of the four analytes namely; L-Dopa, DA, HVA and DOPAC have been studied to reveal a potential for future clinical purposes. The mixed aqueous standard containing 5 mg L⁻¹ each analyte was injected into the HPLC system with aid of aqueous TFA solution and ACN with flow rate of 1.0 mL.min⁻¹ and three different columns was tested (C-18, C8 and HILIC) have been used to compare their performances.

Figure 3.10 shows the actual chromatograms recorded with C8 column.

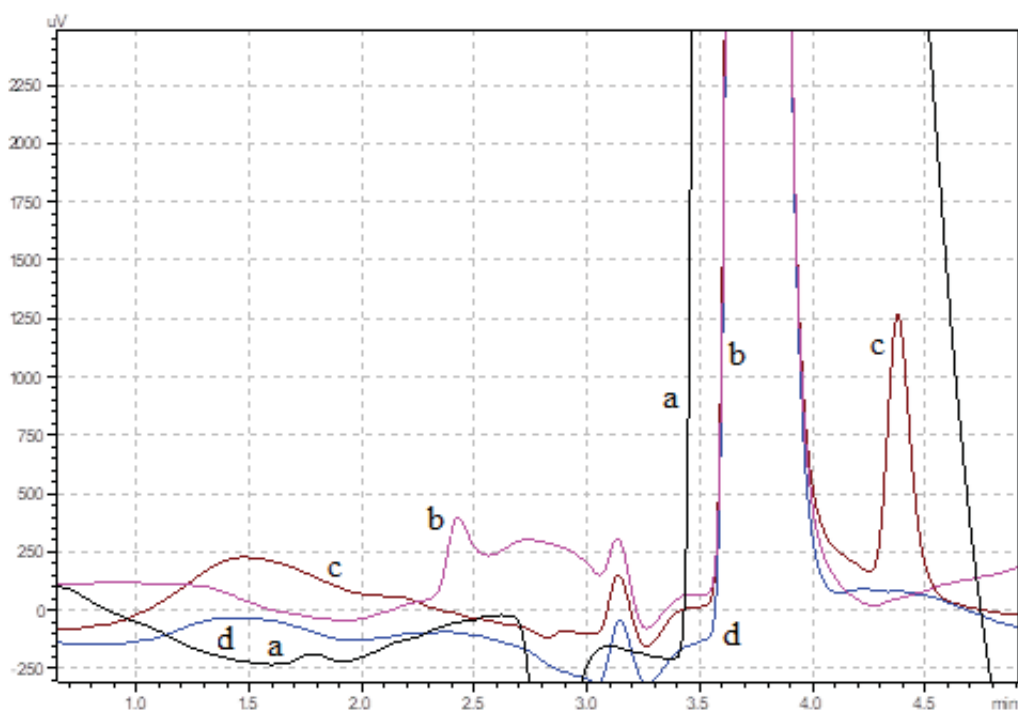


Figure 3.10 HPLC–UV chromatograms of 5 mg L⁻¹ a) L-Dopa, b) Dopamine, c) HVA and d) DOPAC recorded by using C-8 column

As can be seen from the above figure, the analysis of the four analytes suffers from the poor resolution. Therefore, hydrophilic interaction liquid chromatographic column (HILIC) was studied in pursue of better resolution. Figure 3.11 has shown the chromatograms recorded with HILIC column.

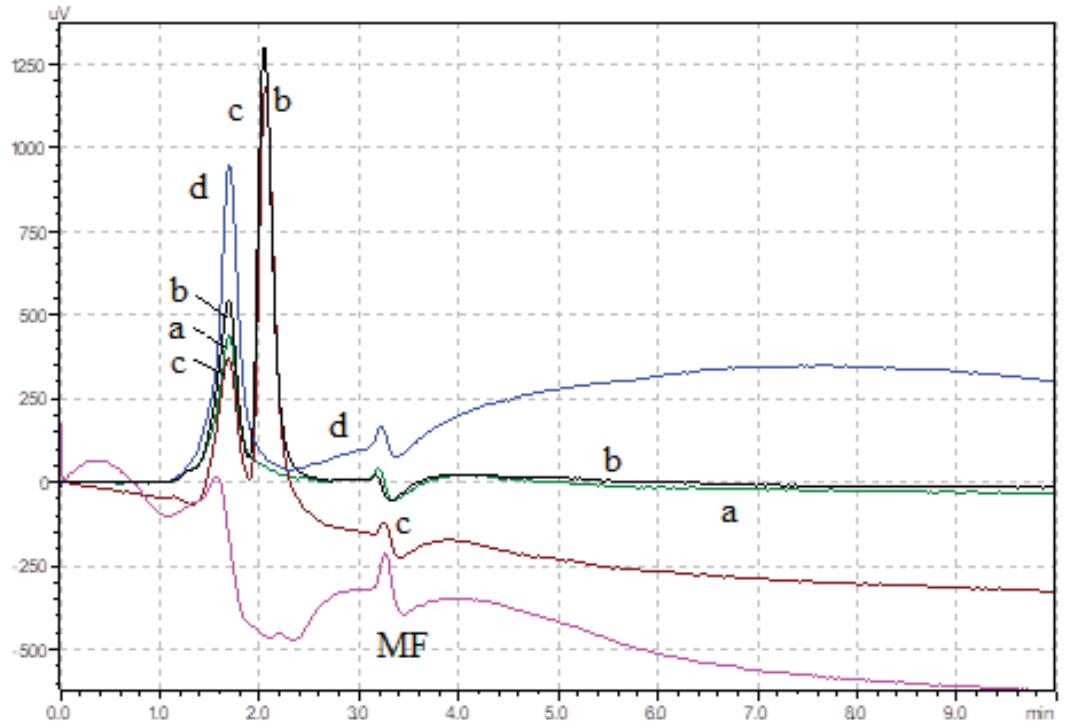


Figure 3.11 HPLC–UV chromatograms of mobile phase (MF), 5 mg L⁻¹ a) L-Dopa, b) DA, c) HVA and d) DOPAC recorded by using HILIC column

As can be concluded from Figure 3.11, overlapping peaks at 1.8 min have been observed for all the analytes and the peak was doubled for the metabolites; HVA and DOPAC giving another peak at 2.1 min. Therefore, C18 column was also searched for better resolution. Figure 3.12 shows the chromatograms recorded at C18 column.

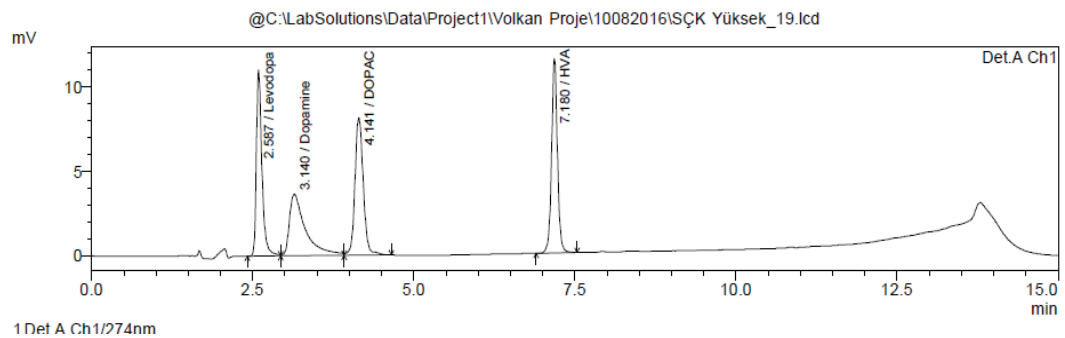


Figure 3.12 HPLC–UV chromatograms of 5 mg L⁻¹ L-Dopa, DA, HVA and DOPAC recorded by using C-18 column

The chromatograms have indicated that C-18 has given the best results in terms of resolution and peak symmetry. Then, the column was selected as C18 and the moved to the next step for optimization of the chromatographic conditions.

The mobile phase composition used for the analysis was optimized. Trifluoroacetic acid is chosen since it has less UV cut-off level and their percentages in the mobile phase might have an impact on the signals.

Figure 3.13-3.16 shows the chromatograms obtained for 0.01%, 0.05%, 0.075% and 0.1 % TFA. Best results have been obtained with 0.05% TFA solution.

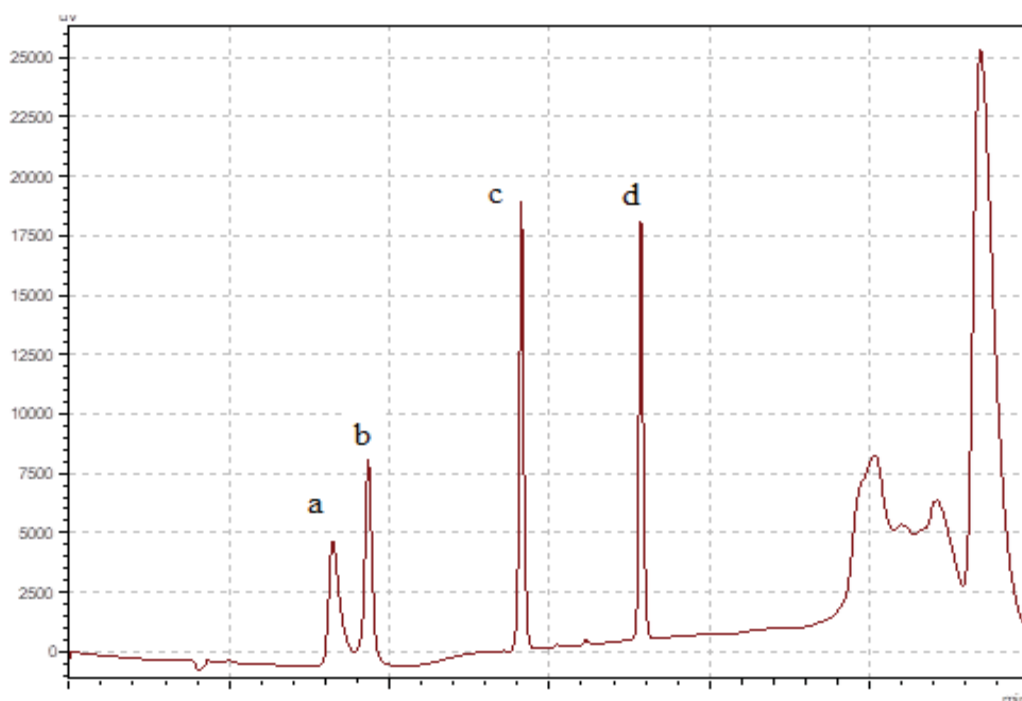


Figure 3.13 HPLC–UV chromatograms recorded for 5 mg L⁻¹ a) L-Dopa, b) DA, c) DOPAC and d) HVA by using C-18 column with a mobile phase solution of 0.01% TFA.

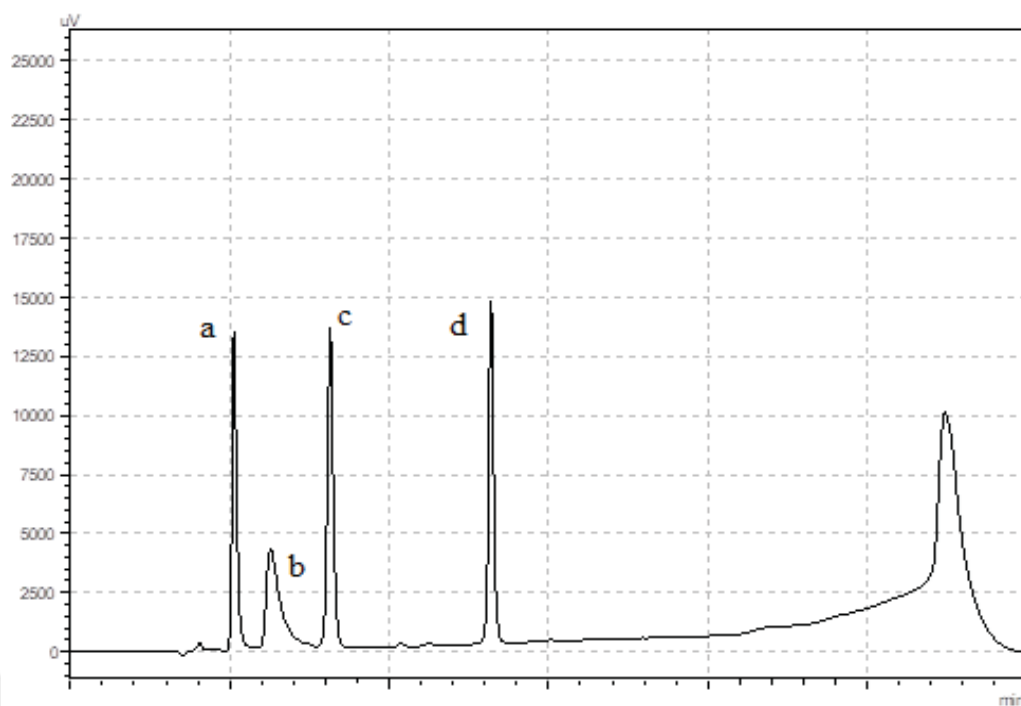


Figure 3.14 HPLC–UV chromatograms recorded for 5 mg L⁻¹ a) L-Dopa, b) DA, c) DOPAC and d) HVA by using C-18 column with a mobile phase solution of 0.05% TFA.

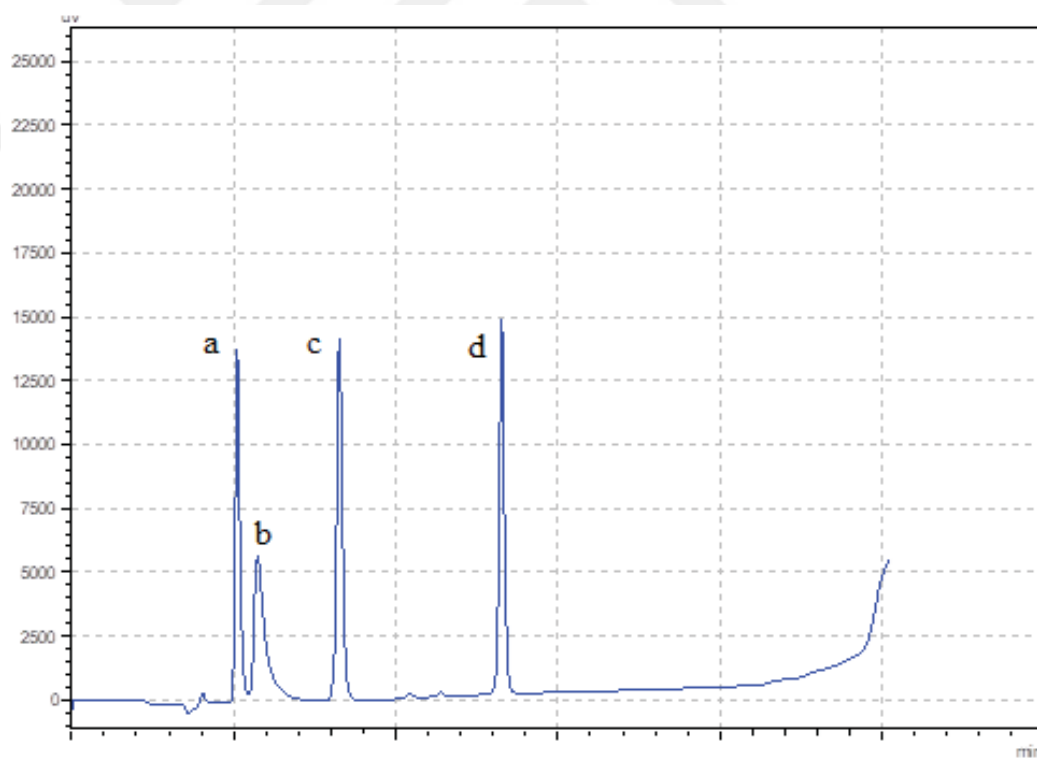


Figure 3.15 HPLC–UV chromatograms recorded for 5 mg L⁻¹ a) L-Dopa, b) DA, c) DOPAC and d) HVA by using C-18 column with a mobile phase solution of 0.075% TFA.

