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Sveučilište u Zagrebu

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NOVE ELEKTROKEMIJSKE METODE U ANALITICI ODABRANIH LIJEKOVA PRIMJENOM SENZORA S UGLJIKOVIM NANOCJEVČICAMA

DOKTORSKI RAD

Mentor: prof.dr.sc. Biljana Nigović

Zagreb, 2018.

The doctoral thesis was submitted to the Faculty Council of the Faculty of Pharmacy and Biochemistry, University of Zagreb in order to acquire a PhD degree in the area of Biomedicine and Health, the field of Pharmacy, the branch of Pharmacy.

The work presented in this doctoral thesis was performed at the Faculty of Pharmacy and Biochemistry, University of Zagreb, under supervision of Prof. Biljana Nigović, PhD, in collaboration with Division of Materials Chemistry of the Rudjer Boskovic Institute.

ZAHVALE / ACKNOWLEDGEMENTS

Mojoj mentorici, prof. dr. sc. Biljani Nigović, koja je svoju strast i predanost prema istraživanju prenijela na mene još za vrijeme studentskih dana. Hvala na uvođenju u svijet znanosti, posebno u područje analitike lijekova, u kojoj sam pronašla onaj segment farmaceutske struke kojim se želim do kraja života baviti. Veliko hvala na nesebičnoj podršci, pomoći i savjetima tijekom svih ovih godina istraživanja i izrade doktorskog rada.

Doc. dr. sc. Mirandi Sertić na pomoći i svim savjetima oko "kromatografskog" dijela ovog doktorskog rada te na prijateljstvu.

Mojoj najdražoj tehničarki Đurđici Nestić koja je svojim bodrenjem i osmijehom uvijek obasjavala laboratorij, pa čak i onda kad bi analiza "zapela".

Svim ostalim djelatnicima Zavoda za analitiku i kontrolu lijekova na ugodnoj atmosferi i druženjima.

Dr.sc. Miri Ristić i njezinim suradnicima sa Zavoda za kemiju materijala Instituta Ruđer Bošković na snimanjima uzoraka elektronskom mikroskopijom.

Mojim dragim prijateljima uz koje je sve bilo puno lakše i vedrije.

Mojim roditeljima i bratu na bezuvjetnoj ljubavi, vjeri i podršci. Posebno hvala mami i tati što su mi omogućili da danas budem sve što jesam i sve što mogu zamisliti.

Danielu, mojoj ljubavi i vjernom pratiocu kroz sve ove godine školovanja. Hvala ti što si mi uvijek bio oslonac i što si uvijek vjerovao u mene te bio pun razumijevanja i kad su neke druge stvari "trpile" zbog sati i sati provedenih u laboratoriju. ☺

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Doktorski rad

Zagreb, 2018.

Mentor:

- prof. dr. sc. **Biljana Nigović** (Farmaceutsko-biokemijski fakultet Sveučilišta u Zagrebu)

Ova disertacija napisana je na engleskom jeziku temeljem članka 15. ***Pravilnika o doktorskom studiju*** *(Farmaceutsko-biokemijski fakultet u Zagrebu, 2017.)*

Disertacija je pisana kao skup objavljenih znanstvenih radova popraćen kritičkim preglednim poglavljem (tzv. skandinavski model), temeljem članka 14. ***Pravilnika o doktorskom studiju*** *(Farmaceutsko-biokemijski fakultet u Zagrebu, 2017.)*

SUMMARY

Due to strict requirements regarding quality control of pharmaceuticals and with the aim of obtaining maximal efficacy and safety of drug therapy for patients, there is a constant demand for developing new reliable analytical methods to determine drug concentrations in complex samples. Electroanalytical techniques, especially voltammetric, represent a powerful analytical tool. Electrochemical nanosensors have recently found extensive applications in pharmaceutical analysis. Therefore, the aim of this doctoral thesis was to develop new electroanalytical methods for the determination of selected pharmaceuticals in dosage forms and biological fluids using novel sensors based on multi-walled carbon nanotubes dispersed in Nafion matrix, as well as their combination with zirconium oxide nanoparticles. Electrochemical behaviour of selected drugs was examined by cyclic voltammetry to get an insight into their redox mechanism at modified electrodes. Experimental conditions and instrumental parameters that could affect the electroanalytical performance of proposed sensors were carefully optimised. New methods were validated according to ICH guidelines. Direct pulse voltammetric techniques (square-wave or differential pulse) were used to determine the content of ropinirole, L-dopa, mesalazine and nebivolol in tablets, except for ondansetron in which case adsorptive stripping procedure was employed. The good recoveries indicated that excipients did not interfere with the assay of active ingredient in any case. In comparison to the high performance liquid chromatographic methods, there were no significant differences regarding the accuracy and precision; however voltammetric techniques offer high sensitivity, rapid response and simplicity. Finally, nanosensors were applied for the simultaneous quantification of selected pharmaceuticals with other co-administered drugs in therapy (such as ondansetron with morphine and ropinirole with L-dopa) or their metabolites (mesalazine and *N*-acetylated metabolite) in human serum samples using adsorptive stripping voltammetry. Integration of the nanoparticles in the sensor improved notably the determination of therapeutic concentrations of nebivolol in serum. Excellent recoveries in the range of 98.7 – 102.6 % were obtained in all cases, without the need for sample pretreatment. A considerable enhancement effect on voltammetric responses of selected drugs is due to synergy of remarkable properties of nanomaterials and cation-exchange polymer that enhanced the preconcentration of positively charged drug molecules.

KEYWORDS: multi-walled carbon nanotubes, zirconium oxide nanoparticles, Nafion, voltammetry, electrochemical nanosensors, ondansetron, morphine, ropinirole, L-dopa, mesalazine, metabolite of mesalazine, nebivolol

SAŽETAK

Uvod: S obzirom na sve složenije zahtjeve u kontroli kvalitete lijekova, a s ciljem osiguravanja maksimalne učinkovitosti i sigurnosti terapije za pacijente, postoji neprestani zahtjev za razvojem novih analitičkih metoda za određivanje lijekova u farmaceutskim dozirnim oblicima i biološkim tekućinama. Elektroanalitičke tehnike su jako osjetljive, selektivne, točne i precizne, a istodobno jednostavne i relativno jeftine s kratkim vremenom analize. Zahvaljujući svojim brojnim prednostima, u posljednje vrijeme pronalaze sve značajniju primjenu u analitici lijekova. Među njima se ističu voltametrijske tehnike, kao najčešće korištene u području kvalitativnih i kvantitativnih ispitivanja lijekova. Razvoj elektroanalitičkih tehnika usmjeren je ponajprije na razvoj novih elektrokemijskih senzora, među kojima se posebno ističu nanosenzori. Ugljikove nanocjevčice, zbog svoje jedinstvene strukture i izvanrednih svojstava, privukle su veliku pozornost u pripremi elektrokemijskih senzora. Stoga su u sklopu ove doktorske disertacije razvijene nove voltametrijske metode za određivanje odabranih elektroaktivnih lijekova slabo bazičnih svojstava primjenom elektrokemijskih senzora s ugljikovim nanocjevčicama. Ugljikove nanocjevčice dispergirane su u polimernom matriksu Nafiona, a u nekim ispitivanjima i dodatno obogaćene nanočesticama cirkonijeva dioksida (ZrO_2). Nakon validacije u skladu s ICH smjernicama, metode su primijenjene za određivanje lijekova u gotovim farmaceutskim oblicima, kao i za simultane analize zajedno primjenjivanih lijekova u terapiji ili lijekova i metabolita u biološkim tekućinama.

Metode: Različite voltametrijske tehnike korištene su u svrhu kvalitativnog i kvantitativnog ispitivanja odabranih lijekova. Cikličkom voltametrijom ispitano je elektrokemijsko ponašanje lijekova radi stjecanja uvida u mehanizam i kinetiku redoks procesa koji se odvijaju na modificiranim elektrodama. Pravokutnovalna voltametrija primijenjena je za određivanje sadržaja ondansetrona, ropinirola, levodope i mesalazina u tabletama. Diferencijalna pulsna voltametrija primijenjena je za određivanje sadržaja nebivolola u tabletama. Tablete su nabavljene u ljekarnama u Republici Hrvatskoj. Kao usporedna tehnika za evaluaciju točnosti i preciznosti novorazvijenih metoda korištena je tekućinska kromatografija visoke djelotvornosti (HPLC) primjenom statističkih alata. Adsorptivna pravokutnovalna tehnika korištena je za simultanu analizu ondansetrona s morfinom i ropinirola s levodopom te mesalazina s glavnim N-acetiliranim metabolitom, dok je adsorptivna diferencijalno pulsna

metoda primijenjena za određivanje nebivolola u uzorcima seruma. Uzorci seruma prikupljeni su od zdravih dobrovoljaca koji nisu uzimali nikakvu drugu terapiju u vrijeme davanja krvi te su potpisali informirani pristanak i bili upoznati sa svrhom korištenja dobivenih uzoraka seruma. Predloženi elektrokemijski senzori pripremljeni su dispergiranjem nanomaterijala u Nafionu te nanošenjem odgovarajućih volumena suspenzije na površinu prethodno ispolirane elektrode od staklastog ugljika, nakon čega je uslijedila evaporacija otapala. Morfologija površine pripremljenih senzora ispitana je elektronskom mikroskopijom (scanning electron microscopy, SEM) te cikličkom voltametrijom. U svrhu obrade podataka za potrebe ovog doktorskog rada korišteni su programski paketi GPES 4,9 μ-Autolab potenciostata te Microsoft office Excel 2010 i OriginPro 7,5 i 9,0.

Rezultati:

Elektrokemijsko ponašanje antiemetika ondansetrona ispitano je prvi put u sklopu ovog doktorskog rada na nemodificiranoj i modificiranoj elektrodi od staklastog ugljika. U odnosu na nemodificiranu elektrodu, modifikacija elektrode ugljikovim nanocjevčicama u polimernom matriksu dovela je do izvanrednog poboljšanja voltametrijskog odgovora ondansetrona zahvaljujući sinergističkom učinku modifikatora. 0,1 M sumporna kiselina odabrana je kao optimalni elektrolit, a najveća osjetljivost dobivena je nanošenjem 5 μL suspenzije koja je sadržavala ugljikove nanocjevčice i Nafion u omjeru 1:1. Razvijena metoda primijenjena je za određivanje sadržaja ondansetrona u tabletama metodom standardnog dodatka. Dobivena vrijednost analitičkog prinosa od 99,7 % ukazuje na dobru točnost metode te prikladnost predloženog senzora za ovu svrhu. Analitički prinos dodane otopine standarda u prethodno analiziranu otopinu tableta od 98,1 % pokazuje da pomoćne tvari u formulaciji ne interferiraju s određivanjem aktivne tvari te nije potreban separacijski korak. Rezultati su uspoređeni s rezultatima razvijene HPLC metode te je statističkom analizom pokazano da nema značajne razlike u izvedbi ovih dviju metoda što se tiče točnosti i preciznosti. Predloženi senzor uspješno je primijenjen i za simultanu analizu s analgetikom morfinom u uzorcima seruma s kojim se nerijetko primjenjuje u terapiji. Dobivena je linearna ovisnost njihovih voltametrijskih odgovora u području koncentracija 1,0 x 10^{-7} – 5,0 x 10^{-6} M za ondansetron i 1,0 x 10^{-7} – 4,0 x 10^{-6} M za morfin nakon akumulacije pri optimalnim vrijednostima E_{acc} = -0,5 V i t_{acc} = 360 s. Razlika potencijala od 430 mV između strujnih vrhova ondansetrona i morfina te niska granica detekcije (3,1 x 10^{-8} za ondansetron i 3,2^{-8} M

za morfin) omogućili su simultano određivanje vrlo niskih koncentracija ondansetrona i morfina kakve se mogu naći u biološkim tekućinama nakon primjene uobičajenih terapijskih doza s vrijednostima analitičkih prinosa 98,7 % ± 1,5 % za ondansetron i 102,6 % ± 2,7 % za morfin.

Nanosenzor predložen u sklopu ovog doktorskog rada pokazao je izvrsnu osjetljivost i selektivnost te je primijenjen u razvoju nove analitičke metode za simultanu analizu ropinirola i levodope. Iako se ropinirol i levodopa vrlo često koriste zajedno u terapiji Parkinsonove bolesti, dosad u literaturi nije bilo analitičke metode za njihovu simultanu analizu. Za optimalnu izvedbu nanosenzora primijenjeno je 5 μL suspenzije koncentracije 1 mg/mL, pri čemu je uzeta 0,5 % etanolna otopina Nafiona. Sva mjerenja u svrhu kvantifikacije provedena su u 0,1 M sumpornoj kiselini. Pri optimalnim uvjetima postignut je linearan odgovor za ropinirol u širokom području koncentracija 1,0 x 10^{-7} – 1,0 x 10^{-5} M te 2,5 x 10^{-7} – 1,0 x 10^{-5} M za levodopu. Velika razlika potencijala od 720 mV omogućila je simultanu analizu ropinirola i levodope uz optimalnu akumulaciju pri E_{acc} = 0 V i t_{acc} = 240 s prije samog voltametrijskog mjerenja. Dobivene su vrijednosti analitičkih prinosa 99,6 % ± 2,2 % za ropinirol i 98,8 % ± 2,3 % za levodopu u složenom matriksu poput seruma bez kompliciranog postupka ekstrakcije prije analize. Nadalje, predloženi senzor primijenjen je i za individualno određivanje sadržaja ropinirola i levodope u gotovim farmaceutskim oblicima. Dobiveni rezultati pokazuju slaganje s deklariranim sadržajima u tabletama. Vrijednosti analitičkih prinosa od 99,2 % za ropinirol i 99,4 % za levodopu ukazuju da pomoćne tvari ne interferiraju, a *t*-test i *F*-test da nema statistički značajne razlike u pogledu točnosti i preciznosti između novorazvijenih voltametrijskih i HPLC metoda.