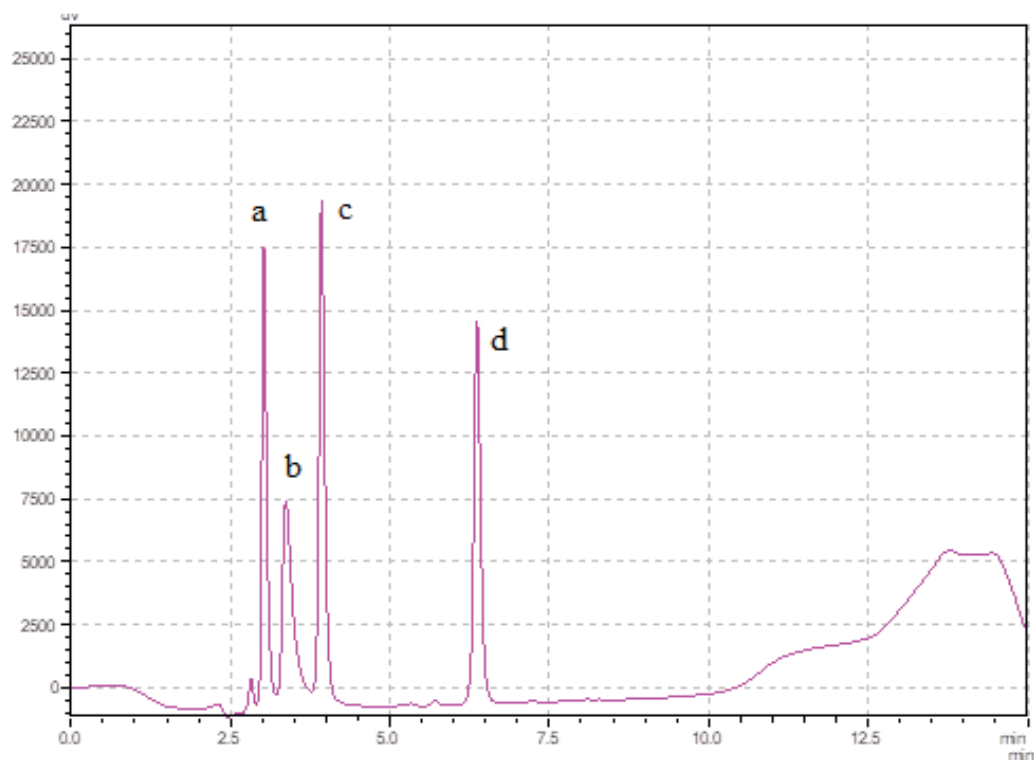


Figure 3.16 HPLC–UV chromatograms recorded for 5 mg L⁻¹ a) L-Dopa, b) DA, c) DOPAC and d) HVA by using C-18 column with a mobile phase solution of 0.1% TFA.

As can be deduced from Figure 3.13-to Figure 3.16, the small changes in the TFA amount in mobile phase has resulted significant shifts in the peak characteristics including retention times and peak symmetry as well. As pointed out above, 0.05% TFA has given the best results in terms of the chromatographic resolution.

Following the optimization step, calibration studies have been carried out in sub ppm to ppm regions and resulting plots have been given in Figure 3.17. According to these figures, the graphs have been found linear in 0.05 to 2.0 mgL⁻¹ concentration range with correlation coefficient close to unity.

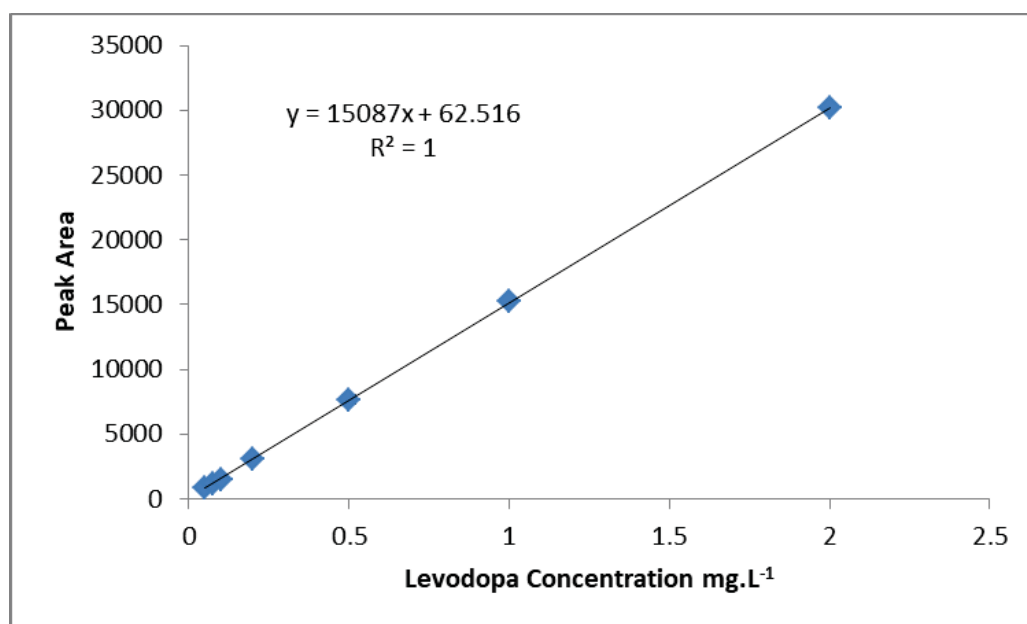


Figure 3.17 Calibration curves obtained for the L-Dopa in the concentration range of 0.05 to 2.0 mgL⁻¹ by HPLC-UV system

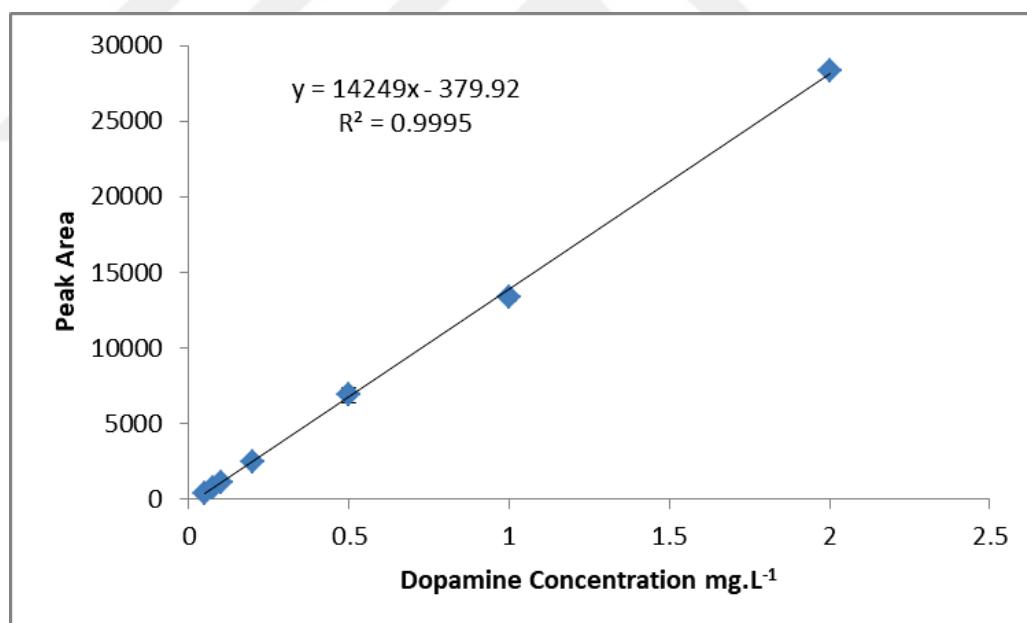


Figure 3.18 Calibration curves obtained for the Dopamine in the concentration range of 0.05 to 2.0 mgL⁻¹ by HPLC-UV system

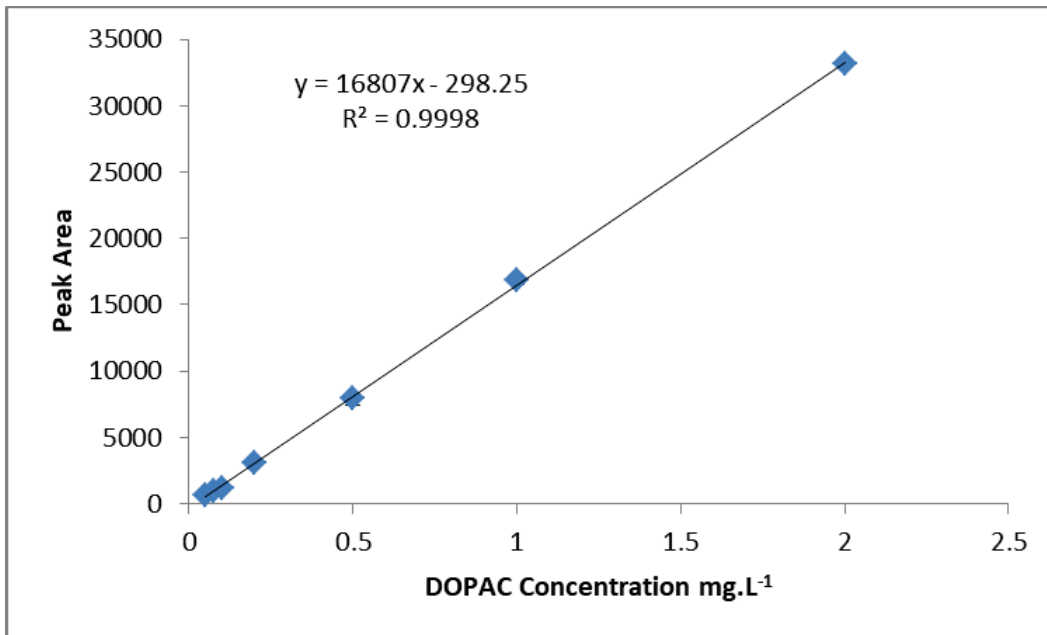


Figure 3.19 Calibration curves obtained for the DOPAC in the concentration range of 0.05 to 2.0 mgL⁻¹ by HPLC-UV system

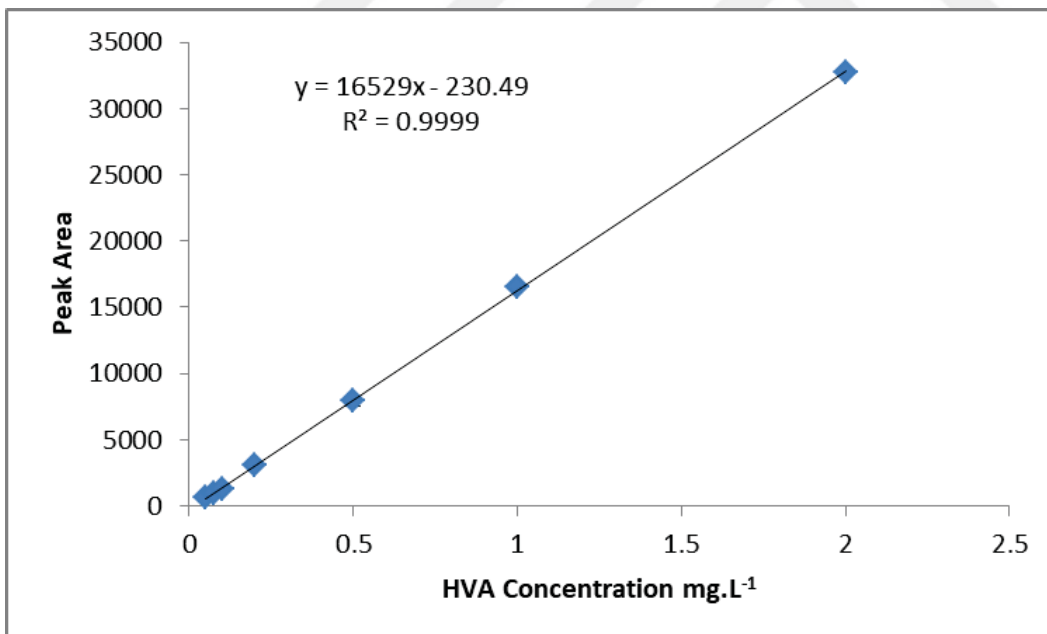


Figure 3.20 Calibration curves obtained for the HVA in the concentration range of 0.05 to 2.0 mgL⁻¹ by HPLC-UV system

Sequentially, the validation studies have been put in the progress in artificial cerebrospinal fluid (ACF) medium as described in Experimental Section. The parameters namely, precision, and accuracy have been investigated at four level; LLOQ, low, middle and high with concentration of 0.075, 0.1, 0.4 and 1.0 for all analytes, respectively. The precision of the method were calculated by using the relative standard deviation of 5 replicates and found in the acceptable range. Table 3.6, 3.7, 3.8 and 3.9 summarize these data for all the analytes.

Table 3.6 Between run and between day precision for L-Dopa

	L-Dopa (Day: 1)				L-Dopa (Day: 2)			
	LLOQ	Low	Middle	High	LLOQ	Low	Middle	High
N ₁	1098	1470	5915	46754	952	1370	5788	46107
N ₂	1091	1403	5763	60777	1001	1303	5625	59974
N ₃	990	1444	5707	56896	994	1415	5572	56335
N ₄	1036	1378	5759	60602	1039	1620	5545	59609
N ₅	1126	1368	5841	58877	968	1347	5665	58414
Mean	1068.2	1412.6	5797	56781.2	990.8	1411	5639	56088
SD	54.55	43.47	81.49	5820.55	33.40	123.63	95.39	5757.4
% RSD	5.11	3.08	1.406	10.25	3.37	8.76	1.69	10.27

Table 3.7 Between run and between day precision for Dopamine

	Dopamine (Day: 1)				Dopamine (Day: 2)			
	LLOQ	Low	Middle	High	LLOQ	Low	Middle	High
N ₁	582	908	5108	43675	504	808	4414	43783
N ₂	643	958	4786	57149	526	813	4635	57223
N ₃	597	922	4912	53489	527	1133	4467	53474
N ₄	597	963	4805	56920	604	1278	4285	56717
N ₅	667	1107	4805	55479	718	945	4751	55916
Mean	617.20	971.6	4883.20	53342.40	575.8	995.40	4510.40	53423
SD	36.06	79.21	135.13	5597.65	88.08	205.99	183.98	5577.6
% RSD	5.84	8.15	2.77	10.49	15.30	20.69	4.08	10.44

Table 3.8 Between run and between day precision for DOPAC

	DOPAC (Day: 1)				DOPAC (Day: 2)			
	LLOQ	Low	Middle	High	LLOQ	Low	Middle	High
N ₁	878	1220	6210	51625	779	1214	5932	56615
N ₂	795	1250	6124	66963	763	1186	5863	65836
N ₃	775	1231	6209	62739	736	1285	5798	61722
N ₄	861	1252	5909	66678	792	1354	5817	65354
N ₅	946	1365	6061	69853	800	1205	5883	64176
Mean	851.0	1263.60	6102.6	63571.6	774.00	1248.8	5858.6	62740
SD	68.5	58.2	125.0	7141.8	25.5	69.7	53.4	3776
% RSD	8.05	4.61	2.05	11.23	3.29	5.58	0.91	6.02

Table 3.9 Between run and between day precision for HVA

	HVA (Day: 1)				HVA (Day: 2)			
	LLOQ	Low	Middle	High	LLOQ	Low	Middle	High
N ₁	781	1237	6143	51278	845	1273	6087	51187
N ₂	854	1309	6046	66815	773	1422	5967	66092
N ₃	757	1464	6030	62641	847	1286	6051	62180
N ₄	776	1279	5849	66671	796	1311	5869	65939
N ₅	787	1209	6082	64470	771	1201	5883	64175
Mean	791.0	1299.60	6030.0	62375.0	806.40	1298.6	5971.4	61915
SD	36.97	99.58	110.10	6437.63	37.47	80.19	97.49	6203
% RSD	4.67	7.66	1.83	10.32	4.65	6.18	1.63	10.02

The accuracy of the method was elucidated by calculated recovery values for all levels and the results have been summarized in Table 3.10. According to these data, all the recovery values were found in the acceptable range of 80–120%.

Table 3.10 Accuracy of the method developed

	Recovery (%)			
	LLOQ	Low	Middle	High
L-Dopa	88.9	88.9	88.9	88.9
Dopamine	93.3	93.3	93.3	93.3
DOPAC	91.2	91.2	91.2	91.2
HVA	82.4	82.4	82.4	82.4

Stability of working standard in HPLC system was tested for 10 hours period. Table 3.11 has indicated the peak areas obtained successive injections at all levels for the initial injection time and after 10 hour. The data have been elucidated with students't test and no significant difference has been observed between the two time intervals in 95% confidence limit.

Table 3.11 Peak areas for low, middle and high concentrations of L-Dopa

Conc. (mg L ⁻¹)	Hour: 0				Hour: 10			
	L-Dopa	DA	DOPAC	HVA	L-Dopa	DA	DOPAC	HVA
Low (0.1)	1574	1101	1209	1283	1397	940	1182	1275
	1407	1310	1283	1242	1512	1100	1308	1524
	1472	1068	1272	1291	1494	975	1276	1359
Mean	1484.3	1159.7	1254.7	1272.0	1467.7	1005.0	1255.3	1386.0
SD	84.18	131.23	39.93	26.29	61.86	84.11	65.49	126.68
Middle (0.4)	5959	5208	6272	6163	5794	4949	6130	6162
	6014	5032	6241	6353	6038	5161	6214	6226
	6010	5289	6274	6159	5852	4851	6991	6118
Mean	5994.3	5176.3	6262.3	6225.0	5894.7	4987.0	6445.0	6168.7
SD	30.66	131.39	18.50	110.87	127.47	158.46	474.71	54.31
High (5.0)	67102	63391	74221	73388	66657	62994	73445	73881
	66348	63018	73266	72530	66218	62757	72674	72503
	60816	57711	67141	66581	60625	57373	66710	66449
Mean	64755.3	61373.3	71542.7	70833.0	64500	61041.3	70943.0	70944.3
SD	3432.3	3177.2	3841.7	3707.2	3363.02	3179.08	3686.10	3953.57

Overall results have revealed that developed method posses a potential for the analysis of these compounds for the targeted analytes in clinical samples. Since the validation studies of the method have been carried out in artifitial cerebrospinal fluid, it can be concluded that the method offers a practical tool for their determination in real samples. Next section describes the studies carries out with voltammetric techniques.

3.2 Voltammetric Studies

Voltammetric studies cover the electrode modification for the individual and simultaneous determination of L-Dopa, Carbidopa and Droxidopa. In addition, electrochemical behavior of metamizole sodium was also investigated and developed method applied to commercial tablets and lab made quality control samples.

Initial voltammetric experiments dedicated to develop new route for PPy-CNT composite films. Ultrasonication has been used in the electrodeposition step to provide uniform CNT distribution and pulsed amperometric technique has been demonstrated for electrodeposition process. Accordingly, the deposit film was characterized with various techniques.

3.2.1 Electrodeposition of PPy-CNT film on the GCE

Today, nanomaterial integrated sensor technologies are in continuous progress due to their widespread applications in clinical, pharmaceutical and environmental analysis. Composite materials developed by coupling the conducting polymers with carbonaceous nanomaterials, carbon nanotubes in particular, have displayed a synergetic effect with greater application potentials in many fields.

However, the structural consistency of the composite suffers from the poor dispersion of CNTs in the coating solution and sonication is the method of choice for preventing agglomeration by accelerating the mass transfer. Present study describes a new synthesis route for the preparation of polypyrrole/CNT composite film via combining the ultrasonication and electrochemical pulsed deposition for the first time.

Electropolymerization of PPy-CNT film was performed by applying the potential pattern as depicted in Figure 2.1 along with the ultrasonication. The resulted chrono-amperogram given in Figure 3.21 for two different voltages applied. As can be seen from the figure, anodic current has continued to rise up to 550 s due to the oxidation of pyrrole monomer.

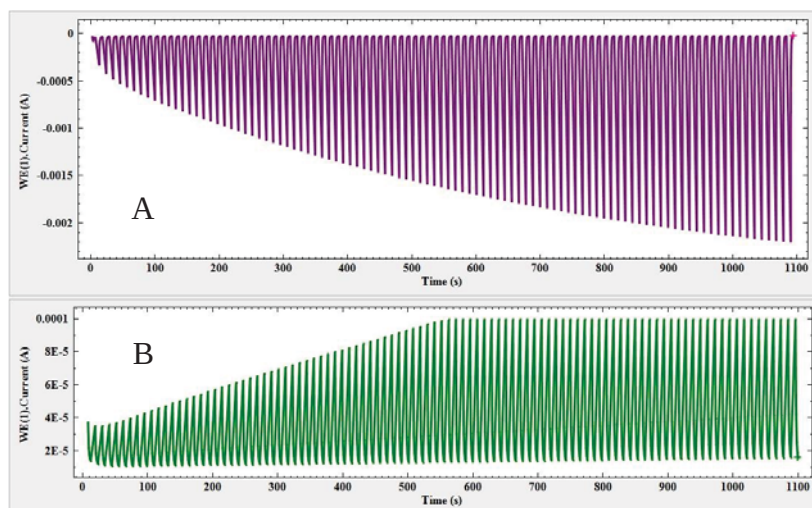


Figure 3.21 Chrono-amperogram recorded while the pulsed deposition method is applied to prepare the PPy-CNT composite electrode at A) -0.25 V and B) 2.2 V.

Structural analysis of composite film electro-synthesized by the pulsed deposition technique was performed by using FTIR technique. Figure 3.22 shows the FTIR spectra of Polypyrrole (PPy), Polypyrrole –carbon nanotube composite film (PPy-CNT) and Polypyrrole –carbon nanotube composite film, obtained with ultrasonication (PPy-CNTu).

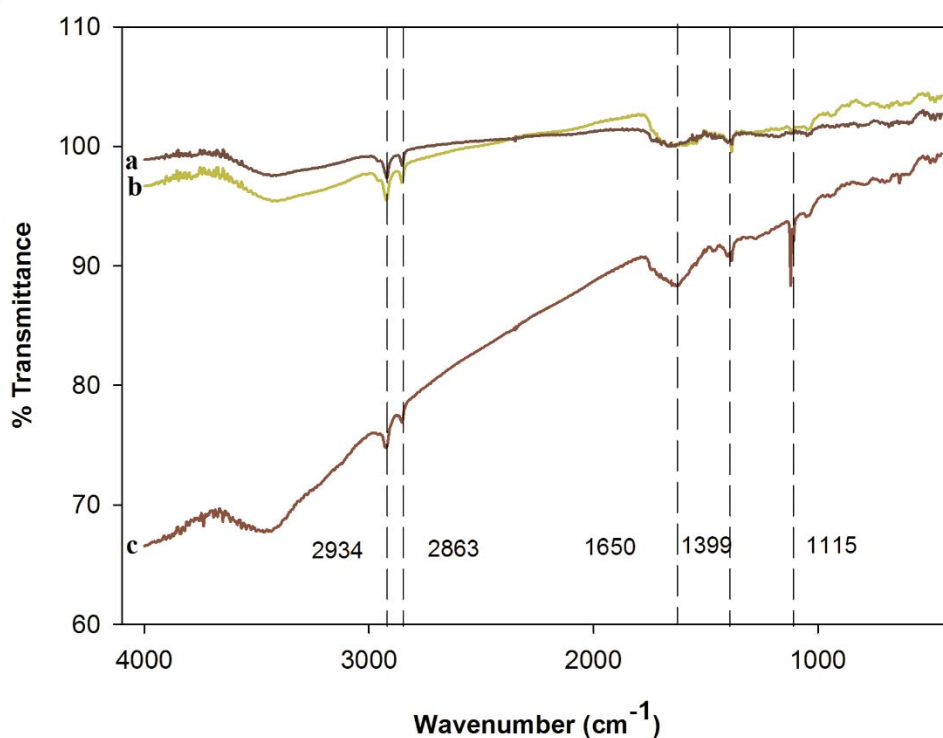


Figure 3.22 FTIR spectrum of electro-synthesized PPy, PPy-CNT composite and uPPy-CNT electrodeposited on a GCE by ultrasonic assisted deposition.

According to the data on the FTIR spectra, the peaks at 2934 and 2863 cm^{-1} are associated with pyrrole ring C–H stretching. The N–H stretching in PPy was observed at 3601 and 1650 cm^{-1} , respectively. Additionally, the peak at 1115 can be attributed to plane vibration of C–N moieties of PPy (Hieu et al., 2009; Rawal et al., 2013; Lee et al., 2010).

Morphology of composite film was also investigated with SEM technique. Figure 3.23 illustrates the SEM images of PPy, PPy-CNT and PPy-CNTu composite films. PPy-CNT film (Figure 3.23B) shows irregular distribution of CNT on PPy film. As it is consisted in Figure 3.23C and D, the use of ultrasonication has leaded more ordered surfaces.

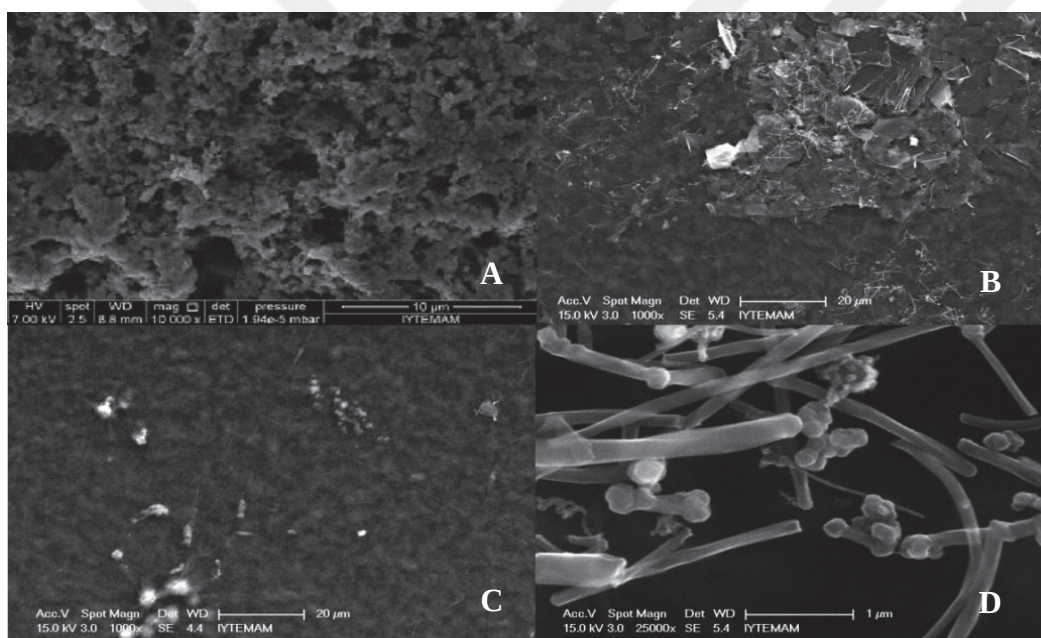


Figure 3.23 The SEM images of A) PPy at 10 000x magnification, B) PPy-CNT, C) PPy-CNTu at 1000x magnification and D) PPy-CNTu with 25000x magnification

Thermal resistance of the film was also tested and Figure 3.24 displays the thermal gravimetric analysis (TGA) results indicating a slight increase in the resistance by applying ultrasonic aided electropolymerization technique.

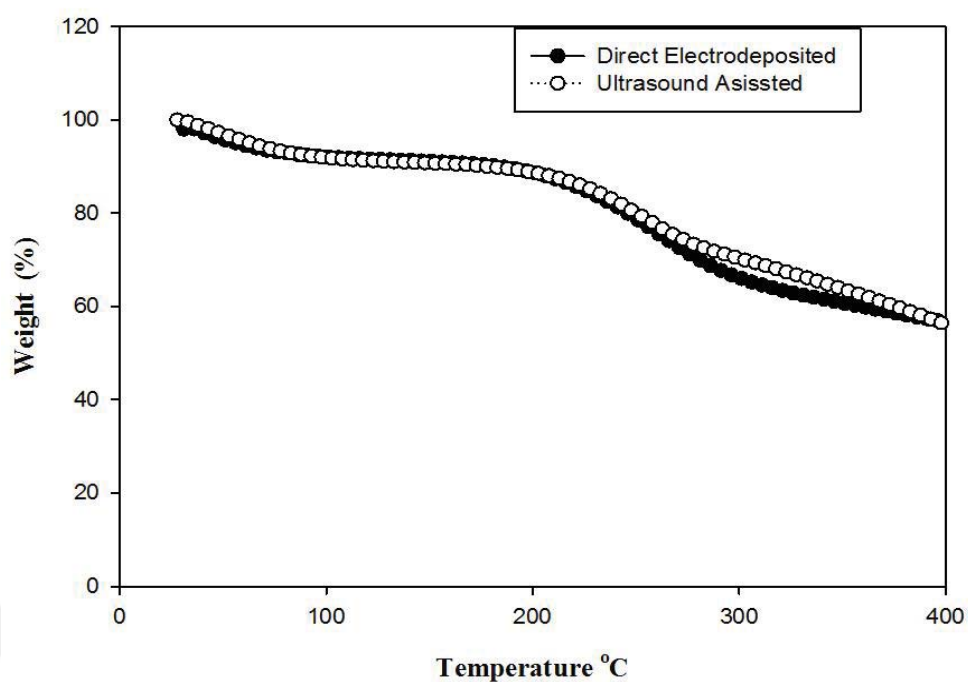


Figure 3.24 Thermograms of deposited PPy-CNT film obtained by applying ultrasonication (dotted line) and without ultrasonication (black dotted line) under N_2 atmosphere

The performance of the pulsed deposited polymeric surfaces as a catalytic film has been checked by cycling the potential between -0.8 - 0.8 V in a solution containing 5 mM $Fe(CN)_6^{3-/2-}$ and resulting voltammograms given in Figure 3.25 have revealed that the reversible peaks observed around 0.1 V for PPy-CNT have shown an increase in height upon modification with CNT. From the curve c in the figure, it is clear that higher signal formation was observed for ultrasonic assisted polymerization probably due to the more homogenous deposition.

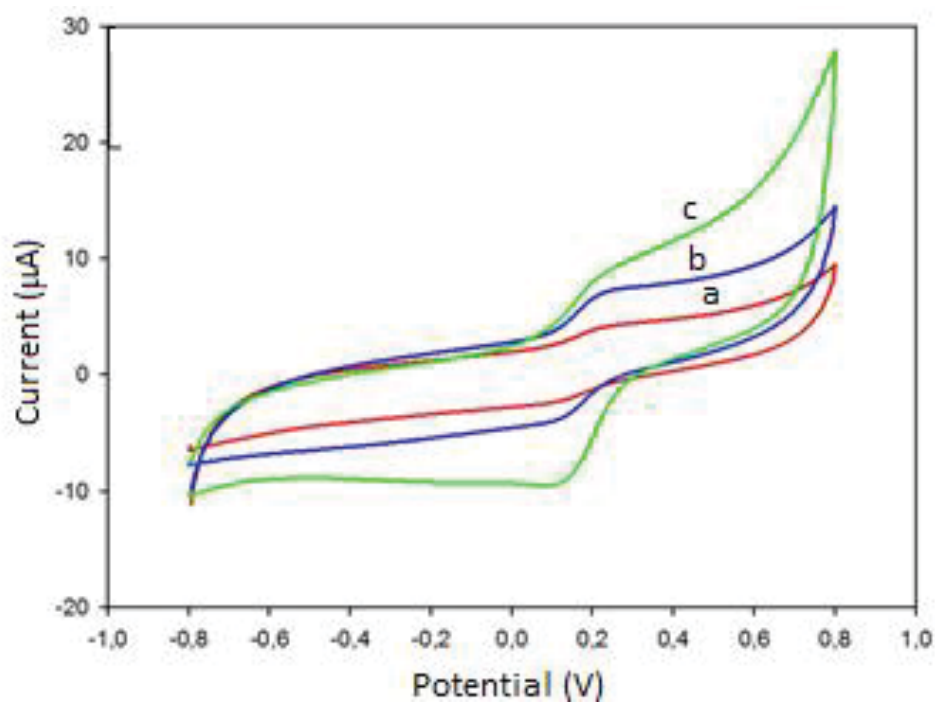


Figure 3.25 Cyclic voltammograms of a) GCE/PPy, b) GCE/PPy-CNT and d) GCE/PPy-CNTu electrodes in 5 mM $\text{Fe}(\text{CN})_6^{3-/2-}$ solution with a scanning rate of 50 mVs^{-1}

Electrochemical impedance measurements have been made in the same solution at 0.1 V and a frequency range of 0.05 -50000 Hz. It is consistent from the Figure 3.26, the conductivity of the bare electrode has decreased upon polymerizing the surface with a PPy coating and the radius of the semicircle has shown an increase as expected.