U sklopu ovog doktorskog rada razvijena je prva elektroanalitička metoda za simultanu analizu mesalazina i *N*-acetiliranog metabolita. Po prvi put ispitano je elektrokemijsko ponašanje metabolita mesalazina. Zahvaljujući svojim odličnim svojstvima predloženi nanosenzor omogućio je selektivno određivanje strukturno vrlo sličnih spojeva kao što su lijek i njegov metabolit zajedno u serumu bez prethodne separacije. Razlika potencijala na senzoru s ugljikovim nanocjevčicama u polimernom matriksu bila je 200 mV. Senzor je pripremljen nanošenjem 3 μL suspenzije ugljikovih nanocjevčica u omjeru 1:1 s 0,3 % etanolnom otopinom Nafiona. Britton-Robinson pufer pH 2,0 pokazao se kao optimalan za simultanu kvantifikaciju. Bilo je potrebno svega 30 sekundi za simultano određivanje mesalazina i metabolita u složenom matriksu poput seruma s visokom osjetljivošću (2,33 x 10^7 za mesalazin i 8,37 x 10^6 μA M^{-1} za metabolit) i bez interferencija s endogenim biološkim

tvarima, što se vidi po vrijednostima analitičkih prinosa 100,1 % ± 2,7 % za mesalazin i 99,3 % ± 2,8 % za *N*-acetilirani metabolit. Direktnom pravokutnovalnom voltametrijom određen je sadržaj mesalazina u dva različita farmaceutska oblika dostupna na tržištu Republike Hrvatske. Dobivene vrijednosti analitičkih prinosa u vrijednosti 100,7 % te 99,8 % omogućuju usporedbu njihove kvalitete i ukazuju na slaganje s deklariranim sadržajima u oba slučaja. Ispitan je utjecaj pomoćnih tvari na voltametrijski odgovor mesalazina i ustanovljeno je da nema interferencije (analitički prinos u rasponu 98,4 – 101,6 % za oba farmaceutska proizvoda). Usporedbom točnosti i preciznosti s postojećim HPLC metodama u literaturi, zaključeno je da nema statistički značajne razlike u njihovim izvedbama.

U sklopu razvoja nove voltametrijske metode za određivanje kardioselektivnog beta blokatora nebivolola u farmaceutskom dozirnom obliku i serumu, senzor predložen u ranijim istraživanjima dodatno je obogaćen nanočesticama ZrO_2 kako bi se integracijom učinaka dvaju nanomaterijala na elektrokatalitičku aktivnost postigao još bolji voltametrijski odgovor lijeka i veća osjetljivost metode za praćenje terapijskih koncentracija lijeka u serumu. Posebna pažnja pridodana je optimizaciji samog nanosenzora. S ciljem dobivanja maksimalne osjetljivosti sinergističkim djelovanjem ugljikovih nanocjevčica, ZrO_2 nanočestica i Nafiona, ispitan je utjecaj omjera koncentracija dvaju nanomaterijala, koncentracije Nafiona u etanolnoj otopini te volumena konačne suspenzije na elektrokemijsko ponašanje nebivolola. Na inovativnom nanosenzoru dobiven je linearni odgovor nebivolola u području koncentracija $1{,}5 \times 10^{-6}$ – $1{,}0 \times 10^{-4}$ M bez akumulacije te u području koncentracija $1{,}0 \times 10^{-7}$ – $6{,}0 \times 10^{-6}$ M s akumulacijom od 240 sekundi neposredno prije voltametrijskog mjerenja. Diferencijalno pulsnom voltametrijom određen je sadržaj nebivolola u tabletama i pokazano je slaganje dobivenih rezultata s deklariranim sadržajem (analitički prinos 98,3 %). Nema statistički značajne razlike u odnosu na rezultate dobivene prethodno razvijenom HPLC metodom. Utjecaj pomoćnih tvari ispitan je metodom standardnog dodatka i ustanovljeno je da nema interferencija (analitički prinos 98,1 %). Očekivane koncentracije nebivolola u serumu nalaze se unutar linearnog područja metode nakon kratke akumulacije na površinu elektrode te je adsorptivna diferencijalno pulsna voltametrija korištena za određivanje nebivolola u uzorcima seruma. Srednja vrijednost analitičkog prinosa 100,8 % ukazuje na odličan potencijal predloženog nanosenzora za praćenje nebivolola u biološkim tekućinama.

Zaključak: Razvoj novih elektroanalitičkih metoda predstavlja važan znanstveni doprinos u analizi onih lijekova za koje ovakve metode nisu dosad uopće razvijene ili nisu razvijene na inovativnim elektrokemijskim senzorima. Nanosenzori predloženi u sklopu istraživanja ovog doktorskog rada znatno su utjecali na poboljšanje osjetljivosti, selektivnosti i reproducibilnosti novorazvijenih metoda zahvaljujući sinergističkom učinku modifikatora. S druge strane, predloženi nanosenzori pokazuju dobru stabilnost u duljem vremenskom razdoblju, a njihova priprema i regeneracija su vrlo jednostavne i brze. Suvremene elektroanalitičke metode temeljene na ovakvim inovativnim elektrokemijskim senzorima imaju velik potencijal za primjenu u složenom matriksu, kao što su farmaceutski dozirni oblici i biološke tekućine, ne samo za istraživanja u akademske svrhe, već i u rutinskim analizama lijekova. Predloženi nanosenzori mogu stoga doprinijeti u procjeni kvalitete gotovih farmaceutskih proizvoda te poslužiti u kliničkoj medicini i farmaciji za praćenje terapijskih koncentracija lijekova u biološkim uzorcima s ciljem postizanja što boljeg farmakološkog učinka i manje toksičnih nuspojava.

KLJUČNE RIJEČI: ugljikove nanocjevčice, nanočestice cirkonijeva dioksida, Nafion, voltametrija, elektrokemijski nanosenzori, ondansetron, morfin, ropinirol, levodopa, mesalazin, *N*-acetilirani metabolit mesalazina, nebivolol

ABBREVIATIONS AND SYMBOLS

A	electrode area
Ac-5-ASA	*N*-acetylated metabolite of mesalazine
AdDPV	adsorptive differential pulse voltammetry
AdSV	adsorptive stripping voltammetry
AdSWV	adsorptive square wave voltammetry
API	active pharmaceutical ingredient
BDD	boron-doped diamond
BDDE	boron-doped diamond electrode
BR	Britton-Robinson
C	concentration
CE	cappilary electrophoresis
CEC	capillary electrochromatography
CMEs	chemically modified electrodes
CNTs	carbon nanotubes
CP	carbon paste
CV	cyclic voltammetry
CVD	chemical vapor depostition
D	diffusion coefficient
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DPV	differential pulse voltammetry
E_{max}	maximum peak potential
E_{min}	minimum peak potential
$E_{p,a}$	anodic peak potential
$E_{p,c}$	cathodic peak potential

EDXS	energy dispersive x-ray spectroscopy
E_{sw}	square-wave amplitude
f	frequency
FIA	flow injection analysis
GC	glassy carbon, gas chromatography
GCE	glassy carbon electrode
HOPG	highly oriented pyrolytic graphite
HPLC	high performance liquid chromatography
$I_{p,a}$	anodic peak current
$I_{p,c}$	cathodic peak current
L-dopa	levodopa
LC	liquid chromatography
MALDI	matrix-assisted laser desorption ionization
MOR	morphine
MWCNTs	multi-walled carbon nanotubes
n	number of electrons
n_a	number of electrons in the rate-determining step
NBV	nebivolol
NPs	nanoparticles
NPV	normal pulse voltammetry
OND	ondansetron
Ph.Eur.	European Pharmacopoeia
ROP	ropinirole
RVC	reticulated vitreous carbon
SEM	scanning electron microscopy
SPAB	2-hydroxy-5-[(4-sulfophenyl)azo]benzoic acid
SPC	screen-printed carbon strips

SPE	solid phase extraction
SPME	solid phase micro extraction
SV	staircase voltammetry, stripping voltammetry
SWCNTs	single-walled carbon nanotubes
SWV	square-wave voltammetry
XRD	X-ray diffraction
5-ASA	5-aminosalicylic acid, mesalazine
α	charge transfer coefficient
v	scan rate
τ	duration of a potential cycle
ΔE	step potential
ΔI	difference in current

TABLE OF CONTENTS

1. INTRODUCTION

1.1. The importance of pharmaceutical analysis

In recent years, there has been extraordinary progress in the discovery, synthesis, sensitive analysis and means of delivery of pharmaceutically active compounds used in the diagnosis, prevention and treatment of human diseases [1]. During the development of a new drug product from the discovery stage to the commercial market, analytical chemistry plays a great role [2]. The pharmaceutical and biomedical analysis is among the most important branches of applied analytical chemistry [3]. Drug analysis is essential in various stages of drug design and development, such as quality control, stability testing, preclinical and clinical trials [4,5]. All these investigations require sensitive and reliable analytical methods in order to measure drugs in pure and formulated forms as well as in complex media such as biofluids [5] with the aim of obtaining data that can contribute to the maximal efficacy and safety of drug therapy and maximal economy of the production of pharmaceuticals [6]. The increased level of regulatory scrutiny under which the pharmaceutical industry should operate has also fostered the continued development of analytical methods [4].

1.2. Electroanalytical techniques in pharmacy

Electrochemistry is a fast-growing field with a number of possible applications in pharmacy. The great diversity of electroanalytical methods allows the application of electrochemistry in various stages of drug research and development [7]. Oxidation and reduction of functional groups are also important transformations in organic synthesis [8] and therefore, electroorganic synthesis of pharmacologically interesting molecules can be used in the early stages of drug research [9]. It represents a cost-efficient and sustainable "green-chemistry" method [8,10,11] with possibility of easily scaling up many electrochemical reactions [12]. Furthermore, electroorganic synthesis can provide outstanding selectivity and reactivity due to accurate potential control [7] and in some cases the formation of products that are not accessible via conventional methods [10]. In recent years, there have been several examples of electrochemical synthesis of potential pharmaceuticals, such as novel 1,3-indandione derivatives as possible antiplatelet agents [13], phenoxazine and diphenoxazine derivatives which were shown through previous examinations to be effective antitumor, antileukemia and antimicrobial material [14], benzoxazoles that represent an important structure of several natural products, pharmaceuticals and biologically active compounds [15]

and benzofuranoquinone derivatives [16] with triad structures of catechol, benzofuran and quinone that were shown to be useful from the point of view of pharmaceutical properties.

In addition to the synthetic approach, electrochemistry can be used at an early stage of drug research for screening the pharmacological activity of a homologous series of newly synthesized molecules [9]. The screening is based on correlation between oxidation or reduction potential of investigated group of compounds with their molecular structure and pharmacological activity [7]. For example, the relationship between antioxidant activity of natural phenolic compounds and their voltammetric behaviour was investigated using electroanalytical approach [17]. Antioxidant capacity of several drug products with acetylsalicylic acid was also evaluated using voltammetry [18]. In early screening of new analogues of clozapine, oxidation potential was correlated with their potential for hematotoxicity. It was shown that only the compounds that did not easily oxidise could be promising antipsychotic candidates with low side effects [19].

Electroanalytical methods can be quite useful to directly measure the stability of certain pharmaceuticals in aqueous solution [20]. Depending on the compounds involved it can be used to monitor either the decomposition of a drug [21–24] or the formation of a decomposition product [25,26].

Electron transfer reactions have an important role in biochemical processes and hence in biotransformation of drugs [7]. Due to similarity in the electrochemical and biological reactions, it can be assumed that the oxidation/reduction mechanisms taking place at the electrode and in the body share similar principles [27]. In such cases, when a relationship between voltammetry and drugs exists, the knowledge of the mechanism of their electrode reactions can help us to elucidate the mechanism of their interaction with living cells [28] and their metabolic fate [29,30] in the human body after administration of the dosage form [31].

Using modern electroanalytical techniques, interactions of drugs with biologically important macromolecules can be studied [7]. The extent to which a drug is bound to plasma proteins may influence its distribution, onset of activity and rate of metabolism and excretion [20]. In pharmacology, the free drug concentration, as opposed to the total drug concentration, is suggested to reflect therapeutic efficacy. Banis et al. examined sensing performance of clozapine using fundamental electrochemical methods with respect to the impact of protein binding, showing lack of significant electrochemical activity resulting from bound clozapine in protein complexes [32]. The decay in the current of paracetamol, after the addition of

protein, also confirmed a decrease in free drug concentration and formation of electroinactive biocomplex [33]. Since the discovery of the electrochemical activity of nucleic acids, interaction of anticancer drugs and other DNA targeted molecules with DNA has been the focus of many electrochemical investigations [34]. Electrochemical approach can provide new insight into rational drug design and would lead to further understanding of the interaction mechanism between anticancer drugs and DNA [35–37]. The interaction mechanism can be investigated in three different ways, by using DNA modified electrode, drug-modified electrode and interaction in solution [38]. Moreover, DNA modified electrodes have recently been often used in developing novel electrochemical biosensors for selective drug determination [39–41].

Although there is an increase in utility of electrochemical methods in different parts of pharmaceutical field, most publications dealing with electrochemistry describe its use in solving analytically oriented pharmaceutical problems [9]. It can be used as an alternative or complementary technique to spectrophotometric and separation chromatographic techniques due to their many advantages [1,4]. Nevertheless, those methods are still commonly employed for the determination of the drug in bulk form, pharmaceutical formulations and biological fluids despite their many disadvantages, such as the need for derivatization, time-consuming extraction procedures, consumption of large solvent volumes, expensive instrumentation and running costs [27].

On the other hand, electroanalytical methods are a powerful and versatile analytical tool that offers high sensitivity, selectivity, accuracy and precision, often in spectacularly reproducible way [6] with relatively simple and inexpensive instrumentation [31] and rapid analysis time [4]. It only requires very small sample volumes, often in microliter range. For these reasons, electrochemistry is suited for the analysis of pharmaceuticals in biological samples for clinical investigations, where small volumes of blood or urine are analysed for low concentrations of drug products and metabolites [1]. Besides in biological fluids, electroanalytical methods are well suited for the determination of drugs in various samples, such as raw material and different pharmaceutical dosage forms, even those involving a complex matrix, due to their very large useful linear concentration range [31].