Further modification with CNT has led to a smaller radius similar to the bare electrode. Ultrasonic assisted polymerization of PPy in the presence of CNT, on the other hand, has given the best results probably due to the homogeneous alignment of CNTs in the polymer matrix.

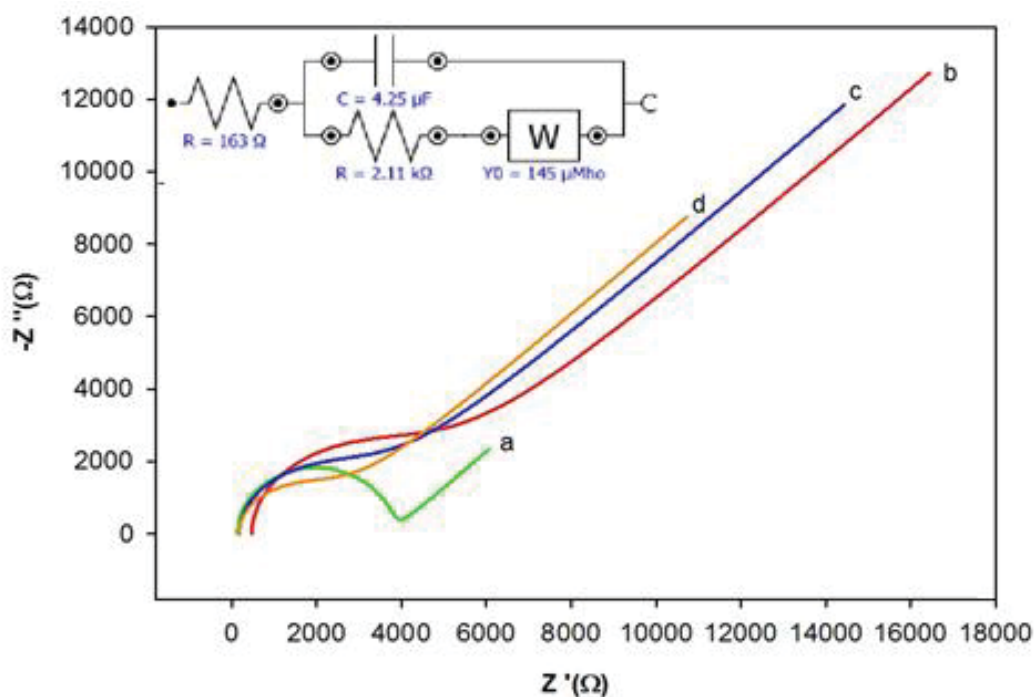


Figure 3.26 Electrochemical impedance spectrum of a) GCE, b) GCE/PPy, c) GCE/PPy-CNT and d) GCE/PPy-CNTu in 5 mM $\text{Fe}(\text{CN})_6^{3-/2-}$ solution, in the range of 0.05 -50000 Hz. Equivalent circuit was shown as an inset.

3.2.2 Electrochemical Determination of Droxidopa at a PPy-CNT/GCE

Having cleared that electrochemical pulsed deposition coupled with ultrasonication has given better electrochemical characteristics, the performance of this electrode was tested by using a rather new drug used for Parkinson disease. The electrode was transferred into the standard solution of droxidopa prepared in Britton-Robinson buffer in a wide pH range.

Figure 3.27 shows the oxidation peaks of Droxidopa at 0.45 V in pH 2.0 medium. The peak potential has shifted in negative direction and the best peak current has observed at pH 3.6 BR buffer solution. The peak current then, has shown a decline in more basic solutions and disappeared at pH 8.5 medium. Further studies have been conducted in pH 3.6 solutions.

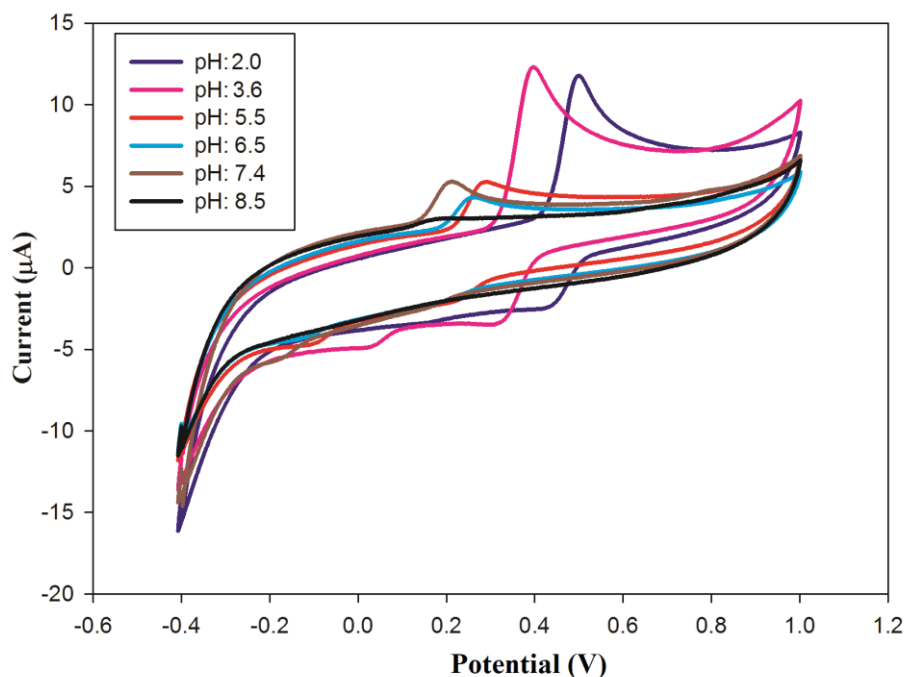


Figure 3.27 Cyclic voltammograms of 100 mg L⁻¹ Droxidopa in different pH media recorded at a scan rate of 50 mV s⁻¹.

The next step includes the optimization of the parameters related to the pulsed deposition parameters namely; pulse number and duration along with the pyrrole concentration.

Figure 3.28 displays the effect of pulse duration applied in deposition of 0.11 M pyrrole monomer as a polymeric film by applying 20 successive pulses on the oxidation peak formation of Droxidopa prepared in 10 mg L⁻¹ in pH 3.6 buffered media. According to these data, 0.5 s pulse duration was selected as the optimum. Then, the effect of pulse number was studied in the deposition of 0.11 M pyrrole monomer as a polymeric film (Figure 3.29). The best results have been obtained with 100 pulse number under these conditions.

The last parameter to be optimized is the pyrrole concentration. Figure 3.30 displays the effect of monomer concentration used in electrode polymerization on the oxidation signal of 10 mg L⁻¹ Droxidopa. According to these results, 0.07 M pyrrole in 0.1 M LiClO₄ solution in DMF has given the highest peak current which reflects the effect of polymeric film thickness on the electrode performance. Overall results related to the optimization were summarized in Table 3.12 for oxidation signal of 10 mg L⁻¹ droxidopa prepared in pH 3.6 BR buffer solution.

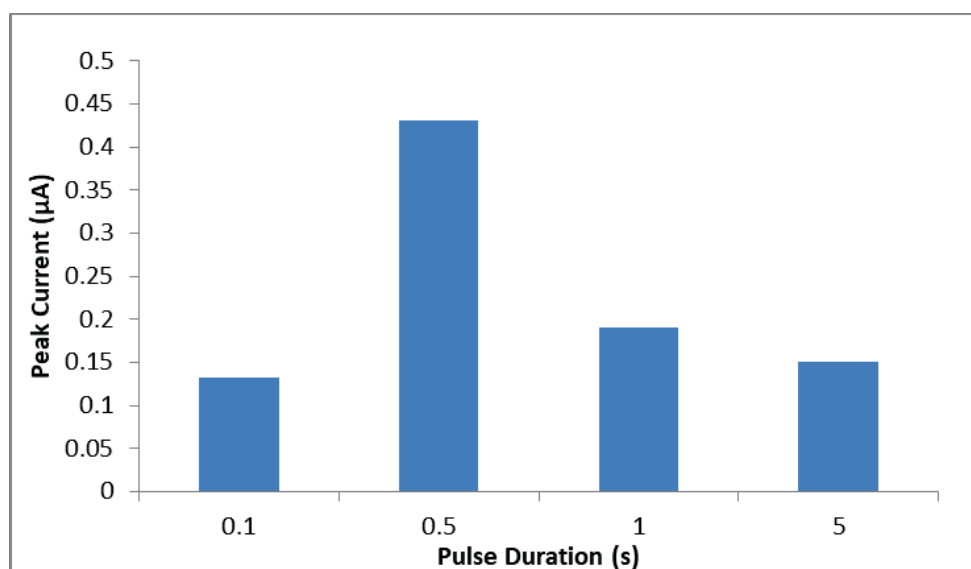


Figure 3.28 The effect of pulse duration on the oxidation signal of 10 mg L⁻¹ Droxidopa

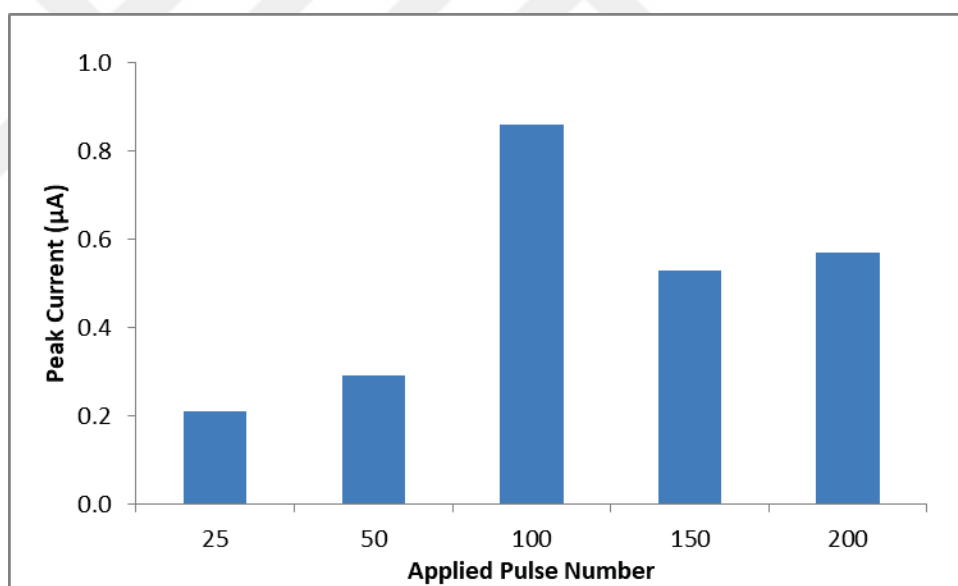


Figure 3.29 The effect of pulse number on the oxidation signal of Droxidopa

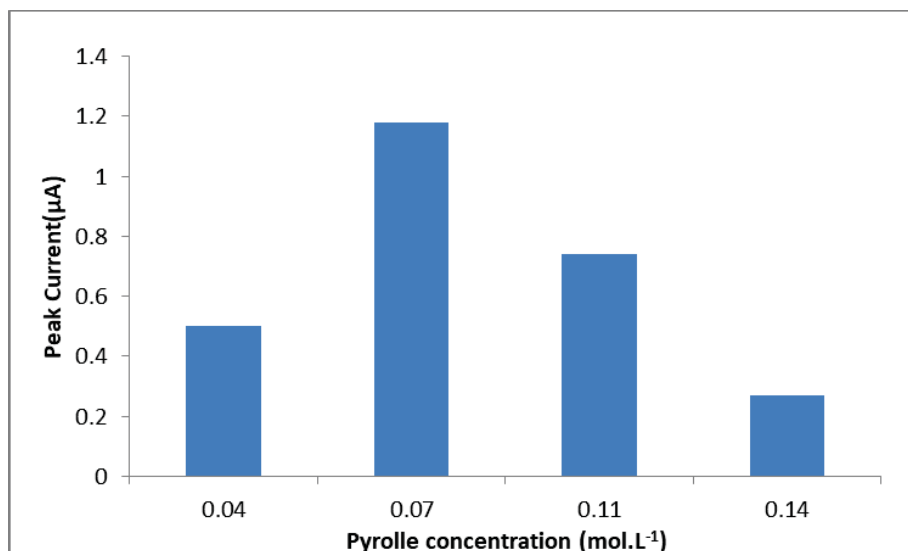


Figure 3.30 The effect of pyrrole concentration on the oxidation signal of Droxidopa

Table 3.12 Effect of several parameters in the preparation of uPPy-CNT/GCE on the oxidation signal of 10 mg L⁻¹ droxidopa at pH 3.6 buffer solution

Parameter		Peak current (μA)
Pulse duration (s)*	0.1	0.13
	0.5	0.43
	1	0.19
	5	0.15
Pulse number**	25	0.21
	50	0.29
	100	0.86
	150	0.53
Pyrrole conc. (M)***	0.04	0.5
	0.07	1.18
	0.11	0.74
	0.14	0.27

*Pulse number: 20, Pyrrole conc: 0.11 M,

**Pulse time: 0.5 s, Pyrrole conc: 0.11 M,

*** Pulse number: 100, **Pulse time: 0.5 s,

Under the optimized conditions, the method was applied to construct the calibration curve for droxidopa. Figure 3.31 shows the voltammograms obtained for 4.0-20 μM levels. When the peak currents have been plotted against the concentration (Figure 3.32), a linear response was obtained with an equation of $y=0.452x -0.15$.

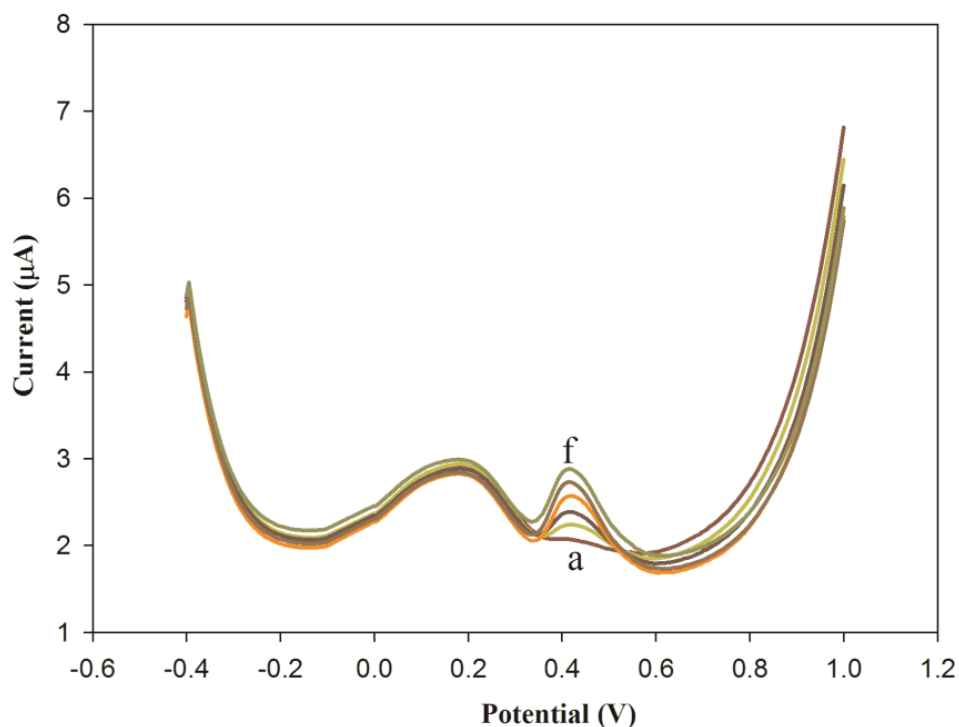


Figure 3.31 DP voltammograms recorded for droxidopa in pH 3.6 BR buffer solution at a GCE/PPy-CNT electrode in a range of 4.0 μM (a) to 20.0 μM (f).

Upon addition of droxidopa for higher concentration regions, another linear range was obtained in 20-120 μM with an equation of $y=0.018x -0.36$. These two linear ranged-calibration curves can also be encountered in the literature for several compounds and can be attributed to the change in the catalytic activity of the electrode surface in higher analyte concentration around the electrode surface. Clearly, lower slope of the curve has resulted to separate linear ranges for droxidopa probably due to the altered surface characteristics of the electrode at high concentrations.

The limit of detection ($S/N=3$) was calculated as 1.3 μM and accordingly limit of quantification was determined as 4.0 μM which is well suited for droxidopa analysis in pharmaceutical preparations. The RSD was also calculated for 4.0 μM as 18% which is below the limit of 20%.

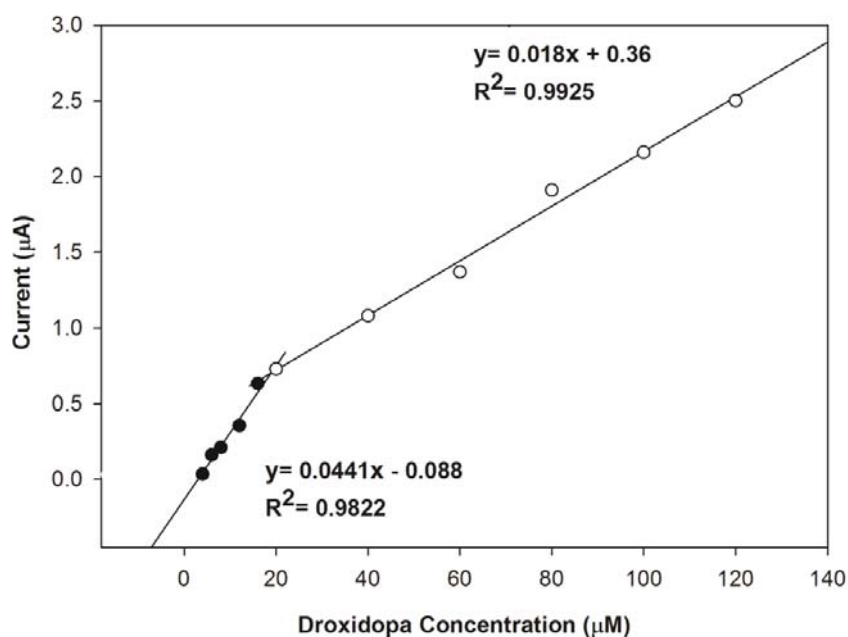


Figure 3.32. Calibration curve obtained for Droxidopa

In comparison to the other electrochemical studies encountered in the literature, Gupta et al. have developed a carbon paste electrode enriched with zinc oxide and an ionic liquid have reported a linear range of 5–310 μM (Gupta et al., 2013). In another study, graphene and ethyl 2-(4-ferrocenyl-[1,2,3]triazol-1-yl) acetate (EFTA) modified carbon paste electrode was used for the determination of droxidopa (Tajic et al., 2013). The electrochemical behavior of droxidopa has revealed that a linear relationship between the anodic peak current and droxidopa concentration in the range 2.0–400 μM has been observed.

In a very recent study, a carbon paste electrode modified with carbon nanotube and 5-amino-2'-ethyl -biphenyl-2-ol(5AEB) was fabricated (Movlaee et al., 2017). The oxidation of droxidopa at 0.36 V was utilized for its simultaneous determination with carbidopa. The linear range was found as 0.12 – 225 μM .

In the light of the literature survey given above it can be stated that the electrode developed with the aid of sonication enhance the electron-transfer between the electrode and the droxidopa which result in the improvement of the voltammetric signal. Finally, the electrode is easy to fabricate and provides a cost-effective alternative for sensitive, selective and rapid quantitation of droxidopa in tablets.

3.2.3 Electrochemical Detection of Carbidopa at a PPy-CNT/GCE

The method described above was also applied for another drug used in Parkinson's disease: Carbidopa. Initially, the dependence of oxidation characteristics on pH was optimized by changing the pH 2.0 to 8.0. The oxidation of carbi on PPy-CNT electrode has resulted two anodic peaks. The cyclic voltammograms of 100 mg L^{-1} carbi in the various pH were shown in Figure 3.23.

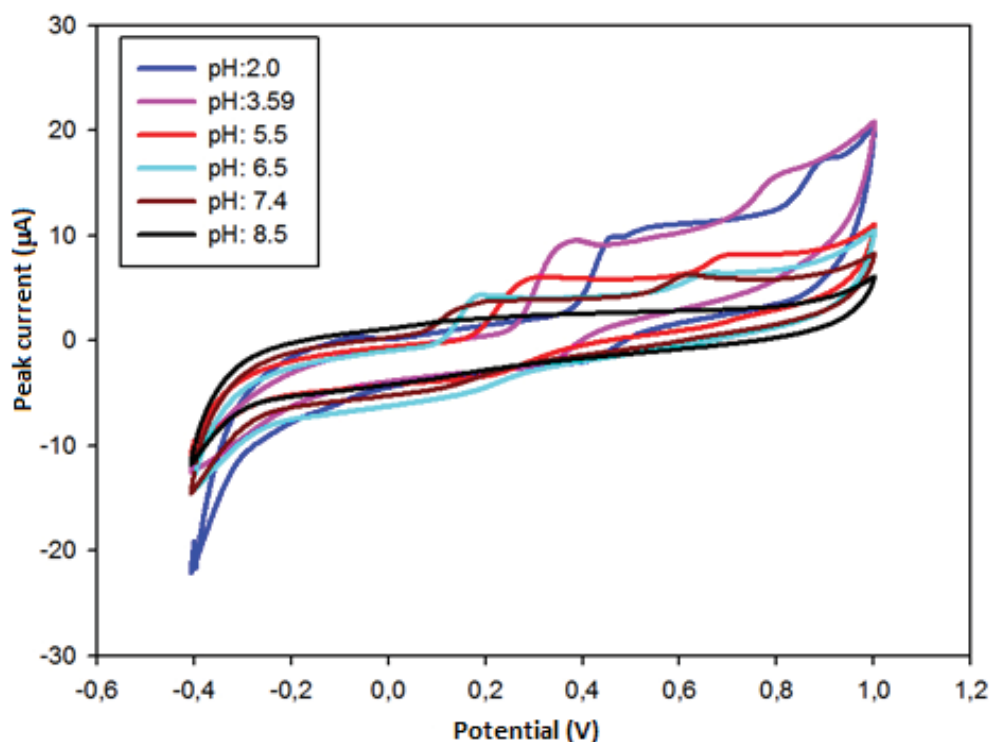


Figure 3.33 Cyclic voltammograms of 100 mg L^{-1} Carbidopa in various pHs in the potential range of $-0.4 - 1.0 \text{ V}$ with scan rate of 50 mV s^{-1} .

As can be followed from the figure, the oxidation peak current has increased by changing the pH from 8.0 to 2.0, while the potential has shifted from 0.18 V to 0.41 V . In addition, the second peak potential shifted more positive values by decreasing pH however, the peak current of this signals are not regular and the 3.59 was chosen the optimum and used for further experiment. The buffer capacity of BR is not sufficient at the pH value of 3.59. Therefore, the 50 mM formic acid – sodium formate buffer was used for further experiment.

Then, other parameters have been optimized for square wave determination of Carbidopa. It is known that electropolymerization process can be affected from monomer concentration, amount of carbon nanotube, applied pulse number.

First of all monomer concentration in the cell was investigated by addition of 100 mg L^{-1} Carbidopa in pH 3.59 formic acid buffer (Figure 3.34). The concentration pyrrole was changed from 0.029 to 0.100 M and the prepared electrodes have been used for the voltammograms of 100 mg L^{-1} Carbidopa. The resulting peak current has increased giving a maximum at 0.043 M while the peak potentials change were negligible. Accordingly, 0.043 M pyrrole concentration was chosen as the optimum and used for further studies.

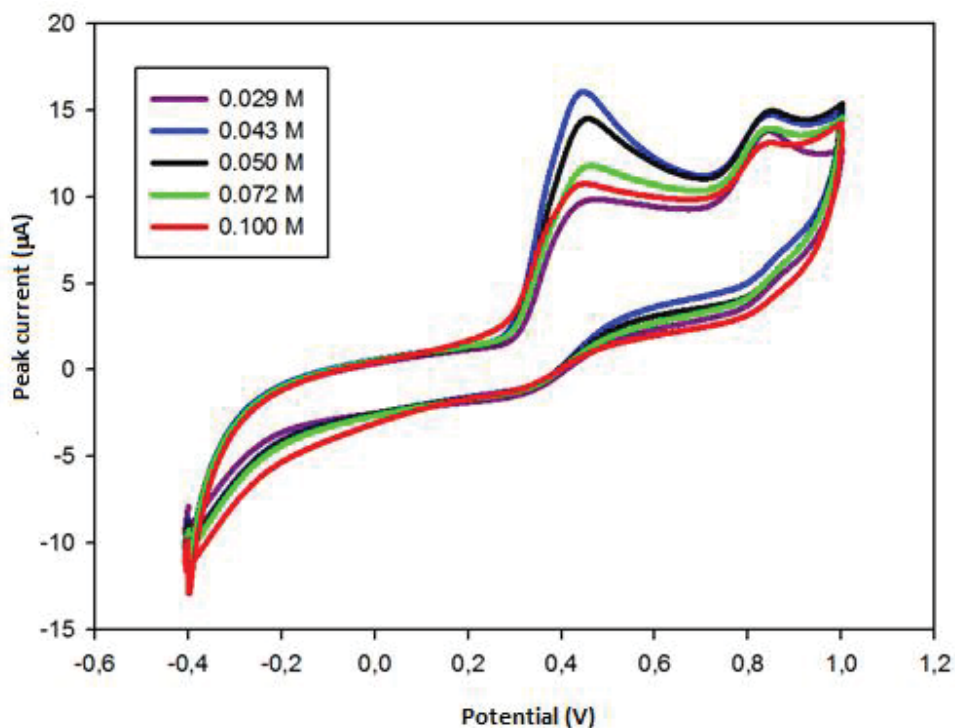


Figure 3.34 The effect of pyrrole concentration on the Carbidopa signal in pH 3.59 formic acid buffer

Then, the amount of the MW-CNT which can be predicted to effect the composition of PPy-CNT composite was optimized. For this purpose, 10 mL DMF solutions containing 0.5, 1.0, 2.0, 3.0 and 4.0 mg MW-CNT amount have been tested and 2.0 mg has given the best result (Figure 3.35). The effect of CNT amount on the peak current was displayed as the percentages of the highest current obtained and given in Figure 3.36.

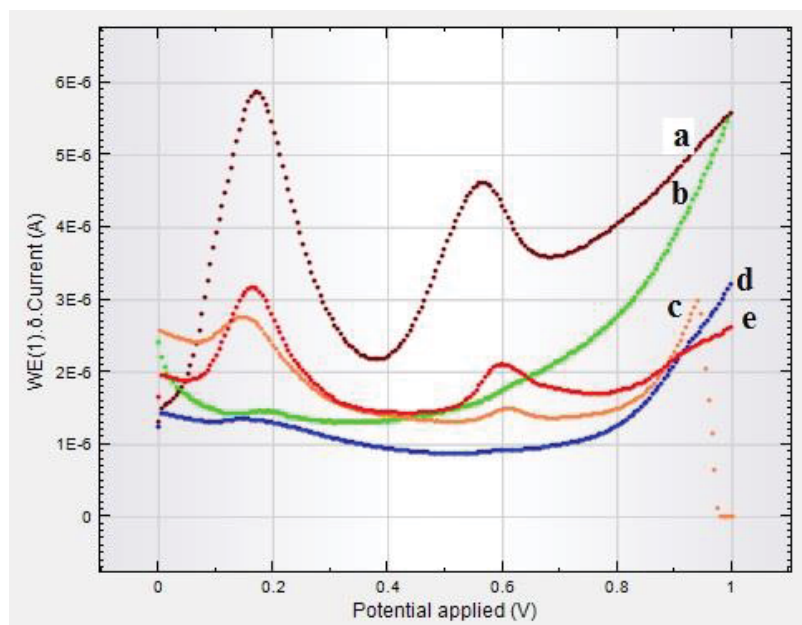


Figure 3.35 The effect of MW-CNT amount on the Carbidopa oxidation peak a) 2.0, b) 0.5, c) 3.0, d) 4.0 and e) 1.0 mg in 10 mL DMF solutions.

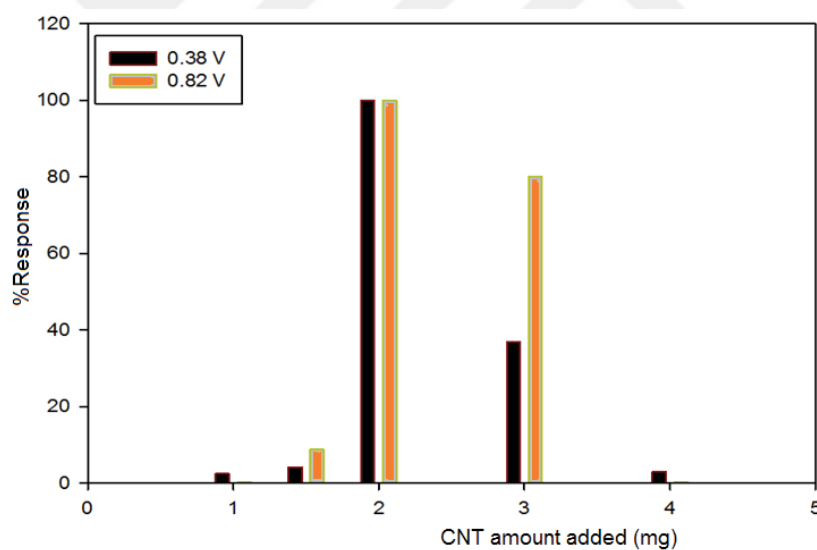


Figure 3.36 The effect of MW-CNT amount on the Carbidopa oxidation peaks as the percentages

According to above data, 2.0 mg of CNT is ultrasonically dispersed in 10 mL of DMF containing 0.07 M pyrrole and 0.1 M LiClO₄ solution for the most sensitive results. The last parameter is the pulse number applied under these conditions. Figure 3.37 indicates that successive 50 pulses has given the best results and applied for further studies.