They are, of course, restricted to determination of compounds, which can undergo electrochemical oxidation or reduction in the available potential range, which depends on the structure of the analysed species [42]. Most of the pharmaceutical active compounds were

found to be as electrochemically active and readily oxidised or reduced in contrast to the excipients of pharmaceutical dosage forms [1]. The principal advantage is that electroanalysis of pharmaceuticals in their dosage forms or biological samples is usually not affected from the possible interferences depending on their oxidation or reduction potentials [1] and generally the separation and extraction procedure is not necessary [43].

They can also be used as electrochemical detectors as they can very well adapt to hydrodynamic conditions such as those encountered in liquid chromatography (LC), in capillary electrophoresis, or in flow injection analysis (FIA) applied to drug compound analysis [42]. Some of them are already implemented in European Pharmacopoeia (Ph. Eur.), such as potentiometry, voltammetry, conductometry or amperometric titration in the determination of water in pharmaceutically active compounds by Karl-Fischer titration [44].

The application of electrochemical techniques in the analysis of pharmaceuticals has greatly increased over the few years [31] and there are many examples demonstrating applicability of these methods in pharmaceutical and biomedical area, with only few selected ones presented here, seeing that the extent of this doctoral thesis makes it impossible to quote all papers dealing with various electrochemical, especially voltammetric determination of drugs [45–66]. Examples include mainly innovative electrode materials and techniques, similar to those used for the research in this doctoral thesis and described in the following chapters.

1.3. Voltammetry

Among the variety of electrochemical techniques available for drug analysis, voltammetry has become the most important and widespread technique [42]. The term voltammetry refers to a group of electroanalytical methods in which information about analyte is aquired by measuring current in an electrochemical cell as a function of applied potential [67]. A plot of potential vs. current is called a voltammogram. Analysis of voltammograms can yield analytical, kinetic and thermodynamic information as well as information on other phenomena such as adsorption on the electrode surface [42]. Some of the most important types of voltammetry, which are used for gaining both qualitative and quantitative information about investigated drugs in this doctoral thesis, are discussed in the following sections.

1.3.1. Cyclic voltammetry

In cyclic voltammetry (CV) the current response of a small stationary electrode in an unstirred solution is excited by a triangular voltage waveform [42], which is shown in Figure 1, a).

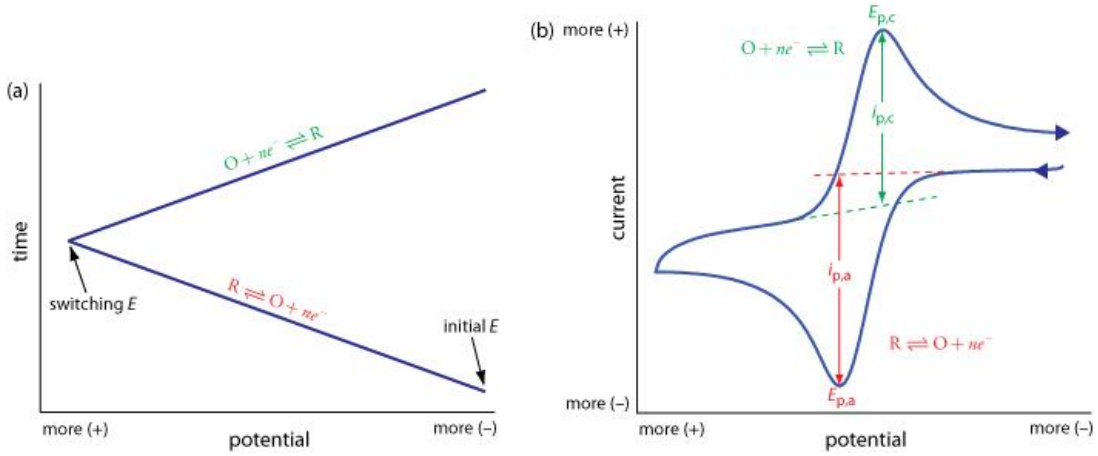

Figure 1. One cycle of the triangular potential-excitation signal (a). The resulting cyclic voltammogram (b). From [68].

The starting potential value is usually selected so that the chemical species under investigation is not initially oxidised or reduced. The potential is then swept to a voltage E_{max} or E_{min} (positive or negative sweep, respectively), at which point the scan direction is reversed back to the original or to a different potential value. The potential window is selected so that includes the oxidation or reduction process of interest. The current vs. applied potential curve is recorded over one sweep (linear sweep), one cycle or multiple cycles [42]. The rate of change of potential with time is referred to as the scan rate [1].

CV is the most widely used technique for acquiring qualitative information about electrochemical reactions [11] of new systems and has also proven very useful in obtaining information about fairly complicated electrode reactions particularly in the field of organic chemistry and pharmaceutical compounds [42]. It is perhaps the most versatile electroanalytical technique in pharmaceutical analysis [27] and often the first experiment performed in an electrochemical study [27,42]. The power of CV results from its ability to rapidly determine formal redox potentials of electroactive species, determine number of

electrons transferred and number of protons involved in redox reaction [9], detect chemical reactions that precede or follow electron transfer or evaluate electron transfer kinetics [11,27].

The important parameters in CV are the magnitudes of the peak currents, $I_{p,a}$ and $I_{p,c}$ and the potentials at which peaks occur, $E_{p,a}$ and $E_{p,c}$ (Figure 1, b)) that provide the basis for analysing the cyclic voltammetric response [11]. A redox couple in which both species rapidly exchange electrons with electrode, i.e. the potential-current profile which is governed solely by diffusion and by Nernst equation, is termed an electrochemically reversible couple. For a reversible couple, the anodic peak current, $I_{p,a}$, is given by the Randles-Sevcik equation (T = 25 ºC):

$$I_{p,a} = 2{,}69 \times 10^5 \, n^{3/2} A \, D^{1/2} \, v^{1/2} \, C$$

where $I_{p,a}$ is the anodic peak current (in amperes), v is the scan rate (V s^{-1}), n is the number of electrons transferred per species, A is the electrode area (cm^2), D is the diffusion coefficient of the electroactive species (cm^2s^{-1}) and C is the concentration of analyte in bulk solution (mol cm^{-3}). For a cathodic peak, $I_{p,c}$ is given by the same expression but with a negative sign [42].

Moving towards irreversible CV systems, when the kinetics has a successively greater effect in slowing the electron transfer, the current peaks become more widely separated. In the irreversible limit, no reverse reaction is observed, which happens with the oxidation of the most pharmaceutical compounds (38). For an irreversible system the peak current is:

$$I_p = 2{,}99 \times 10^5 \, n(\alpha n_a)^{1/2} A \, D^{1/2} \, v^{1/2} \, C$$

where n_a is the number of electrons in the rate-determining step of the electrode process and α is the charge transfer coefficient. The detection limit of CV is usually about 10^{-6} M and it is not sensitive enough to determine drugs in body fluids after therapeutic doses [43], but it can be very useful in optimising analytical conditions [3].

1.3.2. Pulse voltammetric techniques

Many limitations of CV, such as slowness and poor detection limits, were overcome by the development of pulse methods [67]. The potential step is the basis of various pulse techniques and sequence of such potential steps, each with a duration between 20 and 50 ms, is applied to the working electrode [11,42]. Due to the short pulse duration, great increase in sensitivity is achieved when compared to sweep or similar voltammetric methods. By

substantially increasing the ratio between the faradaic and non-faradaic currents compared to sweep techniques and increasing sensitivity, pulse techniques may permit a limit of quantitation at about 10^{-8} M concentration level, and, with correctly chosen parameters, the measured current consists almost solely of the faradaic current [42].

The difference between the various pulse voltammetric techniques is the excitation waveform and the current sampling regime [11]. After developing more sensitive pulse methods, the electroanalytical methods are more regularly used on the drug analysis in their dosage forms and especially in biological samples [1]. The most common types are staircase (SV), normal pulse (NPV), differential pulse (DPV) and square wave (SWV) voltammetric techniques, the most widely used being DPV and SWV [42].

1.3.2.1. Differential pulse voltammetry

Application of fixed-magnitude pulses superimposed on a linear potential waveform to the working electrode results in differential pulse voltammetric techniques [69]. The minimisation of charging current is achieved by sampling the current twice [1,42], before pulse application and at the end of the pulse (Figure 2, a)). The difference in current (ΔI) per pulse is recorded [67] as a function of the linearly increasing excitation voltage (Figure 2, b)).

a) b)

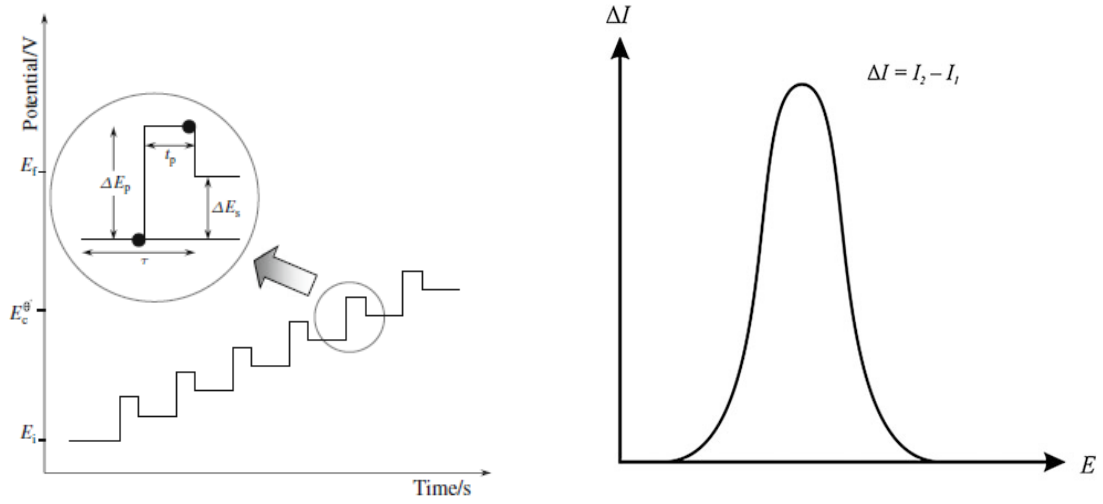

Figure 2. Potential program for differential pulse voltammetry (a). From [69]. Voltammetric profile of ΔI versus staircase potential in DPV (b). From [70].

A consequence of double sampling and representing ΔI against potential is that the DPV curves are peak-shaped and thus are well suited to analytical purposes [43]. Peak height is directly proportional to the concentration of electroactive species [42]. Because of the

derivative shape of differential pulse voltammograms, the resolution of two compounds with similar redox potentials is improved. Individual peak maxima without overlapping can be observed for substances with a small potential difference such as 40-50 mV. In contrast, normal-pulse voltammetry requires a potential difference of about 200 mV for resolving waves [67]. Due to its high sensitivity and the availability of low-cost instruments, DPV is often the choice for the determination of drug-active compounds in their dosage forms and in body fluids [42].

1.3.2.2. Square-wave voltammetry

SWV is a powerful electrochemical technique suitable for analytical application, mechanistic study of electrode processes and electrokinetic measurements. Nowadays it is considered as one of the most advanced voltammetric techniques, which unifies the advantages of pulse techniques (enhanced sensitivity), cyclic voltammetry (insight into the electrode mechanism) and impedance techniques (kinetic information of very fast electrode processes) [71]. In SWV, a waveform composed of a symmetrical square wave and superimposed on a base staircase potential is applied to the working electrode [11] (Figure 3, a)).

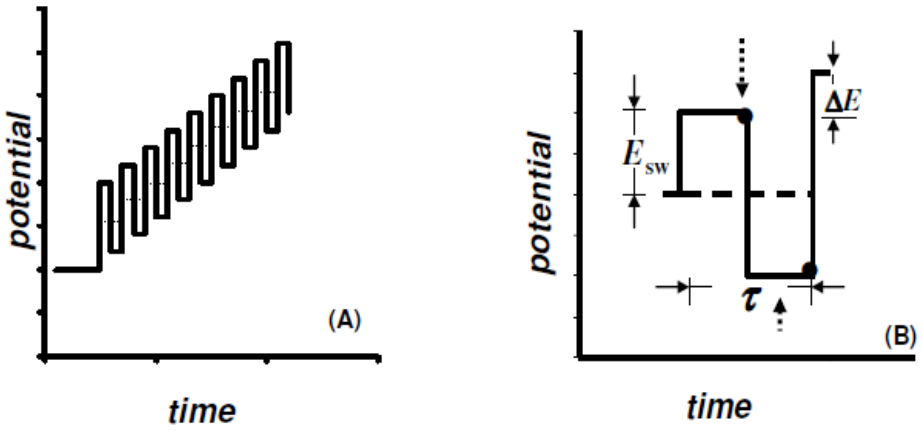

Figure 3. Potential waveform (a) and one potential cycle (b). From [72].

The main parameters of a potential cycle composed of two neighbouring pulses (Figure 3, b)) are the height of the pulses (reffered as the SW amplitude, E_{sw}), SW frequency (f, defined as $f = 1/\tau$, where τ is the duration of a potential cycle) [72] and height of the staircase waveform (potential increment, step potential, ΔE) [73].

The current is sampled twice during each square-wave cycle, once at the end of the forward pulse and once at the end of the reverse pulse (Figure 3, b)). The difference between the two measurements is plotted versus the base staircase potential [11]. Similarity with DPV lies in the fact that the current is sampled at two different times in the waveform [1] and that detrimental effects of charging current are reduced [42], however SWV has few advantages over DPV and other pulse techniques which is why it is employed more often than others in pharmaceutical analysis [1]. The main advantage of SWV is its speed. The analysis time is drastically reduced; a complete voltammogram can be recorded within a few seconds, where in DPV it takes about 2-3 minutes [11].

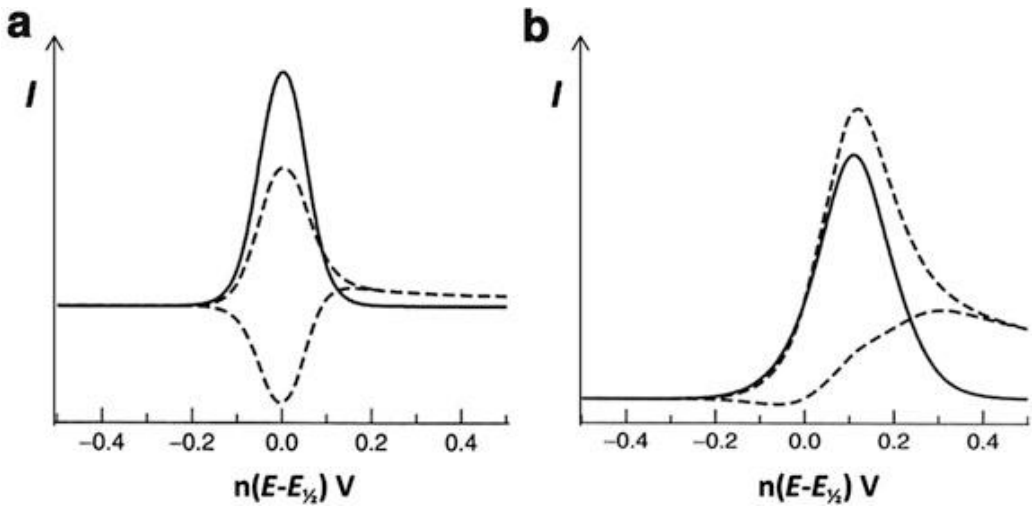

Figure 4. Square-wave voltammetry (SWV): (a) reversible and (b) irreversible reaction. From [42].

The values of the forward and reverse current are subtracted so that the plot of current difference vs. staircase potential yields a peak-shaped voltammogram [42]. The two components of the SWV response, the current response of the forward and reverse series of pulses, can be displayed as well as the net current (Figure 4). Excellent sensitivity arises from the fact that net current is larger than either the forward or reverse components (since it is the difference between them) which is why the peak height is usually quite easy to measure with greater accuracy than in DPV. The sensitivity is usually higher than that of DPV (in which the reverse current is not used) [11], but detection limits for SWV can also be similar to the detection limits for DPV (10^{-7} – 10^{-8} M) [42]. Due to the outstanding resolution of the voltammetric response, SWV is applied for simultaneous determination of compounds with close redox potentials. SWV method was applied to numerous drug active compounds

because of its sensitivity and rapidity and it is very useful for drug analysis in their dosage forms and biological samples because of the low LOD and LOQ values achieved with it [1].

1.3.2.3. Stripping voltammetry

Growing concerns about trace amounts of pharmaceutically active compounds and heavy metal combinations have led to an increase in the monitoring of their low amounts in a variety of matrices such as biological fluids or wastewater samples and environment [42,74]. In the analysis of such dilute samples, it is often necessary to employ some type of preconcentration step prior to the actual quantification. This happens when the analyte concentration is below the detection limit of the instrumental technique applied [43]. Stripping voltammetric (SV) forms a subdivision of voltammetry and constitutes one of the most important groups of electroanalytical techniques [1].

There are different types of SV, classified primarily by the nature of the preconcentration steps and scan direction of the stripping steps: anodic, cathodic, adsorptive (AdSV) or potentiometric stripping voltammetry [42]. Even though those techniques have their own unique features, they all have two steps in common [4]. First, the analyte is accumulated or deposited on the electrode surface at open curcuit or under controlled applied potential. During this accumulation step, the solution is usually stirred to increase solution flow and thus also increasing the deposition rate. After equilibration, if needed, and after stop stirring, the measurement (stripping) step is carried out [42]. Depending on the nature of analyte, different modes of stripping analysis are used, such as linear sweep, NPV, DPV SWV or potentiometric methods [1]. Pulse voltammetric waveforms are especially useful for the stripping step as they effectively correct for background current contributions [4]. Using DPV and SWV in the determination step, following the preconcentration, the detection limits can be lowered down to 10^{-10} – 10^{-12} M [42]. Because of that, SV represents an extremely sensitive electroanalytical technique [11] and can also increase in many cases the selectivity of the method as it is less sensitive to matrix effects from excipients (in drug dosage forms) or endogenous substances (in biological media) [27].

Numerous pharmaceuticals and biomolecules have a strong tendency to be adsorbed from solutions onto an electrode surface [9] so the application of AdSV in the determination of pharmaceuticals in biological fluids and drug dosage forms have been widely reviewed. The primary difference between AdSV and other stripping methods is that preconcentration step of the analyte is accomplished by adsorption on the electrode surface or by specific

reactions at chemically modified electrodes rather than accumulation by electrolysis. To achieve maximum sensitivity with AdSV, optimum conditions for maximum adsorption should be utilized during the accumulation step [4]. The main disadvantage of AdSV is interference from other substances in the sample solution. Such compounds are usually organic but can also be inorganic. Interference effects can often be minimised by applying the right accumulation potential, shorter accumulation time and appropriate electrolyte parameter (e.g. pH, buffer, ionic strength). AdSV techniques are well established and represent a large area with many possible applications in the analysis of pharmaceutically active compounds in their dosage forms or in biological media. AdSV with pulse techniques is highly selective, sensitive and precise for detecting ultra-trace amounts of pharmaceutically active compounds, important in research into drug design, bioavailability and safety [42].

1.4. Electrodes in voltammetry

Modern electroanalytical analyses are usually performed in a vessel containing a three electrode cell system [31], immersed in a solution containing the analyte and also an excess of a nonreactive electrolyte called a supporting electrolyte [67]. The performance of the voltammetric procedure is strongly influenced by the material of the working electrode [11]. As voltammetric methods continue to develop, the range of working electrode materials continues to expand [75]. A great challenge for high-quality electroanalytical determinations is reproducibility, a low background current, an easily renewable electrode surface and a fast electron transfer rate for target analyte [42].

Mercury used to be a very attractive choice of electrode material because it provides a clean, reproducible surface for each determination with every new drop [3]. It also has a high hydrogen overpotential that greatly extends the cathodic potential window (compared to solid electrode materials). Disadvantages of the use of mercury are its limited anodic range (due to the oxidation of mercury at low positive potentials) and its toxicity. The limited anodic potential range of mercury electrodes has precluded their utility for monitoring oxidizable compounds [11]. Moreover, at present time, in many countries the mercury electrodes are considered as undesirable due to the toxic character of mercury and related salts.

Accordingly, solid electrodes with extended potential windows have attracted considerable analytical interest [11]. Despite the difficulty of controlling their surfaces in a reproducible manner, solid electrodes have distinct advantages over the mercury electrode [76]. They have much larger positive potential range and are more mechanically stable and

environmentally friendly. The performance of an electroanalytical method depends on the geometry and material composition of the solid electrode. In the selection of the solid material, the redox behavior of the analyte, the nature of the supporting electrolyte, the pH and the potential region required for the measurement of the redox processes of electroactive drugs have to be considered [42]. Many different solid materials can be used as working electrodes. The most often used are carbon-based and metal electrodes [76].

1.4.1. Carbon-based electrodes

Carbon, in different forms, is the most widely used solid electrode material. The most popular carbon electrode materials are those involving glassy carbon (GC), carbon paste (CP), carbon fiber, screen-printed carbon strips (SPC), reticulated vitreous carbon (RVC), highly oriented pyrolytic graphite (HOPG), carbon films, pencil lead graphite, boron doped diamond (BDD), etc. [42]. Electrochemical reactions on carbon-based electrodes are often slower than at noble metal electrodes [11], the electron transfer kinetics being dependent on structure and surface preparation [11,42], but they have a wider useful potential range than metal electrodes in the negative and particularly so in the positive direction, due to slow kinetics of carbon oxidation [75].

The high degree of delocalisation of π electrons, together with weak van der Waals forces, provide good electrical conductivity and influence the electrical double layer, molecular adsorption and electrode polarity [76]. The high surface activity of carbon materials explains their susceptibility to electrode fouling by adsorption of organic compounds and especially by electroactive pharmaceutical compounds. Many pretreatment cleaning procedures have been developed, depending on the type of carbon, both renewing the surface and activating carbon-based electrodes [42], and play a role in increasing the π electron transfer rates [75]. The successful application of carbon electrodes in electroanalytical studies of electroactive pharmaceutical compounds is due to the high chemical and electrochemical stability of carbon materials, relatively high hydrogen and oxygen overpotential in different electrolytes, a broad working potential range and availability of different carbon materials [42], as well as their low background current, rich surface chemistry and low cost [11].

1.4.1.1. Glassy carbon electrode

Glassy carbon electrode (GCE) represents the most commonly used carbon electrode in electroanalysis since the 1980s. The structure of GCE consists of graphitic planes randomly organised as tetrahedral domains in a complex topology linked by short oxygen-containing

bridges, closely related to that of a vitreous material, with high lustre and glass-like fracture characteristics, hence the designations of glassy carbon, vitreous carbon or pyrolytic carbon [42]. GCE has remarkable physico-chemical properties, such as high strength, high thermal and electrical conductivity [76] and also high chemical inertness [11] making it probably the most inert carbon-based material [42]. It is mechanically polishable, extremely impermeable to gases and liquids and compatible with all common solvents [75]. It is well known that the GCE electrode possesses six-membered aromatic ring structures (sp^2 hybridized carbon) in which functional groups such as benzoquinone/ hydroquinone, phenolic and carboxylic acids are present [77]. These influence reactivity, and thence also chemical derivatisations that are possible on GCE [42]. The formation of these oxygen-containing functional groups is common for almost all carbon surfaces of sp^2 hybridized materials due to their reaction with oxygen and water [78]. This surface chemistry can be very complex and has to be considered during the study of the structure-function relationships at carbon-based electrodes [42].

1.5. Chemically modified electrodes

As far as solid electrodes are concerned, their perfection consists of the development of electrochemical sensors, searching for and using new electrode materials [76]. Chemically modified electrodes (CMEs) consist of conducting or semiconducting materials modified using different reagents with the objective of obtaining specific surface properties for electroanalytical applications [42]. For electroanalytical purposes, such deliberate alteration of electrode surfaces aims at improving sensitivity, selectivity and/or stability [79]. It also very often enables preferential accumulation of analyte or acceleration of electron transfer reactions [11]. The unmodified electrodes are called "bare", "native" or "virgin" electrodes [42] .

1.5.1. Carbon nanotubes-based electrochemical sensors

Nano-sized materials, also called nanostructured materials, have been shown to have a number of novel and interesting physical and chemical properties which led to rapid growth of using nanotechnology in the sensor fields [80]. Among them various families of novel carbon materials with unique properties and advanced electrochemical performances emerged and rapidly established themselves as reliable alternatives in electroanalysis due to their nano-sizes and/or special structures [81]. Electrochemical nanosensors have recently found extensive applications in pharmaceutical and biomedical industries with some great advantages such as lower detection limits, wider linear response range, sensitivity, good

stability and reproducibility when compared with other sensors and techniques. Carbon nanotubes (CNTs) represent an increasingly important group of nanomaterials and they have recently received enormous attention in the preparation of electrochemical sensors [80] and biosensors [82].

CNTs are built from sp^2 carbon units arranged in graphene sheets, which have been rolled up to form a seamless hollow tube [83]. Based on the number of layers, structures of CNTs are classified into two types (Figure 5): single-walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs) [84,85]. SWCNTs, as the name suggests, consist of a single hollow tube with diameters between 0.4 and 2 nm while MWCNTs are composed of multiple concentric nanotubes 0.38 nm apart where the final MWCNT has diameters of 2–100 nm [84]. At present, the three main methods employed for CNTs synthesis are arc-discharge, laser ablation, and chemical vapor deposition (CVD) [86].

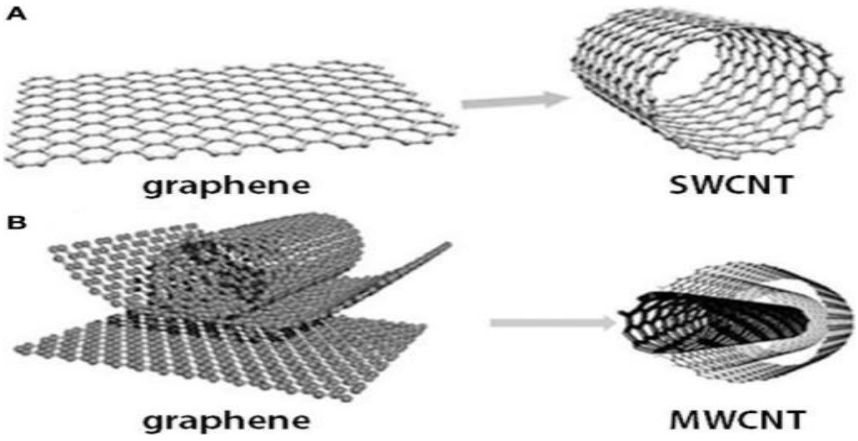

Figure 5. Rolling a sheet of graphene to form SWCNT (a) and MWCNT (b). From [87].

CNTs, a novel carbon form, has been exploited as an electrode material in electroanalysis as a result of its unique structure [83] and extraordinary electronic, chemical and mechanical properties. Their impressive structural, mechanical and electronic properties are due to their small size and mass, their incredible mechanical strength [84], high surface-to-volume ratio [88], chemical stability [89] and their high electrical and thermal conductivity [84]. The conductivity of the tubes, as a consequence of their electronic properties, is important for the role of these unique nanomaterials in electrochemistry. MWCNTs are regarded as metallic conductors, a highly attractive property for an electrode. The situation is

not so clear for SWCNTs which can be metallic or semiconducting depending on their chirality [81] and diameter [80].