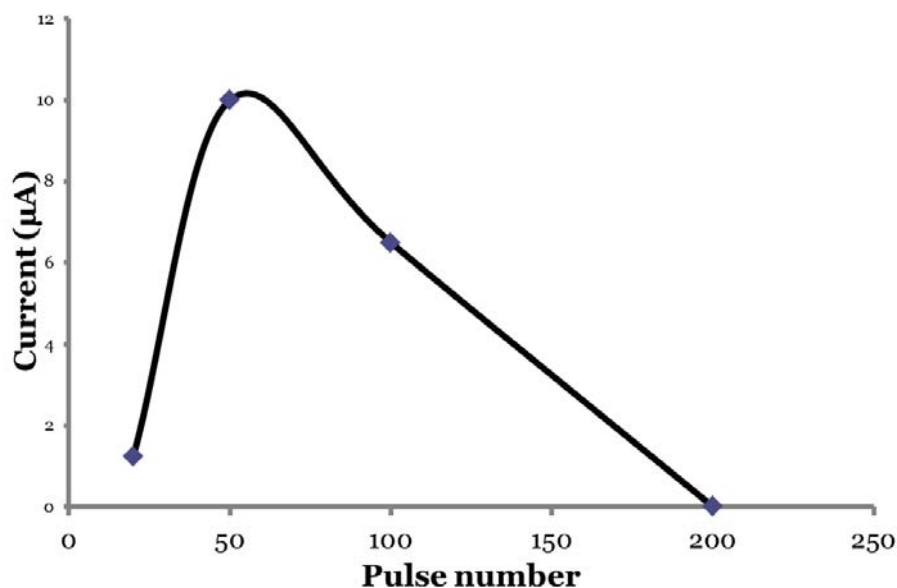


Figure 3.37 The effect of pulse number on the first oxidation signal of Carbidopa

Under the optimized conditions, relation between electrode response and concentration was investigated. The electrode showed two linear range; being 0.5 to 5.0 mg L⁻¹ with a linear equation of $y = 0.1918x - 0.1606$ and a second linear range of 10 to 30 mg L⁻¹ with an equation of $y = 0.0788x + 0.3274$ (Figure 3.38). According the graph the limit of detection and quantification was estimated as 0.17 and 0.5 mg L⁻¹ respectively.

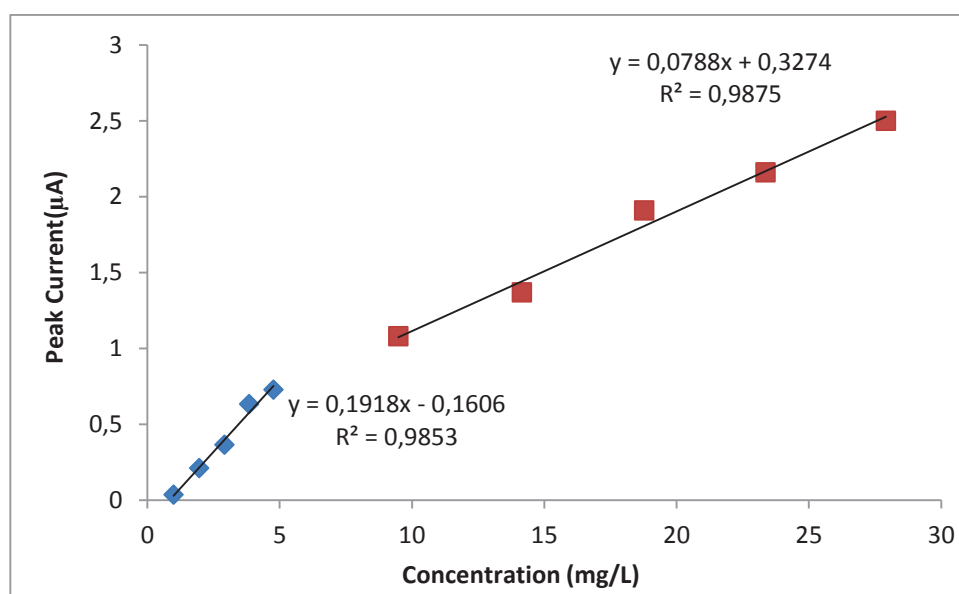


Figure 3.38 Calibration curves of Carbidopa in a concentration range of 0.5–5.0 and 5.0–30 mg.L⁻¹

3.2.4. Simultaneous Determination of Carbidopa and L-Dopa

Before sample application, the interference of L-Dopa that administered with carbidopa was tested. Figure 3.39 indicates SWV voltammograms of carbidopa, L-Dopa and their mixture. According to the Figure 3.39, both substances have an anodic peak at 0.4 V which prevents their simultaneous determination selectively.

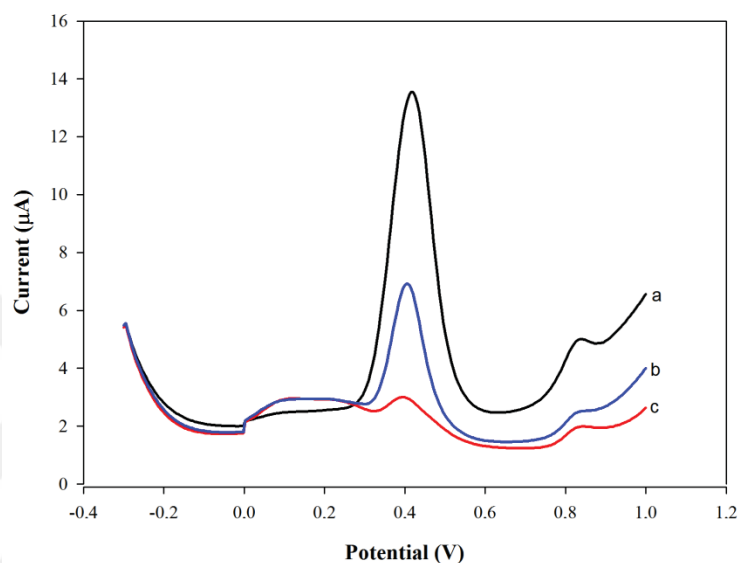


Figure 3.39 Square wave voltammograms obtained in pH: 3.59 formic acid buffer a) 100mg L⁻¹ Carbidopa b) 100mg L⁻¹ L-Dopa and c) 10mg L⁻¹ Carbidopa and 10 mg L⁻¹ L-Dopa

In such interventions, partial least square (PLS) offers a powerful chemometric tool which can separate the signals in such complex system. In this technique, calibration, validation and sample data set were recorded and processed with MINITAB 1.6 software. The actual voltammograms are given in Figure 3.40 and the PLS response plot for carbidopa and L-Dopa shown in Figure 3.41.

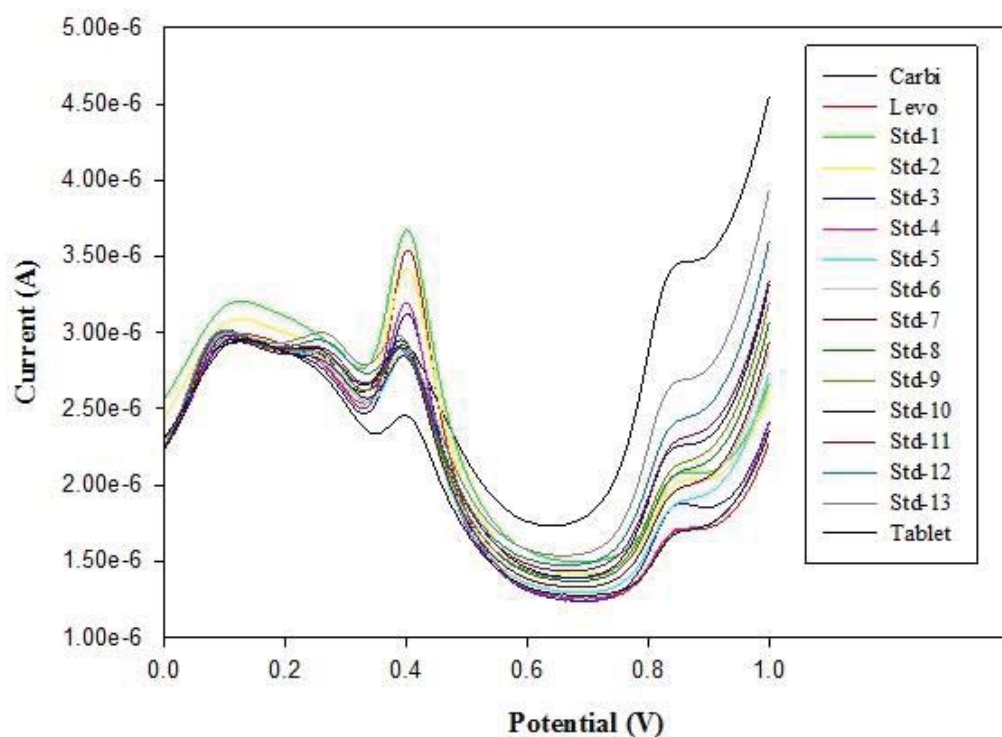


Figure 3.40 The voltammograms obtained for 100 mgL⁻¹ Carbidopa, 100 mgL⁻¹ Levodopa, Std-1 (5 mgL⁻¹ Carbidopa, 95 mgL⁻¹ Levodopa), Std-2 (10 mgL⁻¹ Carbidopa, 90 mgL⁻¹ Levodopa), Std-3 (15 mgL⁻¹ Carbidopa, 85 mgL⁻¹ Levodopa), Std-4 (20 mgL⁻¹ Carbidopa, 80 mgL⁻¹ Levodopa), Std-5 (30 mgL⁻¹ Carbidopa, 70 mgL⁻¹ Levodopa), Std-6 (40 mgL⁻¹ Carbidopa, 60 mgL⁻¹ Levodopa), Std-7 (45 mgL⁻¹ Carbidopa, 55 mgL⁻¹ Levodopa), Std-8 (60 mgL⁻¹ Carbidopa, 40 mgL⁻¹ Levodopa), Std-9 (70 mgL⁻¹ Carbidopa, 30 mgL⁻¹ Levodopa), Std-10 (80 mgL⁻¹ Carbidopa, 20 mgL⁻¹ Levodopa), Std-11 (85 mgL⁻¹ Carbidopa, 15 mgL⁻¹ Levodopa), Std-12 (90 mgL⁻¹ Carbidopa, 10 mgL⁻¹ Levodopa), Std-13 (95 mgL⁻¹ Carbidopa, 5 mgL⁻¹ Levodopa) and the commercial tablet

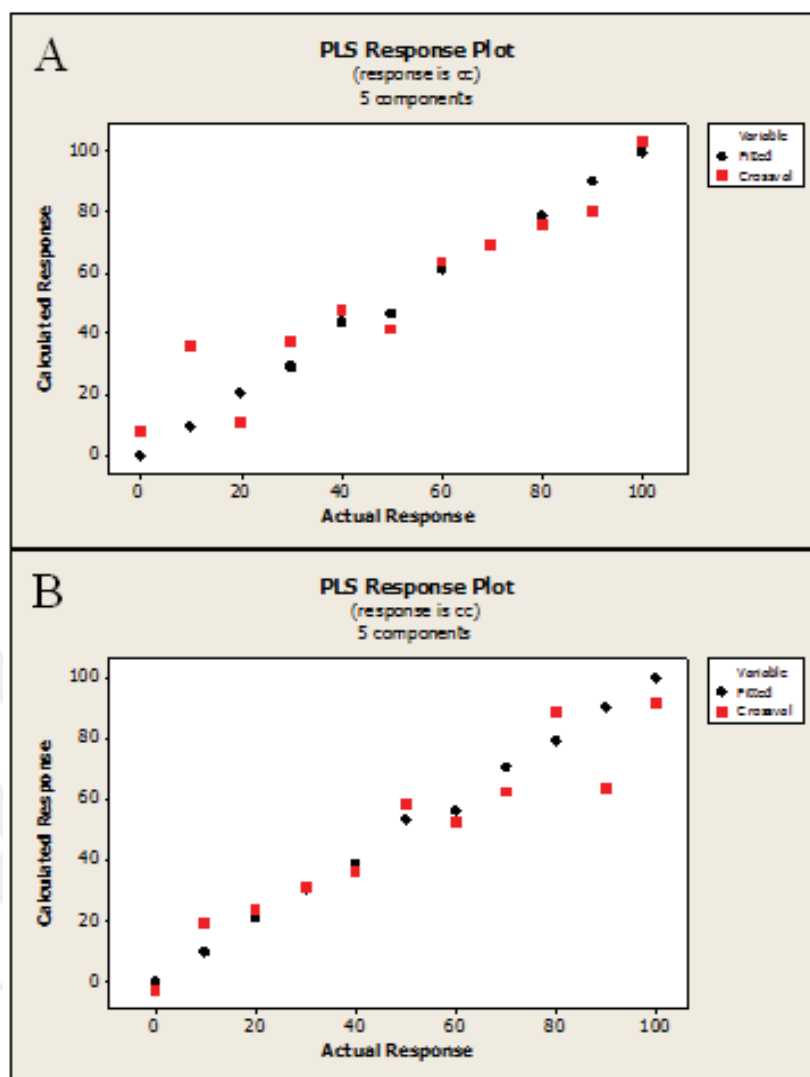


Figure 3.41 PLS response plots for A) Carbidopa and B) L-Dopa

The calculated concentration and actual response are in good agreement both two systems according to the figure. Satisfactory recovery values have been obtained as given in Table 3.13.

Table 3.13 Elucidation of Method Performance

Analyte	Found		Label Value		Recovery (%)	
	Carbidopa	L-Dopa	Carbidopa	L-Dopa		
QC-1	13	86	15	85	87	101
QC-2	55	45	55	45	100	100
QC-3	79	21	20	80	98	105
Sinemet Tablet	27.5	225	25	250	110	90

3.2.5. Simultaneous Determination of Carbidopa and Droxidopa

In this section, simultaneous determination of carbidopa and droxidopa has been tested by using the electrode described above. However, as can be followed from the Figure 3.29 individual peaks for Carbidopa and Droxidopa cannot be obtained. Alternatively, signal was processed by PLS method and experimental data obtained was fitted to the model as given in Figure 3.42 and 3.43. As can be followed from Table 3.14, satisfactory recoveries have been calculated.