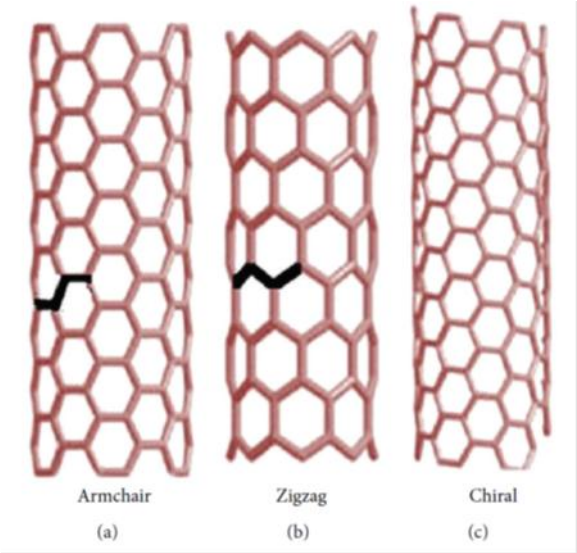

Figure 6. Carbon nanotube structures of armchair, zigzag and chiral configurations. From [84].

The chirality of SWCNTs relates to the angle at which the grafene sheets roll up and hence the alignment of the π-orbitals [80]. There are three distinct ways for the molecule to do the rolling, depending upon its direction: armchair, zigzag, and chiral (Figure 6.). Thus, metallic nanotubes are achiral and in arm-chair configuration, while chiral and achiral zigzag tubes are semi-conducting [84].

CNTs are have been proved to possess distinct electrochemical behaviour from other carbon-based electrode materials because of which they are intensively exploited in electrochemistry [90]. CNTs-based electrodes have been reported with various advantages, such as negligible surface fouling [81,90], increased heterogenous electron transfer (ET) rates [81] and consequently decreased over-potential [90], enhanced electroanalytical signals [91] shown as increased voltammetric currents [84] and also increased sensitivity and therefore lowered detection limits [90]. Analytical methods based on voltammetric stripping techniques have also benefited from the unique properties of CNTs-modified electrodes, mainly due to the stronger adsorption of many organic compounds on CNTs in comparison with conventional working electrode materials [91].

An important part of the impressive success of the use of CNTs for electroanalytical applications is probaby due to the ability of this nanomaterial to promote electron transfer in electrochemical reactions [91]. CNTs are closed structures that represent two well defined regions with clearly different properties, the tube and the cap (the tips and sidewalls), which is half-fullerene-like molecule with topological defects that in this case are mainly pentagons [92] (Figure 7).

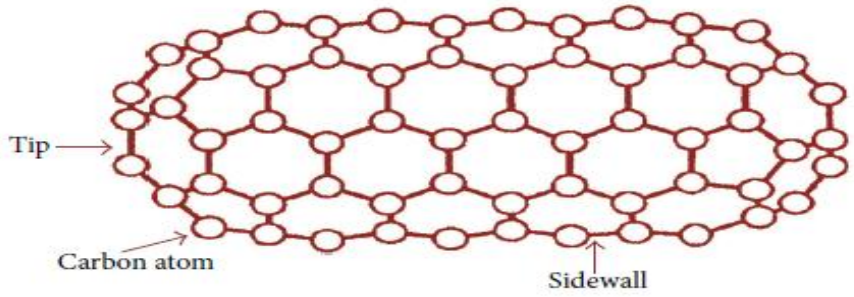

Figure 7. A carbon nanotube with closed ends. From [84].

The electrocatalytic effect of CNTs has been attributed to the activity of edge-plane-like graphite sites at the CNTs ends. MWCNTs have a sidewall structure similar to graphite basal plane of HOPG, and the open-end is similar to the edge plane of HOPG. Accordingly, electron transfer rates similar to a graphite edge-plane electrode can be expected [91]. The presence of pentagonal defects produce regions with charge density higher than those observed in the region of hexagonal graphite, either in planar or in tubular structures demonstrating the connection between topological defects and CNTs electroactivity. It was proposed by Compton's group that the reason for enhanced electrocatalytic activity of CNTs is due to the presence of edge-plane like sites located at the end and in the "defects" areas of the tubes [93].

It has been demonstrated that the electrocatalytic activity of MWCNTs strongly depended on the CNTs fabrication mode [91] and a higher electrochemical reactivity of CNTs was achieved when they were produced in CVD manner [94]. With carbon electrodes the rate of electron transfer has been shown many times to be very surface dependant hence the electrochemical activity is also influenced by the electrode preparation [90]. The protocols of pretreatment are typically based on the oxidation of CNTs under different conditions. Activation by treatment in acidic solutions has been widely used [92]. Modified electrodes with CNTs which were pretreated with concentrate acids in order to open nanotubes and to

create carboxylic groups (Figure 8), exhibited an intense catalytic activity towards the electrochemical oxidation [91].

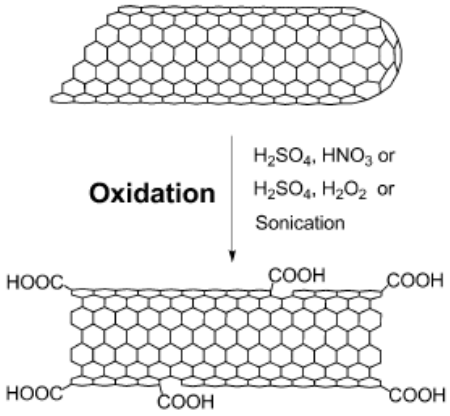

Figure 8. Functionalisation of CNTs with strong acids. From [95].

Besides improving the electron transfer properties, this type of acidic purification is also used for eliminating metallic impurities [92]. Most widely used method in commercial preparation of CNTs is CVD in the presence of metal catalysts [81] which can usually cause for CNTs to have metallic, nanographitic and amorphous carbon-based impurities. These impurities can dramatically influence the electrochemical properties of CNTs and influence the electrochemical responses of a large number of substances on CNTs-based electrodes [96].

Electrocatalytic activity of CNTs also depends on the dispersing agent used to immobilize them on the electrode surface [91], however one of the problems for the preparation of sensors based on the use of carbon nanotubes is to achieve a homogenous dispersion in usual solvents [92]. Before application to analytical assays, CNTs first must be modified to facilitate homogenous dispersion [97]. First step in the modification of CNTs with aim to improve their dispersion in various solvents has already been achieved upon purification of the nanotubes, mentioned above [80]. The ends of CNTs, being terminated in oxygenated species are quite hydrophilic but the walls, which comprise the vast majority of the tube, are highly hydrophobic. This hydrophobicity presents a major challenge when it comes to dispersing and manipulating CNTs to give controlled modification of electrode surface. In aqueous solution or in polar solvents tubes have a tendency to rapidly coagulate

(agglomerate). As a consequence, dispersing tubes is usually performed in non-polar organic solvents such as dimethylformamide (DMF) or with the aid of surfactants or polymers [92]. Using a polymer/CNTs composite is one way of improving the adhesion properties and the film reproducibility since CNTs poor adhesion to the electrode surface and the CNTs dispersion difficulty in most solvents results in the inconsistent electrocatalytic reaction at the electrode [90]. The preparation of hybrid composite materials, based on integration of CNTs with other materials possessing well-known electrochemical significance, has led to modified electrode surfaces that exhibit special properties due to the synergistic effect from the individual components. Materials used for such purposes mostly include polymers and metal nanoparticles, but composites with cyclodextrines, microparticles or more recently ionic liquids, have also been reported [91].

1.5.1.1. Carbon nanotubes/Nafion composite film

The polymer that has been often used as dispersing agent for CNTs is Nafion [90]. CNTs can be homogeneously dispersed in Nafion solution because of the hydrophobic side chains and polar head groups of Nafion. CNTs/Nafion composite thin film modified electrodes have their attractive effects in electroanalytical applications [98]. In general, Nafion is the most extensively investigated ion-exchange polymer used for preparing CMEs [20]. It is generated by copolymerization of a perflourinated vinyl ether comonomer with tetrafluoroethylene [99] (Figure 9). Perfluorinated sulfonated cation exchanger has been widely used as an electrode modifier due to its attractive permselective, ion-exchange and antifouling properties [11].

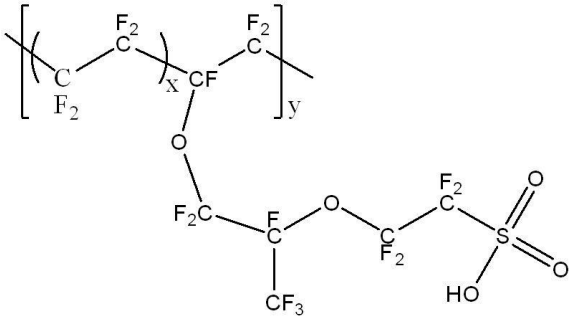

Figure 9. Chemical structure of Nafion. From [100].

Nafion is chemically and thermally inert, non-electroactive, conductive and insoluble in water and is, therefore, particularly suitable for the modification of electrodes [101].

Previously reported results have shown that Nafion membrane can enrich metal cations, improve voltammetric signal-to-noise ratios, and increase the electrode's resistance against fouling by surface-active compounds effectively [102]. The modification of GCE by Nafion is a quite simple and almost standardized way of enhancing electrode performances. After usual polishing procedures of the GCE electrode, the modification is typically obtained by dropping few microliters of alcoholic solution of Nafion onto the electrode surface, and by some subsequent drying procedure [103].

One important reason for the application of Nafion modified electrodes in electroanalytical chemistry is their ability to preconcentrate positively charged molecules, which increases the sensitivity of the method. The accumulation mechanism of Nafion can be explained through an electrostatic interaction due to the hydrophilic negatively charged sulfonate groups in the polymer structure, whereas its ionic selectivity for hydrophobic organic cations is achieved through hydrophobic interactions with the hydrophobic fluorocarbons of the film. These two factors give Nafion selectivity for cations, and especially high selectivity for hydrophobic organic cations [104].

In recent years, CNTs/Nafion-based electrodes have been increasingly used to solve demanding electrochemical problems and often offer advantages over other types of electrode materials [80]. The nano-hybridization by polymers is regarded as an effective technology for obtaining the stable colloidal dispersion while simultaneously preserving the inherent properties of carbon [105]. It is well known that homogeneous CNT dispersion and strong interfacial adhesion with the matrix are critical issues in the development of polymer/CNTs composites in order to attain improved properties. The combination of polymers with carbon nanomaterials for the preparation of chemical sensors is opening up exciting areas of research due to their biocompatibility, excellent sensitivity and selectivity [106].

1.5.1.2. Metal nanoparticles and carbon nanotubes-based nanohybrids

Nowadays, due to the fast development of nanotechnology, various nanomaterials, including carbon nanotubes, nanoparticles of metals and metal oxides, as well as their hybrids have been introduced to construct electrochemical sensors [107]. In recent years, metal nanoparticles (NPs) have attracted much more attention in electroanalysis because of their unusual physical and chemical properties [108]. These unique properties can often be advantageous in electroanalytical techniques [109] and can result in an enhanced mass

transport, high surface area and high electrocatalytic activity [110]. This is all owing to their nanosize and is not typical for the corresponding bulk material [111].

Generally, metal nanoparticles have excellent conductivity and catalytic properties, which make them suitable for enhancing the electron transfer rate [111] for compounds which have sluggish redox process at bare electrodes. The introduction of nanoparticles with catalytic properties into electrochemical sensors and biosensors can decrease overpotentials of many analytically important electrochemical reactions, and even realize the reversibility of some redox reactions, which are irreversible at common unmodified electrodes [108].

Incorporation of nanoparticles to CNTs electrode matrices has demonstrated to enhance the electrocatalytic efficiency of many electrochemical processes [91]. An interesting class of carbon nanotube derivatives results from deposition of metal nanoparticles on the tubular surfaces. Furthermore, uniform dispersion of metal nanoparticles decorated on CNTs surfaces can yield ideal nanocatalysts for application in chemically modified electrodes [110]. Recently, there have been a growing number of electrochemical sensors that incorporate different metal nanoparticles together with CNTs, solubilized in Nafion to form nanocomposites for electrochemical detection [91].

1.5.1.3. Other analytical applications of carbon nanotubes in pharmacy and medicine

Although CNTs application in voltammetric techniques is especially favorable and numerous publications in literature discuss about the use of CNTs as electrode materials or modifiers of conventional working electrodes in analytical voltammetry [86], CNTs have also contributed in different areas of drug analysis. In the field of analytical chemistry, nanomaterials and especially CNTs can be used as matrices in matrix-assisted laser desorption ionization (MALDI), as stationary phases in gas chromatography (GC), high performance liquid chromatography (HPLC) or capillary electrochromatography (CEC), as pseudostationary phases in capillary electrophoresis (CE) and as new solid-phase extraction (SPE) materials [112]. Due to their strong interaction with other molecules, particularly with those containing benzene rings, CNTs surfaces possess excellent adsorption ability. Nonfunctionalized or functionalized CNTs have been successfully employed in extraction techniques, such as SPE and solid phase micro-extraction (SPME) alone or in conjugation with classical SPE sorbents for the analytical extraction of drugs in different media such as biological fluids or drug preparations [84]. Due to their chirality they can be used also as chiral selectors, with or without further modification, for the enantioseparation of

pharmaceuticals [113]. The excellent electroanalytical capabilities of CNTs modified electrodes have also found important application in electrochemical detection systems coupled with separation techniques such as liquid chromatography (LC) and capillary electrophoresis [91]. Furthermore, CNTs have attracted great attention as suitable electrode materials to be applied for the construction of biosensing platforms for *in vivo* and *in vitro* diagnostics and therapeutic monitoring [89].

As a new and emerging nanomaterial, the use of CNTs as drug delivery carriers has brought great attention in recent years [114]. The main applications of CNTs in pharmacy and medicine include drug, biomolecule and gene delivery to cells or organs, tissue regeneration, and biosensor diagnostics and analysis. Many studies have demonstrated that when bonded to CNTs, these molecules are delivered more effectively and safely into cells than by traditional methods [84]. Moreover, CNTs have been recently revealed as a promising antioxidant. It was observed that the presence of –COOH groups would increase the free radical scavenging activity of SWCNTs and that carboxylated SWCNTs are at least as good as, or even better, free radical scavengers than their nonfunctionalized partners. Their antioxidant property has been used in anti-aging cosmetics and sunscreen creams to protect skin against free radicals formed by the body or by UV sunlight [84]. As with any new technology, there are concerns over the application of nanomedicine, stemming from the lack of proper knowledge and the need for the establishment of regulatory measures [114]. Identification of pharmacological and toxicological profiles is of critical importance for the use of nanoparticles as drug carriers in nanomedicine [115]. Despite a large number of studies performed in the past several years to explore the potential toxic effects of CNTs, the results are still often contradictory [116] .

2. Multi-walled carbon nanotubes/Nafion composite film modified electrode as a sensor for simultaneous determination of ondansetron and morphine

Talanta 122 (2014) 187–194

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Multi-walled carbon nanotubes/Nafion composite film modified electrode as a sensor for simultaneous determination of ondansetron and morphine

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ARTICLE INFO

Article history:
Received 19 November 2013
Received in revised form
17 January 2014
Accepted 22 January 2014
Available online 31 January 2014

Keywords:
Ondansetron
Morphine
Multi-walled carbon nanotubes
Nafion
Modified glassy carbon electrode

ABSTRACT

The electrochemical behavior of ondansetron was studied on the multi-walled carbon nanotubes/Nafion polymer composite modified glassy carbon electrode (MWCNTs–Nafion/GCE). The oxidation peak potential was shifted from 1.32 V to 1.18 V compared to the bare electrode indicating excellent electrocatalytic activity of immobilized film toward drug molecule. The modified electrode exhibited a remarkable enhancement effect on voltammetric response due to the synergistic effect of nanomaterial and cation-exchange polymer on the electron transfer rate, the effective electrode area and the accumulation capability. After optimizing the experimental parameters, adsorptive stripping procedure was used for the determination of ondansetron in pharmaceutical formulation. The results were satisfactory in comparison with those obtained by high-performance liquid chromatography. In addition, the MWCNTs–Nafion/GCE exhibited high selectivity in the voltammetric measurements of ondansetron and co-administrated drug morphine with potential difference of 430 mV. The response peak currents had linear relationship with drug concentration in the range of 1.0×10^{-7}–5.0×10^{-6} M and 1.0×10^{-7}–4.0×10^{-6} M with detection limits 3.1×10^{-8} and 3.2×10^{-8} M for ondansetron and morphine, respectively. The electrode was successfully applied for simultaneous electrochemical sensing of both drugs in human serum samples after selective accumulation at the electrode surface.

1. Introduction

Ondansetron (OND) is a selective 5-HT_3 receptor antagonist widely used in the treatment of emetogenic side effects accompanying cancer chemotherapy or radiotherapy (Scheme 1). It has also become the first line therapy for the prevention of post-operative nausea and vomiting [1]. In addition, OND is used to ameliorate pruritus, irritable bowel syndrome, diarrhea associated with cryptosporidiosis or diabetes, chronic refractory diarrhea, anxiety and sleep disorders, alcohol dependency, opiate withdrawal syndrome, vertigo, Tourette's syndrome and psychosis in advanced Parkinson's disease [2]. The studies on the use of OND in new indications continue and, therefore, the development of a sensitive analytical method to evaluate the quality of its pharmaceutical product or to determine drug concentration in biological fluids is highly required.

At present, several analytical methods have been employed for the determination of OND in biological samples or pharmaceutical preparations, such as HPLC combined with mass spectrometry [3–5] or UV detection [6–8], supercritical fluid chromatography/tandem mass spectrometry [9], HPTLC [10], spectrophotometry [11], radioimmunoassay [12] and capillary zone electrophoresis [13]. However, most of these methods are time-consuming, solvent-usage intensive, expensive and involve tedious sample preparations or, on the other hand, suffer from poor selectivity.

Morphine (MOR) is a widely used and highly effective analgesic agent for the treatment of acute and chronic pain associated with cancer, recommended by the World Health Organization. OND often coexists with MOR in biological fluids as these drugs are co-administered for control of pain, nausea and vomiting in patients undergoing surgical procedure and chemotherapy. To prevent overdose-induced toxicity, monitoring of the therapeutic levels of OND and MOR concentrations in biological samples of patients is a critical issue in clinical medicine. Therefore, it is necessary to establish simple, rapid and sensitive analytical methods for detection and quantitation of OND and MOR in biological matrix for clinical, toxicological and pharmacological studies. Recently, some new electrochemical methods have been proposed for morphine detection [14–19]. However, up to now, only one analytical method has been reported in the literature on the simultaneous determination of OND and MOR [20]. Two analytes were separated within 20 min and determined in

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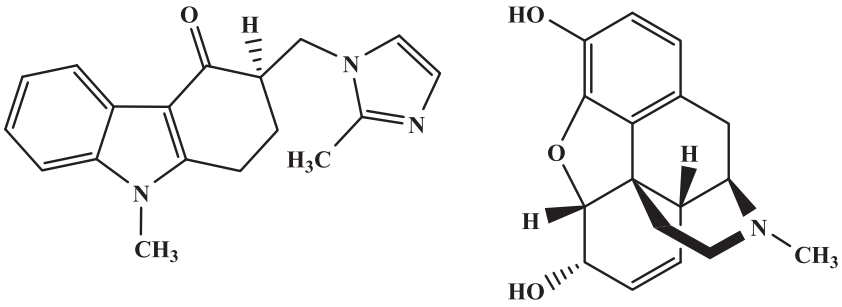
http://dx.doi.org/10.1016/j.talanta.2014.01.026



OND MOR

Scheme 1. Chemical structures of ondansetron (OND) and morphine (MOR).

pharmaceutical dosage form by HPLC method with UV detection. Consequently, a cheap, highly sensitive and selective method is of great demand for simultaneous determination of both drugs.

Electrochemical detection of an analyte is a very elegant method in pharmaceutical analysis due to its high sensitivity, rapid response, simple operations and low cost [21,22]. In spite of that, the redox behavior and electrochemical determination of OND in pharmaceutical formulation or human fluids have not been reported in the literature to this date. Our preliminary results showed that the voltammetric response of OND at the glassy carbon electrode (GCE) was not satisfactory because of slow heterogeneous electron transfer. In the light of these findings, it was a challenge to find a new electrode material for determination of OND using voltammetric technique.

In recent years, carbon nanotubes (CNTs) have attracted increasing attention in the electrode preparation due to their unique structure and extraordinary electronic properties [23]. CNTs have huge surface area, significant mechanical strength, high electrical conductivity and efficient electrocatalytic activity. On the other hand, Nafion has been extensively applied as an electrode modifier due to its excellent antifouling capacity, high permeability to cations and strong adsorption ability. The hydrophilic negatively charged sulfonate group in Nafion film enables selective preconcentration of positively charged drug molecules through electrostatic interaction, whereas, the hydrophobic fluorocarbon network of the polymer gives a selectivity for the hydrophobic part of the molecule [24].

Based on the above advantages of nanomaterial and cation-exchange polymer, a simple and sensitive electrochemical sensor was developed for simultaneous trace analysis of OND and MOR. To take advantage of the remarkable properties of MWCNTs in electrochemical sensing application, the MWCNTs should be properly functionalized and uniformly immobilized at electrode surface. However, the MWCNTs are inclined to form agglomerates due to strong Van der Waals interactions when they are dispersed in water or organic solvents [23]. To overcome this difficulty, Nafion was selected as the binder in electrode fabrication procedure. In the presence of Nafion, the MWCNTs were homogeneously dispersed into ethanol via ultrasonication. Consequently, a stable and uniform film was easily achieved at the electrode surface after organic solvent evaporation.

The electrochemical behavior of OND on the multi-walled carbon nanotubes/Nafion polymer composite modified glassy carbon electrode (MWCNTs–Nafion/GCE) strongly revealed selective interfacial accumulation of positively charged drug molecule and excellent electrocatalytic activity toward target, thus improving the sensitivity and voltammetric signal-to-noise ratios as well as the stability of the resulting film. The proposed method was applied for the quantification of OND in real drug samples. The ability of the modified electrode for voltammetric response of MOR was also evaluated. Finally, the MWCNTs–Nafion/GCE was successfully used as an electrochemical sensor for the simultaneous determination of OND and MOR at trace levels in biological samples.

2. Experimental

2.1. Apparatus

Voltammetric measurements were performed using a μ-Autolab potentiostat (Eco Chemie, Utrecht, The Netherlands) controlled by GPES 4.9 software. A conventional three-electrode system was employed, comprising a bare GCE (3-mm diameter, Metrohm, Switzerland) or MWCNTs/Nafion film modified GCE as a working electrode, a platinum wire as a counter electrode and an Ag/AgCl/3 M KCl (Metrohm) as the reference electrode. All electrochemical experiments were carried out at room temperature (23 ± 1 °C). When required, stirring was applied using a computer-controlled stirrer at ca. 300 rpm.

Scanning electron microscopy (SEM) measurement was performed on a Jeol JSM-7000 F microscope (Jeol Ltd., Tokyo, Japan). High-performance liquid chromatographic experiments were carried out using an Agilent 1100 Series LC system equipped with a diode array detector (Agilent Technologies, Waldbronn, Germany).

2.2. Chemicals

OND, kindly donated by Pliva (Zagreb, Croatia), was used as received without any further purification. Ondantor® (Sandoz) film-coated tablets, containing ondansetron hydrochloride dihydrate equivalent to 8 mg of OND, were supplied by local pharmacy. MOR was obtained from the Agency for Medicinal Products and Medical Devices (Zagreb, Croatia). Multi-wall carbon nanotubes ($>98\%$, O.D. 6–13 nm, length 2.5–20 μm) and Nafion (5 wt% solution in a mixture of lower aliphatic alcohols and water) were from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical grade quality. Ultra-pure water used for the preparation of standard solutions and buffers was obtained by a Milli-Q system (Millipore, Bradford, USA).

2.3. Preparation of MWCNTs/Nafion electrode

Before use, the received MWCNTs were sonicated in concentrated HNO_3 for 4 h to generate carboxylic acid-functionalized MWCNT surface. The suspension was filtered through a cellulose nitrate membrane with a 200 nm pore size. The solid powders were washed thoroughly with double distilled water to remove any residual acid and then dried under vacuum at room temperature. The immobilizing suspension was prepared by dispersing 1 mg of functionalized MWCNTs in 1 mL of 0.5% (m/v) Nafion solution prepared in ethanol. The mixture was then sonicated for

30 min to get a stable and well-distributed MWCNTs–Nafion suspension. Prior to modification, the GCE was polished with aqueous slurry of 0.05 μm alumina powder on a smooth polishing cloth, thoroughly rinsed with water and then ultrasonically cleaned in water for 30 s. Finally, the electrode was washed with purified water and dried. The cleaned GCE was uniformly coated with 5 μL of the MWCNTs–Nafion suspension and air-dried at room temperature. Then, the modified electrode was carefully rinsed with purified water. At the beginning of experiment, the modified electrode was scanned by two successive cyclic voltammetric sweeps between 0 and 1.4 V at 100 mV/s in a blank solution of 0.1 M H_2SO_4. The relative electrochemical surface areas of the MWCNTs–Nafion/GCE and bare GCE were determined by cyclic voltammetry (CV) between −0.2 and 0.7 V in 1×10^{-3} M ferricyanide solution as a redox probe in 0.1 M KCl electrolyte at different scan rates (v). From the slope of the plot of anodic peak current versus $v^{1/2}$, the electro active area was calculated. The modified MWCNTs–Nafion/GCE showed a surface area 5.2 times greater than the bare GCE. The MWCNTs–Nafion/GCE can be stored in air or kept in distilled water for several weeks without the loss of activity. For comparison, a Nafion modified GCE was prepared using the same procedure described previously, but without MWCNTs, and denoted as Nafion/GCE.

2.4. Electrochemical measurement procedures

Stock solution of OND (1×10^{-3} M) was prepared in purified water and stored under refrigeration. Stock solutions of MOR (1×10^{-3} M) were freshly prepared for daily use with purified water. The working standard solutions of OND and MOR were obtained by serial dilution of stock solutions with a supporting electrolyte just before voltammetric measurements. The studies were carried out in 0.1 M H_2SO_4 solution and Britton–Robinson buffer (0.04 M in each of acetic, phosphoric and boric acids) adjusted to the desired pH with 0.2 M sodium hydroxide solution. The oxidative behavior of OND was investigated by CV in the scan range from 0.0 V to 1.5 V. Square-wave voltammetry (SWV) was used for the determination procedures of OND and MOR. The sensor was immersed in the sample solution and preconcentration of both analytes was carried out at potential −0.5 V with a 360 s accumulation step. After 5 s equilibrium period, the voltammogram was recorded in the SWV mode (frequency, 75 Hz; potential step, 8 mV; amplitude, 25 mV) from 0.7 to 1.4 V. Following each measurement, the MWCNTs–Nafion/GCE was transferred to the solution of supporting electrolyte to regenerate the electrode surface by applying single positive-going SWV potential scan from 0.7 to 1.4 V.

2.5. Pharmaceutical dosage form assay procedure

To prepare the solutions of OND commercial pharmaceutical product, ten tablets were weighted and crushed to a fine powder. An accurately weighted powdered sample of the drug formulation equivalent to 3.6 mg of active ingredient was transferred into a 10.0 mL calibrated flask and dispersed in water. The tablet solution was sonicated for 15 min to provide complete dissolution of the active ingredient. After sonification, the sample was filtered through 0.45 μm Acrodisc GHP filters (Gelman, Ann Arbor, USA). An aliquot of filtrate was then transferred into a calibrated flask and diluted with water to yield a final drug concentration of 1.0×10^{-4} M. A series of dilutions were made with the supporting electrolyte to cover the working concentration range. Solutions were subjected to voltammetric measurements as previously described and the content of OND in the pharmaceutical preparations was determined by the standard addition method. For recovery studies, aliquots of the OND standard solutions were added to real samples prepared from tablets.