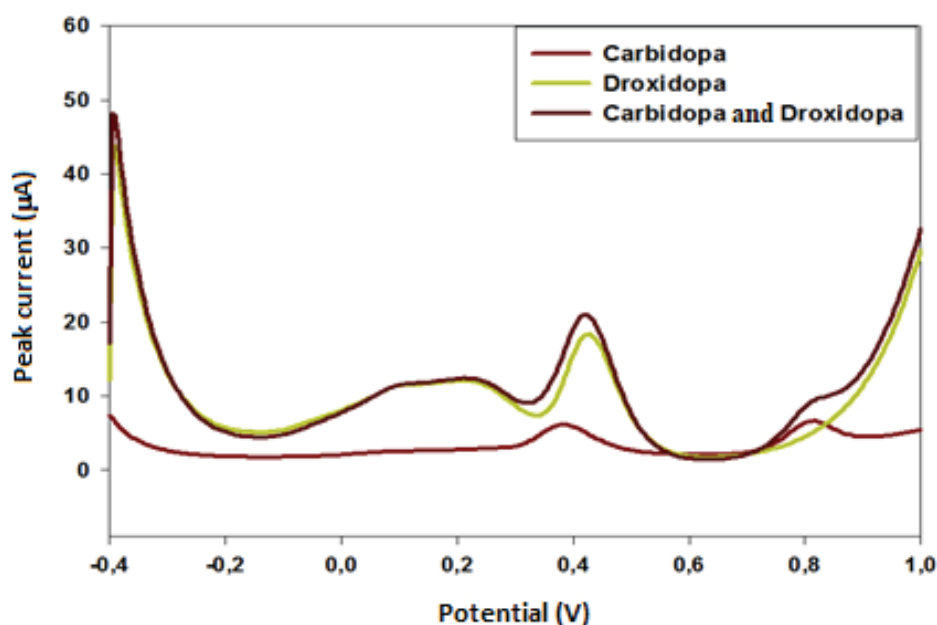


Figure 3.42 DP voltammograms of Carbidopa and Droxidopa alone and simultaneously

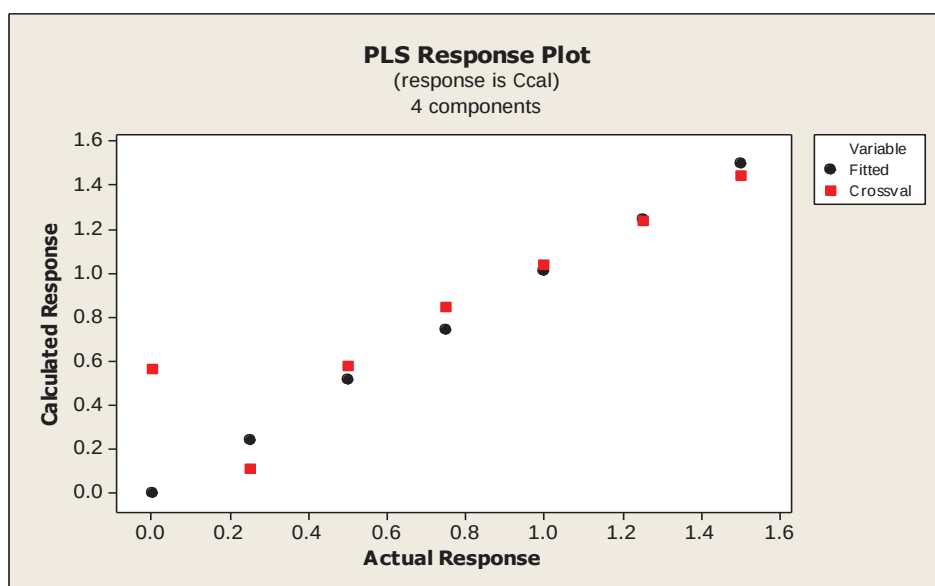


Figure 3.43 PLS Response Plot for Carbidopa

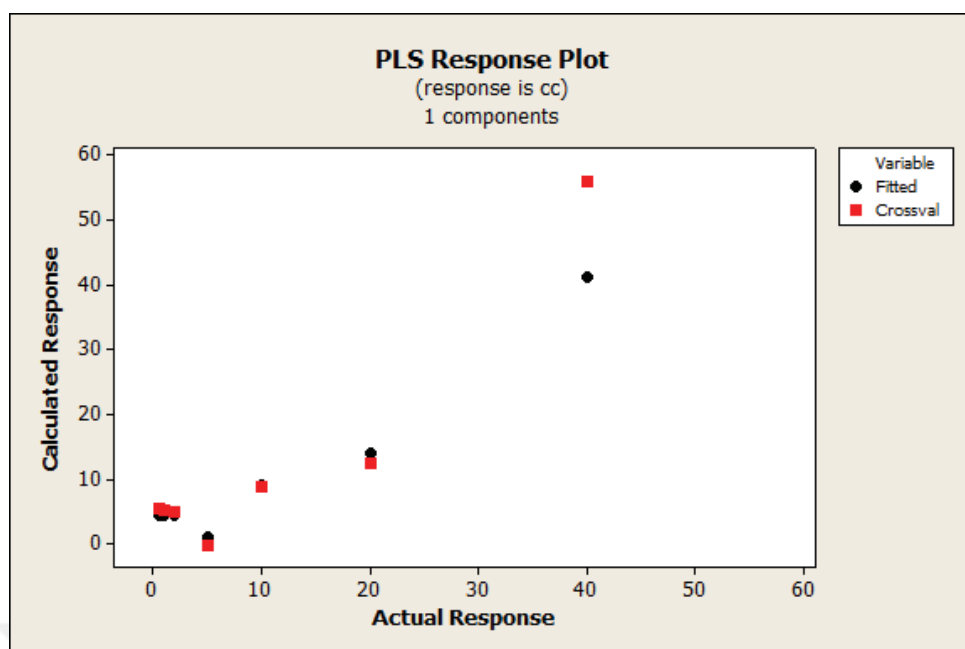


Figure 3.44 PLS Response Plot for Droxidopa

Table 3.14 PLS Results of Quality Control Samples

Sample	Found (mg L ⁻¹)		Nominal Value (mg L ⁻¹)		Recovery (%)	
	Carbidopa	Droxidopa	Carbidopa	Droxidopa	Carbidopa	Droxidopa
QC-1	0.15	0.15	0.16	0.16	105.4	105.4
QC-2	0.2	0.2	0.21	0.21	103.5	103.5
QC-3	1.25	1.25	1.24	1.24	99.2	99.2

According to above data, the method can be safely applied to their simultaneous analysis since high recovery values have been obtained. The performance of the electrode was also tested for another important compound as given in the next section.

3.2.6 Voltammetric Behaviour of Metamizole

The electrode developed was also applied for the determination of an antianalgesic drug; metamizol sodium (Met). Dependence of oxidation characteristics of the Met on the medium pH was investigated by changing the pH from 2.0 to 12.0. Anodic signal of the Met has displayed a quasi-reversible behavior on the PPy-CNT/GCE electrode. Cyclic voltammograms of 40 mg L^{-1} Met in the various pH were shown in Figure 3.45. The oxidation peak current has decreased by increasing the pH from 2.0 to 12.0, and two or more peaks have appeared in basic media. Therefore, pH 2.0 was selected for the further experiments.

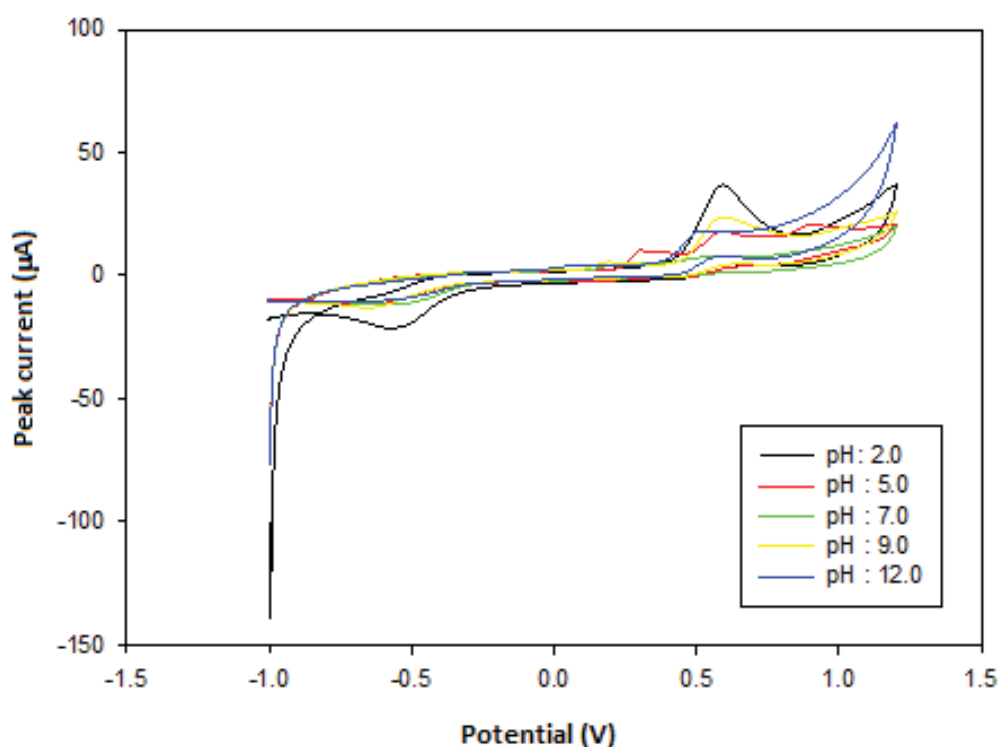


Figure 3.45 Cyclic voltammograms of 40 mg.L^{-1} in various BR buffer in the potential range of -1.0 – 1.2V with scan rate of 50 mVs^{-1} .

The GCE/PPy-CNTu electrode performance towards oxidation peaks of 100 mg L^{-1} of Metamizol Sodium was compared to that of bare GCE and as can be deduced from Figure 3.46, the catalytic activity of the electrode is clearly consistent with the higher peak formation.

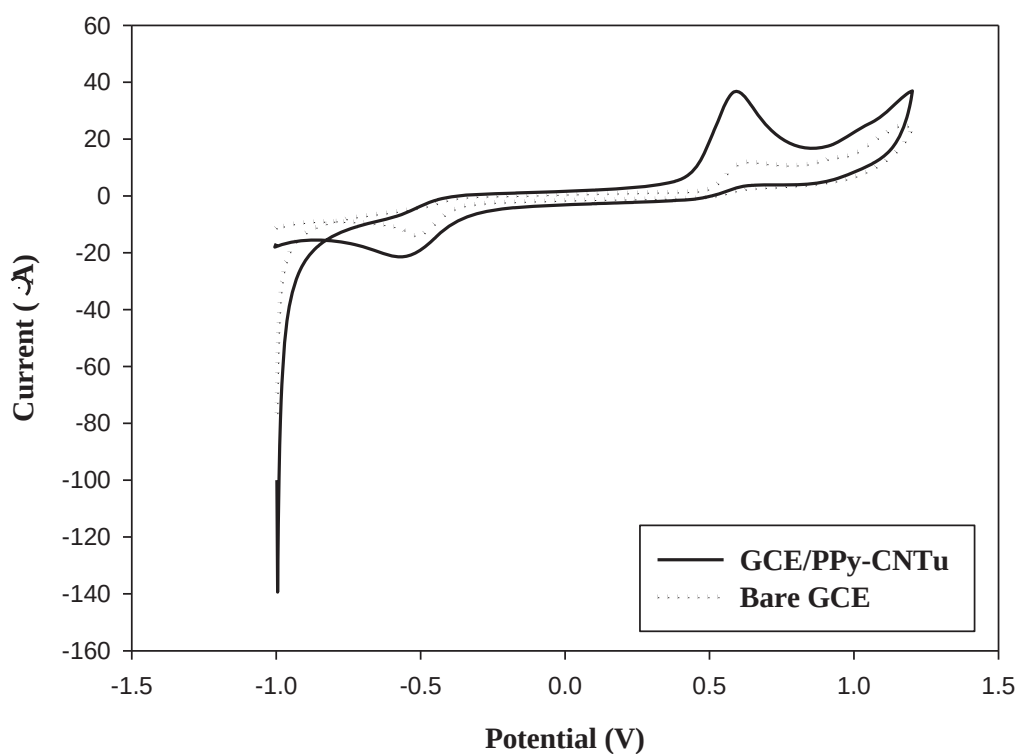


Figure 3.46 Comparison of voltammetric behavior 100 mg.L⁻¹ of Metamizol Sodium at GCE/PPy-CNTu and bare GCE

Since electrochemical behavior of polymeric film strongly depends on the thickness, deposition parameters have been checked. The effect of film thickness was controlled by changing the pulse number in deposition period. For comparison 20, 35 50 80 and 100 pulse number was tested and the results were shown in the Figure 3.47.

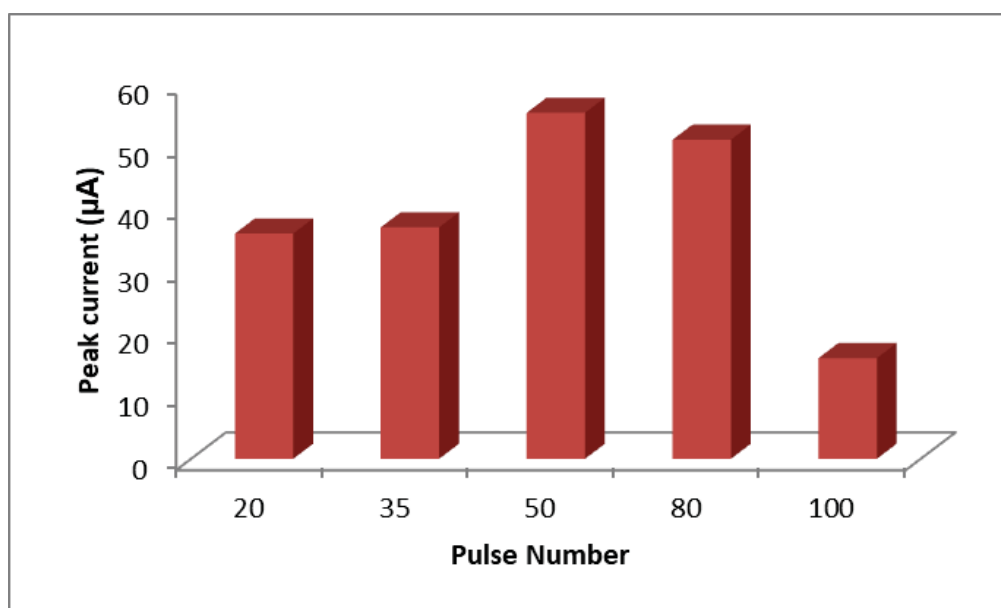


Figure 3.47 Dependence of peak current on pulse numbers

Under the optimized conditions, relation between electrode response and Met concentration was investigated. The voltammograms and the calibration curve have been shown in the Figure 3.48. The electrode have displayed a linear response in a concentration range of 5.0 to 500.0 mg L⁻¹ with a linear equation of $y = 0.0519x - 0.1414$. According the graph, the limit of detection and quantification were estimated as 16 and 5.0 mg L⁻¹, respectively.

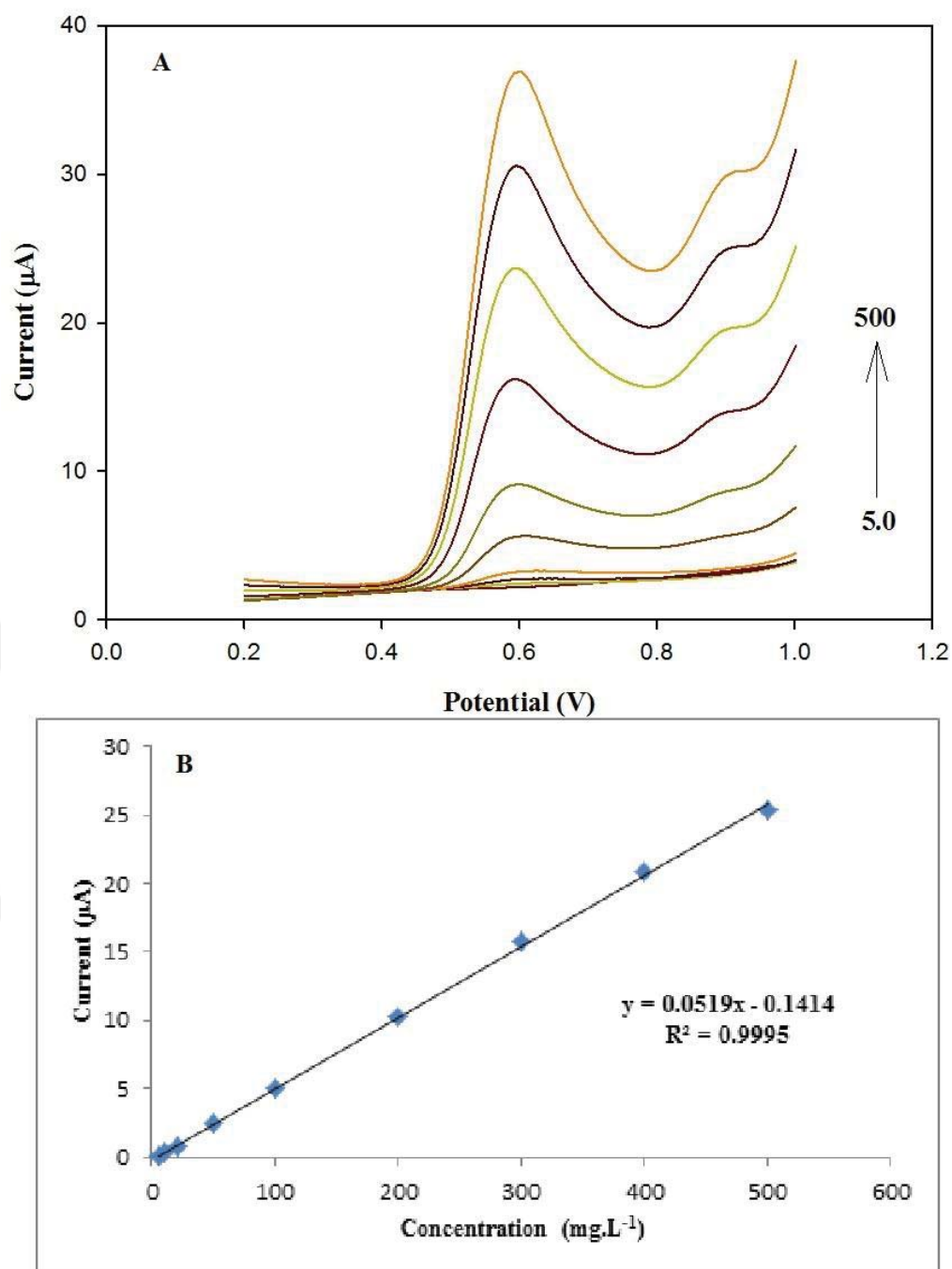


Figure 3.48 A) Calibration voltammograms and B) calibration curves for 0.5 to 500 $\text{mg}\cdot\text{L}^{-1}$ Met.