For the comparison study, HPLC experiments were carried out using a chromatographic column Zorbax C18, 250 × 4.1 mm, particle size 5 μm (Agilent Technologies, Waldbronn, Germany). The mobile phase, consisted of acetonitrile, ultra-pure water and tetraethylammonium hydroxide at the ratio of 50:50:1 (v/v/v), was filtered through cellulose nitrate filter (0.45 μm, Sartorius, Goettingen, Germany). The HPLC analyses were carried out at constant temperature (25 °C) with a flow-rate of 0.7 mL/min under isocratic conditions. For chromatographic analysis, standard solutions of OND were made by dilution with the mobile phase and filtered through a 0.2 μm Acrodisc GHP filters (Gelman, Ann Arbor, USA). The 10 μL aliquots were injected into the HPLC system for analysis. The DAD detector recorded UV spectra in the range from 190 to 400 nm and chromatogram was obtained at 248 nm. All chromatographic data acquisition and processing was performed using ChemStation software (Agilent Technologies, Waldbronn, Germany).

2.6. Determination of OND and MOR in biological samples

Human serum samples were obtained from healthy volunteers abstained from any medications during the week preceding the study and were stored frozen until assay. Serum samples were fortified with the appropriate aliquot volume of OND and MOR standard solutions to achieve final concentrations (5.0×10^{-7} and 1.0×10^{-7} M) that are found in serum after the treatment with therapeutic daily doses of 16 and 80 mg for OND and MOR, respectively [25,26]. An aliquot of serum sample containing OND and MOR was mixed with acetonitrile (1:1) to remove serum proteins effectively. After vortexing for 60 s, the mixture was then centrifuged for 6 min at 6000 rpm. Appropriate volumes of this supernatant were transferred into the volumetric flask and diluted with the supporting electrolyte and analyzed in the voltammetric cell. Quantitations in biological fluids were performed using the standard addition method by adding three successive aliquots of drug standard solutions.

3. Results and discussion

3.1. Electrochemical response of OND at GCE

In this paper, the redox behavior of OND was studied for the first time and initially, we started with the investigation of the electrochemical oxidation of drug molecule at a bare GCE by cyclic voltammetry. A poorly defined anodic current response was observed during the scanning from 0 to 1.5 V (Fig. 1, curve a). On the reverse potential scan, there was no corresponding reduction peak, suggesting that the electrode reaction of OND is totally irreversible. The voltammetric response was broad due to slow electron transfer. In addition, the peak potential of OND obtained at GCE had a relatively high value, close to +1.32 V in 0.1 M H_2SO_4 solution, which resulted in the merging of the signal current with the background current. Therefore, the effect of pH on the electrochemical response of OND was studied over the pH range of 2.0–9.0 in BR buffer. However, the peak potential of the oxidation process remained almost constant in the pH interval 2.0–7.0 (E_p (V) = 1.37–0.009 pH). As the pH value was increased above 7, the oxidation peak shifted to less positive potentials, but it was split into two broad and overlapped waves, together with a decrease in peak currents.

The effect of scan rate on the oxidative peak currents was also evaluated. Variation of the scan rate in the range of 20–500 mV/s resulted in a linear relationship between the anodic peak current

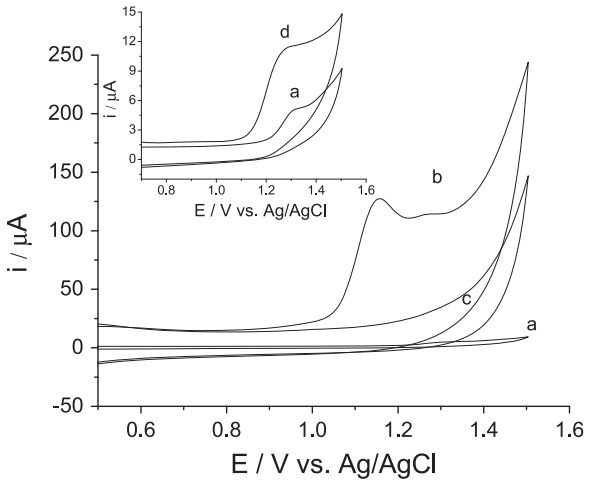


Fig. 1. Cyclic voltammograms of ondansetron (1×10^{-4} M) at a bare GCE (a) and the MWCNTs–Nafion/GCE (b) together with corresponding background recording (c) in 0.1 M H_2SO_4. Inset: cyclic voltammograms of ondansetron at a bare GCE (a) and the Nafion/GCE (d). Scan rate: 100 mV/s.

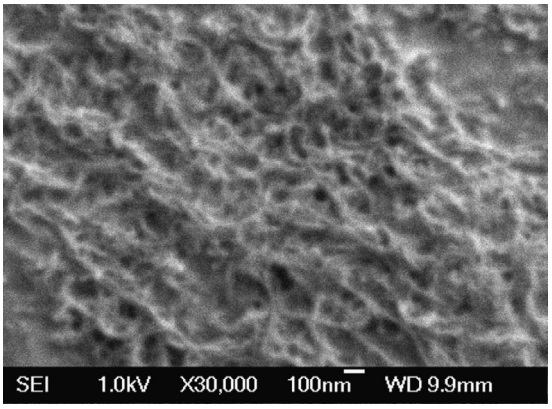


Fig. 2. The surface image of MWCNTs–Nafion/GCE produced by scanning electron microscopy. Scale bar: 100 nm.

and the square-root of a scan rate. This indicates that the electrode process was controlled by diffusion rather than adsorption. Additionally, a plot of logarithm of peak current versus logarithm of scan rate gave a straight line $\log i=0.456\log \nu$ –0.326 ($r=0.993$) with a slope very close to the theoretical value of 0.5, an expected value for an ideal reaction of solution species [27]. Therefore, the preliminary results clearly revealed that the voltammetric response of OND at bare GCE was not satisfactory for the analytical application due to slow electron transfer reaction, reduced sensitivity since the adsorption of drug molecule could not be purposely used as a preconcentration step, electrode fouling problems and unstable analytical signal.

3.2. Electrochemical behavior of OND on modified electrode

To improve the electrochemical response of OND, the electrode was modified with nanomaterial and cation-exchange polymer. The surface morphology of MWCNTs–Nafion/GCE was characterized by SEM. As shown in Fig. 2, the MWCNTs were well dispersed in the Nafion and entrapped in the polymer matrix. The nanocomposite film deposited at the surface of the GCE formed a three-dimensional structure and reticular formation revealing a much larger surface area than electrode geometric area.

The cyclic voltammograms of 1.0×10^{-4} M drug solution in 0.1 M H_2SO_4 obtained at the MWCNTs–Nafion/GCE, Nafion/GCE and a bare GCE with a scan rate of 100 mV/s are presented in Fig. 1. When the bare GCE surface was coated with 0.5% Nafion film, a well-shaped oxidation wave ($i_p=9.6$ μA) appeared at 1.29 V (inset of Fig. 1). Nafion as a cation-exchanger attracted positively charged drug molecules from the bulk solution to enhance the anodic signal. However, the electroanalytical performance of the MWCNTs–Nafion /GCE for measuring OND was advantageous over that observed at the Nafion/GCE and bare GCE. Compared with Nafion/GCE, the voltammetric response of OND intensively increased at MWCNTs–Nafion/GCE with a current of 30.5 μA and a peak potential of 1.18 V. The oxidation peak potential of OND was shifted to less positive values by 140 mV compared with that of using a bare GCE indicating faster electron transfer reaction at the electrode surface and excellent electrocatalytic activity of immobilized film toward drug molecule. The experimental results clearly indicated a remarkable enhancement effect on the electrochemical response of OND obtained from the combined contribution of Nafion and CNTs. This is due to the larger surface area of the modified electrode as well as the synergistic effect of the electronic conductivity and electrocatalytic activity of MWCNTs with ionic conductivity and ion-exchange capacity of Nafion. OND is a weak basic compound with pK_a 7.4 and therefore, the molecule is completely protonated in the experimental conditions under which voltammetric measurements were performed. It is evident that Nafion enhances the preconcentration of drug molecules through electrostatic interaction with negatively charged sulfonate group in Nafion polymer structure, leading to a considerable improvement in the analytical sensitivity. Another effect of Nafion observed here was fixing CNTs onto GCE surface tightly and enhancing the stability of the electrochemical sensor. On the other hand, the sensor exhibited a higher sensitivity for OND determination owing to the excellent properties of MWCNTs, thus indicating an improvement in the electrode kinetics and a decrease in the oxidation potential substantially. The OND oxidation observed at the high potential using GCE was more facile at the MWCNTs–Nafion/GCE due to the shift in peak potentials to less positive values, thus placing the electrochemical oxidation outside of the solvent window.

For evaluating the reaction character of OND at the MWCNTs–Nafion/GCE, cyclic voltammetry was performed in a 5.0×10^{-5} M OND solution with different scan rates. The peak potential was positively shifted from 1.116 to 1.241 V when increasing the scan rates from 20 to 500 mV/s. The dependence of the peak potential is linear with logarithm of the scan rate, which could be expressed as a regression equation: E_p (V)$=0.0927\log \nu$ (mV/s)$+0.989$ ($r=0.996$), allowing the calculation of $\alpha n=0.638$ (inset of Fig. 3). Using the value of the charge transfer coefficient (0.66) obtained from the difference between the peak potential (E_p) and the half wave potential ($E_{p/2}$), taking the equation $\alpha=(47.7/E_p-E_{p/2})$ [28], the number of electrons exchanged was calculated to be $n=0.96$. The voltammetric response of OND at the modified electrode is probably initiated by one-electron transfer to form the cation radical at nitrogen atom in the imidazole ring. On the other hand, peak currents were linearly increased with the scan rates from 20 to 200 mV/s, and their relationship obeyed the following regression equation i_p (μA)$=0.227\,\nu$ (mV/s)$+7.838$ ($r=0.997$). This result indicated that the electrochemical reaction of OND at the MWCNTs–Nafion/GCE was a surface-controlled process [27]. Therefore, to obtain the highest sensitivity of the method, the adsorption of OND at the modified electrode surface was purposely used in subsequent analytical measurements as an effective accumulation step prior to the voltammetric quantitation of the drug.

3.3. Selection of the experimental conditions

The electrochemical oxidation of OND was examined in different electrolyte solutions using CV. Various types of the supporting

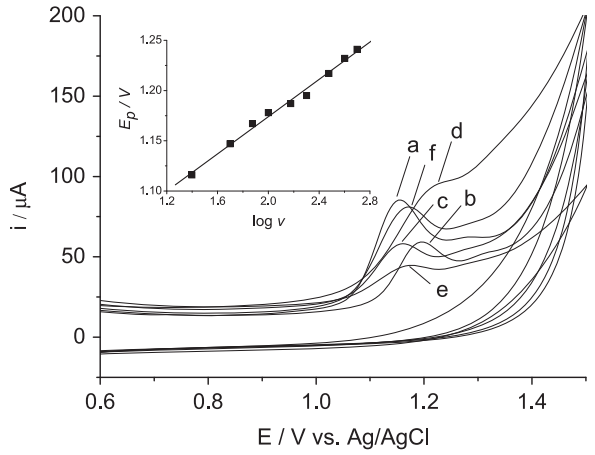


Fig. 3. Influence of supporting electrolyte on ondansetron oxidation at the MWCNTs–Nafion/GCE: 0.1 M H_2SO_4 (a), 0.5 M H_2SO_4 (b), Britton–Robinson buffer pH 2 (c) and pH 4 (d), 0.1 M CH_3COOH (e) and 0.01 M HCl (f). Scan rate: 100 mV/s; $c = 1 \times 10^{-5}$ M. Inset depicts peak potential vs. logarithm of scan rates from 20 to 500 mV/s in 0.1 M H_2SO_4.

electrolytes, including BR buffer pH 2.0–6.0, H_2SO_4, HCl and CH_3COOH, were tested for the electroanalytical measurements, as shown in Fig. 3. The comparison of voltammograms showed that only in lower pH values OND could exhibit higher peak current. The drug molecule is completely protonated on the nitrogen atom in the imidazole ring and could be more effectively attracted to the modified electrode surface. A lower peak potential and highest peak current was achieved in 0.1 M H_2SO_4 and, therefore, this solution was selected as the suitable supporting electrolyte for the drug determination using the MWCNTs–Nafion/GCE.

To improve the sensitivity, SWV was employed for measuring OND due to the most favorable response compared to other voltammetric modes such as differential pulse voltammetry and linear-sweep voltammetry. Variation of the peak current for OND recorded at the MWCNTs–Nafion/GCE was monitored while changing instrumental parameters, i.e. frequency (25 Hz $\leq f \leq$ 100 Hz), pulse amplitude (5 mV $\leq a \leq$ 50 mV) and potential step (2 mV $\leq \Delta E_s \leq$ 10 mV). The most favorable response was achieved using a combination of a frequency of 75 Hz, a pulse amplitude of 25 mV and a potential step of 8 mV.

The thickness of the cast film on the GCE surface, which was determined by the amount of MWCNTs–Nafion suspension, had certain effects on the current response of OND. The volume of MWCNTs–Nafion suspension coated on the electrode changed the properties and functions of the electrode surface. When the volume of MWCNTs–Nafion suspension added to the GCE surface increased from 3 to 5 μL, the oxidation peak current enhanced notably, but with further increase of the amount of MWCNTs–Nafion suspension to 9 μL, the oxidation peak current conversely showed gradual decline. The Nafion film became thicker and lowered the electrical conductivity of MWNTs and, consequently, retarded the electron transfer rate of OND oxidation as well as the mass transportation between the drug and the electrode resulting in the higher electric resistance. In the beginning, on increasing the amount of the modifier, the sites of electron exchange increased and the adsorption of OND was enhanced. However, the accumulation capacities of the film depend on the amount of Nafion, leading to an increment of the ion-exchange capacity of the film up to a certain limit, which is determined by the nature of the analyte and its diffusion through the film. The voltammetric signal increased up to 0.5% of Nafion concentration in the immobilizing suspension. The SWV response of OND reached the maximum when the composite film was uniformly coated on the electrode surface using 5 μL of homogenous MWCNTs–Nafion suspension.