The performance of the method developed was tested on the analysis of novalgine tablets. The tablets were dissolved in buffer solution and filtered as detailed in Section 2.3 prior to each measurement. Standard addition method was used to avoid any interference from the other intergradient of the tablet. Required amount of tablet solution was transferred into voltammetric cell and standard addition curves were plotted.

Figure 3.49 shows the standard addition voltammograms and curves. The Met content of single tablet was found as 498 mg and the recovery was calculated as 99 %, by taking into account the label value (500mg).

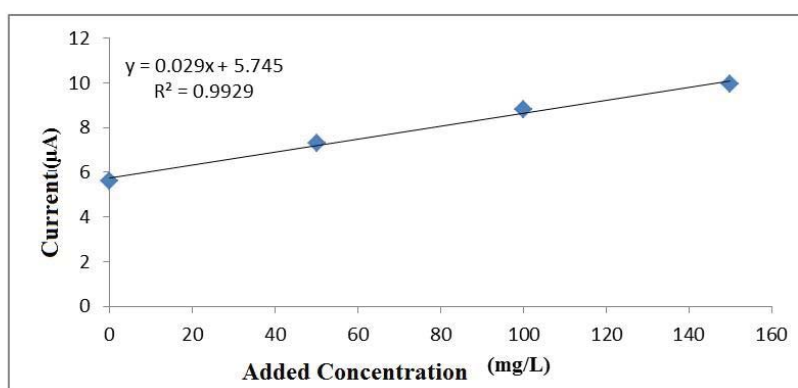
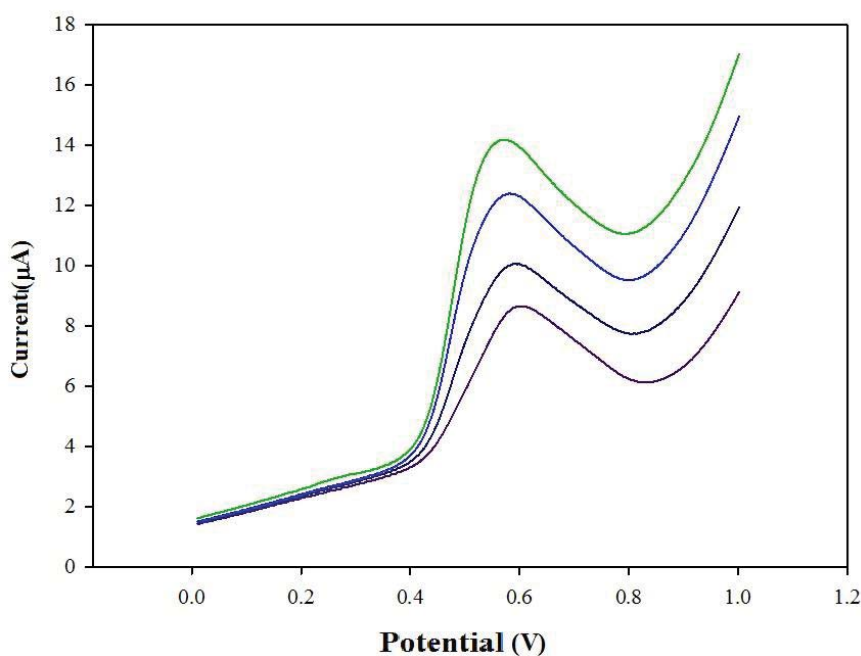


Figure 3.49 Standard Addition voltammograms (up) and the calibration curves (bottom)

4. Conclusion

The main objective of the thesis is to develop easy, quick, selective and sensitive methodologies for the analysis of some selected pharmaceutically important compounds. Many analytical methods are widely used in quality control analysis and medical diagnosis. Probably most common methods are based on chromatography. By this means, some parts of this thesis dealt with development and validation of chromatographic methods for the antiparkinson drugs and some catecholamines.

LC-MS/MS method was developed for the ternary determination of Carbidopa, L-Dopa, and Droxidopa by using C-18 and HILIC column for the first time. Performances of two analytical columns namely; C-18 and HILIC were tested and better resolution was observed with C-18 while similar sensitivities have been obtained for both columns. Good characteristics of the method developed are promising for the many applications not only in biological samples where trace analysis required.

The second chromatographic part of the thesis has been dedicated to the determination of some catecholamines namely, L-Dopa, Dopamine, HVA and DOPAC by HPLC-UV analysis. Three analytical column and different aqueous mobile phases were tested and well separated peaks were obtained. The method developed was further validated in ACF media. Since, the concentration level of dopamine, its precursor L-dopa and its metabolites is very important to screen some disorders, this part of the thesis can lead many application in clinic analysis related with brain.

Although the most wide analytical method used in pharmaceutical analysis are based on chromatography, electrochemical methods are promising due their simplicity, cheap instrumentation and high sample throughput for the determination of some electroactive assays. But, the interferences of other electroactive species in dosage forms and clinical samples can suffer the method accuracy. Thus, modification of the electrodes became crucial step for the selective and sensitive analysis.

This part of the thesis describes a novel methodology for the preparation of PPy-CNT films. This methodology combines the electrochemical pulse deposition and ultrasonication.

By using this route, homogenous PPy-CNT film was obtained and characterized. Then, the electrode developed was applied some drug assay namely, Carbidopa, L-Dopa, Droxidopa and Metamizole Sodium. Modification parameters of the electrode were optimized individually and successful results have been obtained. However, simultaneous analysis of Carbidopa, L-Dopa, Droxidopa have suffered from their similar electrochemical behavior on PPy-CNT/GCE electrode. PLS method was the method of choice to overcome this drawback.

The data obtained from the standard and sample voltammograms were uploaded to the MiniTab software and results were calculated according to PLS model. Obtained analytical results have been tested with the nominal values by analyzing quality control analysis and results were found to be promising. The content analysis of Sinemet Tablet was carried out by this method and the results were in good agreement with label value. Although the higher detection limits according to chromatographic methods, voltammetric method is rapid, cheap and practical for these analysis.

The last application of the electrode developed is analysis of metamizole sodium. The method developed, exhibited linear response in 1000 fold concentration range. In addition, high recovery and low RSD values indicate the accuracy and precision of the method developed. Thus, the method can be used in control of assay content in tablets.

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Cirruculum Vitae

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EDUCATION

Country	University	School	Department	Degree	Graduate Year
Turkey	Ege University	Faculty of Science	Chemistry	Bachelor's	2009
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Turkey	Ege University	Graude School of Naturel and Applied Science	Analytical Chemistry	Doctoral Course	Continue

THESIS

Thesis	Name	Supervisor	Year
Bachelor's Degree	Removal of toxic spieces in aqueous solution by EDI	Prof.Dr.Ümran Yüksel	2009
Master of Science	Head-Space Voltammetry: A Novel Voltammetric Method For Volatile Organics And A Case Study For Phenol	Prof.Dr.F.Nil ERTAŞ	2012

PROJECTS

1	Tarımsal sulamada Kullanım için Bor içeren sulardan Membran filtrasyon, iyon değıştirme ve sorpsiyon-membran filtrasyon hibrit yöntemleriyle Bor giderilmesi ve kazanılması Proje No : 2008 G 0192	Researcher	Ulusal Bor Arařtırma Enstitüsü
2	Erzincan University – Research Project “Erzincan Yöresindeki Doğal Su Kaynaklarının Su Kalitesinin İncelenmesi” 15/02/2011 - 15/02/2012 Project Number :	Researcher	Erzincan University
3	Ege University – Research Project “Head-Space Voltammetry: A Novel Voltammetric Method For Volatile Organics And A Case Study For Phenol” 2011 – 2012 Project Number :11 Fen055	Researcher	Ege Uni.
4	Europe Union Project “Biofuels from Solid Wastes “August, 2012 – March, 2013 Project Number: PIRSES GA-2009-217550	Researcher	Okayama University
5	Tubitak 1002 Hızlı Destek Droxidopa Ve Carbidopa’nın Yan Yana Tayinine Yönelik Voltammetrik Yöntem Geliştirilmesi ve Validasyonu Project Number: 114 Z 153	Coordinator	Ege University

PUBLICATIONS

A1. Head space voltammetry: A novel voltammetric method for volatile organics, and a case study for phenol, Özdokur, K.V., Pelit, L., Ertas, H., Timur, S., Ertas, F.N., <i>Talanta</i> 98(2012)34–39 DOI: org/10.1016/j.talanta.2012.06.032
A2. Preparation and characterization of sodium dodecyl sulfate doped polypyrrole solid phase micro extraction fiber and its application to endocrine disruptor pesticide analysis, Korba, K., Pelit, L., Pelit, F.O., Özdokur, K.V., Ertaş, H., Eroğlu, A.E., Ertaş, F.N. <i>Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences</i>, 929 (2013) 90-96 DOI: org/10.1016/j.jchromb.2013.04.023
A3. Molybdenum oxide/platinum modified glassy carbon electrode: A novel electrocatalytic platform for the monitoring of electrochemical reduction of oxygen and its biosensing applications, Çakar, I., Özdokur, K.V., Demir, B., Yavuz, E., Demirkol, D.O., Koçak, S., Timur, S., Ertaş, F.N., <i>Sensors and Actuators, B: Chemical</i> 185 (2013) 331-336 DOI: org/10.1016/j.snb.2013.04.106
A4. Pyranose oxidase and Pt-MnOx bionanocomposite electrode bridged by ionic liquid for biosensing applications Özdokur, K.V., Demir, B., Yavuz, E., Ulus, F., Erten, Ç., Aydın, I., Demirkol, D.O., Pelit, L., Timur, S., Ertaş, F.N., <i>Sensors and Actuators, B: Chemical</i>, 197 (2014) 123–128 DOI: .org/10.1016/j.snb.2014.01.122
A5. Electrochemical preparation, characterization of molybdenum-oxide/platinum binary catalysts and its application to oxygen reduction reaction in weakly acidic medium Yavuz, E., Özdokur, K.V., Çakar, I., Koçak, S., Ertaş, F.N., <i>Electrochimica Acta</i>, 151 (2015) 72–80 DOI: org/10.1016/j.electacta.2014.11.006
A6. Development of pulsed deposited manganese and molybdenum oxide surfaces decorated with platinum nanoparticles and their catalytic application for formaldehyde oxidation, Özdokur, K.V., Tatlı, A.Y., Yilmaz, B., Koçak, S., Ertaş, F.N., <i>International Journal of Hydrogen Energy</i>, 41 (2016) 5927-5933 DOI: org/10.1016/j.ijhydene.2016.02.127
A7. Polythiophene–Clay Composite Solid-Phase Microextraction Fiber: Preparation, Characterization, and Application to the Determination of Methanol in Biodiesel, Pelit, L., Yılmaz, B., Tatlı, A.Y., Pelit, F., Dizdaş, T.N., Özdokur, K.V, Ertaş, H., Ertaş, F.N., <i>Analytical Letters</i> 49 (2016) 896–906 DOI: org/10.1080/00032719.2015.1039016
A8. Synthesis and characterization of a polyoxometalate-based ionic liquid catalyst for delignification of wood biomass, Ozdokur, K.V., Moniruzzaman, M., Yanik, J., Ono, T., <i>Wood Science and Technology</i> 50 (2016) 1213–1226 DOI: 10.1007/s00226-016-0844-y
A9. A novel ethanol biosensor on pulsed deposited MnOx-MoOx electrode decorated with Pt nanoparticles Ozdokur, K.V., Demir, B., Atman, E., Tatlı, A.Y., Yılmaz, B., Demirkol, D.O., Koçak, S., Timur, S., Ertas, F.N., <i>Sensors and Actuators, B: Chemical</i> 237 (2016) 291–297 DOI: org/10.1016/j.snb.2016.06.100
A10. Determination of Carbidopa, Levodopa, and Droxidopa by High-Performance Liquid Chromatography–Tandem Mass Spectrometry, Özdokur, K.V., Engin, E., Yengin, Ç., Ertaş, H., Ertaş, F.N., <i>Analytical Letters</i>, 2017 in press.

ORAL PRESENTATIONS IN INTERNATIONAL CONGRESS

1	Head- Space Voltammetry: A Novel Voltammetric Method For Volatile Organics and a Case Study For Phenol <i>Aegean Analytical Chemistry Days AACD, 2012, Izmir Turkey</i>
2	Molybdenum Oxide/Platinum Modified Glassy Carbon Electrode And its Biosensing Applications, İ. Çakar, K.V. Özdokur, B. Demir, E. Yavuz, D. Odacı Demirkol, S. Koçak, S. Timur, F.N. Ertaş 2013, ECM-10, Konya, Turkey
3	Determination of Carbidopa, Levodopa and Droxidopa in Pharmaceutical Preparations byLiquid Chromatography Tandem Mass Spectrometry, 2016, AACD, Çanakkale
4	Simultaneous Determination of Levodopa And Carbidopa At A Polypyrrole-Carbon Nanotube Composite Electrode Prepared By Ultrasound Assisted Pulsed Electropolymerization, NyNA 2017 Barcelona, Spain

ORAL PRESENTATIONS IN NATIONAL CONGRESS

1	Elektrokimyasal Puls tekniğiyle Hazırlanan, Pt ile dekore edilmiş MnOx-MoOx Yüzeylerin Etanol Biyosensörü olarak Kullanılması, 2016, Ulusal Analitik Kimya Kongresi, ISPARTA
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POSTER PRESENTATIONS IN INTERNATIONAL CONGRESS

1	Desalination Performance of a Small Scale SWRO System for Geothermal Water – Effect of Applied Pressure. ICAST-2010 İZMİR
2	Removal of Cu ²⁺ ions from aqueous solution by Electro-deionization method –Effect of Cu ²⁺ concentration and electrode solution (H ₂ SO ₄) concentration. EMCC-6 -2010 ANTALYA
3	Manganese Oxide Modified Electrodes and Their Applications For Dissolved Oxygen Reduction, ECM-9 2011 İZMİR
4	Electrochemical Reduction of Oxygen by Molybdenum/Platinum modified Glassy Carbon Electrode and its Biosensor Applications for Glucose AACD 2012 IZMİR
5	Determination Of Endocrine Disruptor Pesticides In Water Samples By Using Polythiophene Coated SPME Fiber By Head-Space SPME Sampling AACD 2012 İZMİR
6	Preparation And Characterization Of Sodium Dodesyl Sulphate Doped Polypyrrole Solid Phase Micro Extraction Fiber And Its Application To Endocrine Disruptor Pesticide Analysis, AACD, 2012, İzmir
7	Manganese-Oxide/Platinum Modified Glassy Carbon Electrode: An Electrocatalytic Platform For The Monitoring Of Electrochemical Reduction Of Oxygen And Its Biosensing Application, K.V. Özdokur, İ. Aydın, E. Yavuz, F. Ulus, Ç. Erten, B. Demir, L. Pelit, S. Timur, F.N. Ertaş, 6. BBKA, 2013, TRABZON
8	Electrocatalytic Reduction of Dissolved Oxygen at Molybdenum-oxide and Pt Nanoparticle Modified Glassy Carbon Electrodes, E. Yavuz, S. Koçak, K.V. Özdokur, F.N. Ertaş, 6BBKA, 2013, Trabzon
9	Preparation of Cobalt oxide And Platinum Nanoparticle Modified Electrodes And Their Electrocatalytic Activity For ORR, NyNA 2017, Barcelona, Spain
10	Tungsten Oxide Modified Electrodes: A Promising Platform For Biosensors, NyNA 2017, Barcelona, Spain
11	Preparation of Vanadium - Ruthenium Oxide / Gold Composite Electrodes And Their Use In Hydrazine Oxidation, NyNA 2017, Barcelona, Spain

AWARDS

1	Poster award (2 nd best poster)	AACD, 16-20 Sept,2012
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