3.4. Adsorption characteristics of OND on modified electrode

Since the modification of the electrode surface with a MWNTs–Nafion layer led to a strong interfacial accumulation of OND, the possibility of the analyte preconcentration before voltammetric measurement was investigated in order to obtain lower detection limits. Fig. 4 shows square-wave voltammograms for 2.5×10^{-6} M solutions of OND without accumulation and after a 360 s accumulation step at the MWCNTs–Nafion/GCE. To achieve the maximum sensitivity and the optimum conditions for the maximum adsorption, the influence of accumulation potential on the stripping peak current was evaluated from −1.0 to 0.9 V using SWV mode. When the accumulation potential shifted from 0 to 0.9 V, the stripping peak current greatly decreased. The results showed that the oxidation peak current increased as the accumulation potential became more negative. The optimum deposition potential of −0.5 V was selected for subsequent sensitive determination of OND. Square-wave voltammograms with increasing accumulation times (between 30 s and 480 s) were recorded for solutions containing OND at concentration level of 1×10^{-6} M. The accumulation time significantly affected the voltammetric response of OND at the MWCNTs–Nafion/GCE. The oxidation peak current enhanced greatly with the increase of the accumulation time within 360 s (Fig. 5). The linear relationship between the voltammetric response and the deposition time pointed to a constant adsorption of positively charged drug molecules attracted on the surface of the MWCNTs–Nafion/GCE. Further increment of accumulation time period showed a deviation of the peak current from linearity, thus indicating surface adsorption saturation. Therefore, the value of 360 s was considered as optimum to achieve the highest possible sensitivity in acceptable analysis time.

3.5. Simultaneous determination of OND and MOR

To develop a new electroanalytical method for simultaneous determination of OND and MOR, the voltammetric response of MOR was also studied at the MWCNTs–Nafion/GCE. Fig. 6 shows the SWV responses from the electrochemical oxidation of OND and MOR in 0.1 M H_2SO_4 solution following preconcentration for 360 s at −0.5 V. The proposed sensor exhibited potent electron mediating behavior followed by well-separated oxidation peaks towards OND and MOR at 1.17 and 0.74 V, respectively. The large

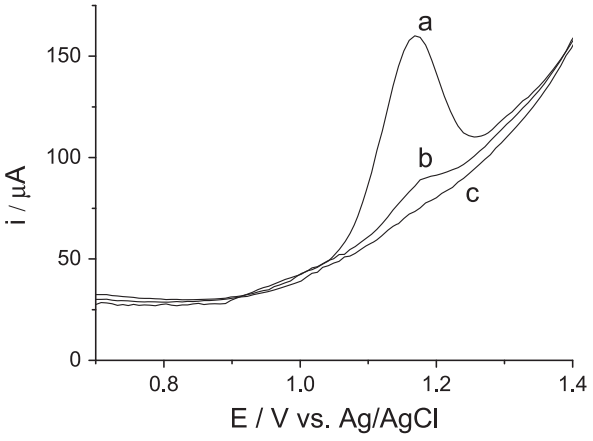


Fig. 4. Square-wave voltammograms of ondansetron (2.5×10^{-6} M) at the MWCNTs–Nafion/GCE in 0.1 M H_2SO_4 after accumulation time of 360 s (a), without accumulation (b) and blank solution (c). SWV settings: frequency of 75 Hz, amplitude of 25 mV and potential step of 8 mV, $E_{acc} = -0.5$ V.

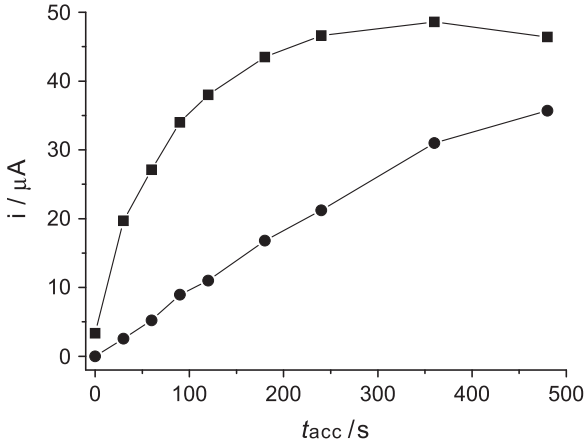


Fig. 5. Effect of the accumulation time on the oxidation peak current for ondansetron (1×10^{-6} M) (circles) and morphine (1×10^{-6} M) (squares). SWV settings and accumulation conditions same as in Fig. 4.

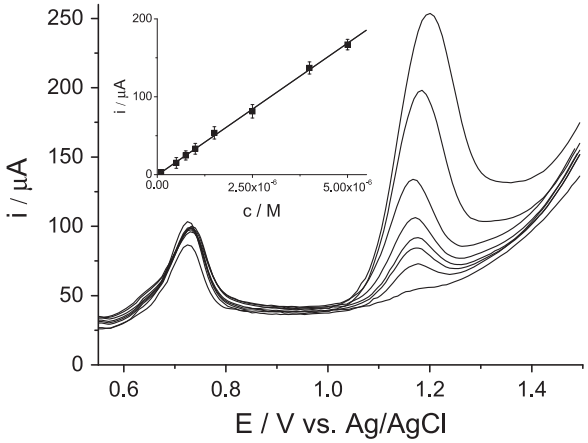


Fig. 6. Adsorptive stripping square-wave voltammograms of ondansetron at the MWCNTs–Nafion/GCE recorded in 0.1 M H_2SO_4 at different concentrations (1×10^{-7}–5×10^{-6} M) in the presence of morphine (1.5×10^{-6} M). SWV settings and accumulation conditions same as in Fig. 4. Inset shows a calibration graph for the quantification of ondansetron.

separation of the peak potentials allows simultaneous determination of OND and MOR in their mixture.

On the basis of previous investigation of MOR oxidation mechanism using GCE, the anodic voltammetric signal of MOR at the MWCNTs–Nafion/GCE can be ascribed to oxidation of phenolic group which involves one electron transfer [29]. The tertiary amine of MOR has a pK_a of 8.4, and the phenolic group has a pK_a of 9.4. Therefore, MOR was also completely in the cationic form under used voltammetric condition and MOR molecule, like OND, showed adsorption at the MWCNTs–Nafion/GCE. As shown in Fig. 5, the accumulation time of 120 s provided for MOR the largest peak current in the linearity range indicating faster adsorption of MOR then OND on the surface of the modified electrode. However, the value of 360 s was used as the optimum for simultaneous quantification of both drugs because the peak current of MOR increased up to a maximum at $t_{acc}=360$ s which was selected previously as the optimal value for OND.

The oxidation peak of OND at 1.17 V in 0.1 M H_2SO_4 solution after 360-second accumulation time showed a linear response at the MWCNTs–Nafion/GCE for concentration in the range of 1.0×10^{-7}–5.0×10^{-6} M. The calibration plot is described by the following regression curve: i_p (μA)$=3.38\times10^7 c$ (M)–0.43, $r=$ 0.999. Since the presence of MOR may affect the quantification of OND due to its competitive adsorption, the variation of adsorptive stripping SWV peak current versus OND concentration in the presence of MOR (1.5×10^{-6} M) was also checked using MWCNTs–Nafion/GCE (Fig. 6). The sensitivity of the modified electrode towards the oxidation of OND in the presence of MOR is 3.27×10^7 μA M^{-1}, which is very close to the value observed in the absence of MOR (3.38×10^7 μA M^{-1}), indicating that the oxidation of OND and MOR at the MWCNTs–Nafion/GCE, as well as their adsorption at electrode surface in the accumulation step, are independent. The detection limit (LOD) and the quantification limit (LOQ) estimated from the calibration curve as LOD$=3s/b$ and LOQ$=10s/b$, where s is the standard deviation of the intercept and b is the slope of the calibration curve, were calculated to be 3.1×10^{-8} M for LOD and 9.6×10^{-8} M for LOQ. Compared with other methods, the linearity range obtained at proposed electrochemical sensor for OND was in the similar concentration range as for HPLC methods with UV detection and HPTLC [6,8,10]. The LOD and LOQ values are better than those obtained in capillary electrophoresis and spectrophotometry [11,13], but are higher than LODs reported for chromatographic methods coupled to mass spectrometry [3–5]. However, the LC–MS analytical procedures demand expensive and sophisticated equipment that is not available in many laboratories. The proposed voltammetric method using the MWCNTs–Nafion/GCE offers several advantages over chromatographic techniques applied only to the quantitative determination of a drug, including short analysis time, simplicity of operation and lower running cost. It should be mentioned once again that in the literature there are no studies on electrochemical behavior of OND or its voltammetric determination.

Fig. 7 shows the square-wave voltammograms for the solutions containing various concentrations of MOR and constant concentration of OND (1.5×10^{-6} M). It can be seen that the related peak currents increased by increasing MOR concentration, while there are no significant changes in the peak current and potential of OND. The calibration graph obtained for anodic peak current of MOR at the MWCNTs–Nafion/GCE was linear within the concentration range of 1.0×10^{-7}–4.0×10^{-6} M (inset of Fig. 7). The regression equation was expressed as i_p (μA)$=2.90\times10^7 c$ (M)$+$ 11.99 with a correlation coefficient of 0.999. The calculated LOD and LOQ values were found to be 3.2×10^{-8} and 9.8×10^{-8} M, respectively.

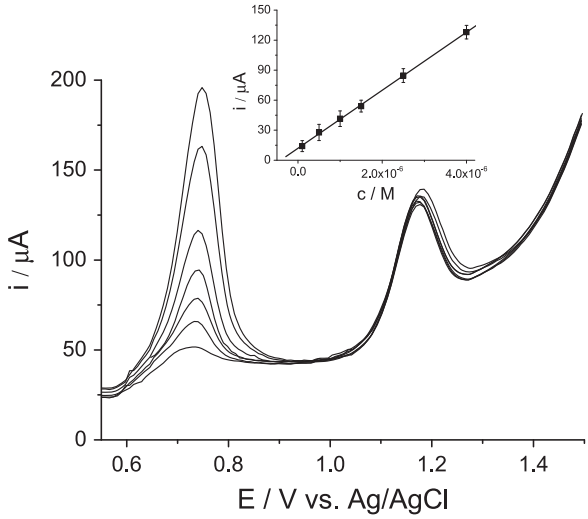


Fig. 7. Adsorptive stripping square-wave voltammograms of the mixture containing morphine at different concentrations (1×10^{-7}–5×10^{-6} M) and ondansetron (1.5×10^{-6} M) recorded at the MWCNTs–Nafion/GCE in 0.1 M H_2SO_4. SWV settings and accumulation conditions same as in Fig. 4. Inset shows a calibration graph for morphine.

In comparison to HPLC–UV procedure developed for the simultaneous quantification of MOR and OND mixtures in 0.9% sodium chloride injection, the MWCNTs–Nafion/GCE showed wider linear range for the quantification of both drugs [20]. The detection and quantification limits of OND and MOR obtained at this sensor are of the same order as for the HPLC method, but the proposed electroanalytical method is simpler, cheaper and faster. In HPLC method the separation time of 20 min was necessary for the analysis. In addition, the HPLC method requires a high percentage of organic solvent making it less environmental friendly.

3.6. Stability and reproducibility of the modified electrode

The stability of the MWCNTs–Nafion/GCE was checked by measuring the adsorptive stripping SWV response in 2.5×10^{-6} M OND and MOR solution over a period of four weeks. Before measurements, the modified electrode was scanned between 0.5 and 1.4 V in the solution of supporting electrolyte until the SWV response was stable. When the nanocomposite modified electrode was stored in the air at room temperature for 10 days, the peak potential of both drugs was unchanged and the current response of OND and MOR decreased by about 2.6% and 3.2%, respectively. The high mechanical strength of the MWCNTs-interspersed in the polymer film could be the reason for the high stability of the proposed electrochemical sensor. Repeating the experiment after a longer time period, it was found that the current responses decreased about 15% in three weeks for both drugs.

To evaluate the repeatability of the electrode response, the same modified electrode was used for six successive measurements of 1.0×10^{-6} M OND and MOR solution. After each measurement, the surface of the MWCNTs–Nafion/GCE was regenerated by applying single positive-going SWV potential scan from 0.5 to 1.5 V in a blank solution of 0.1 M H_2SO_4. The recorded oxidation peak potentials for OND (mean $E_p=1.17$ V) and MOR (mean $E_p=0.74$ V) were unchanged. The RSD values of the peak current for OND (mean $i_p=35.4$ μA) and MOR (mean $i_p=36.9$ μA) were 1.9% and 1.2%, respectively. These results indicated that the MWCNTs–Nafion/GCE was stable in repeated measurements for selective determination of OND and MOR compounds. The reproducibility of the modified electrode was characterized by three replicate measurements of 1×10^{-6} M OND and MOR over three days using freshly prepared standard solutions. The RSDs of the anodic peak current for OND and MOR were not greater than 2.5% and 1.7%, respectively. Additionally, a series of five sensors prepared repeatedly in the same manner were also tested and the RSDs of peak potentials and currents for both drugs were in the range 0.5–0.7% and 2.5–3.1%, respectively, indicating good fabrication reproducibility.

All experiments confirmed that the prepared MWCNTs–Nafion composite film has good durability, homogeneity in deposition, reproducibility, more active sites and strong adherence to electrode surface. These characteristics are suitable for long-term electrochemical sensing applications.

3.7. Interference

The influence of potential interfering species, which are likely to be in biological samples or are co-formulated with the active pharmaceutical ingredient, on the voltammetric responses of OND and MOR was examined. A fixed amount of 1×10^{-6} M OND and MOR, spiked with various excess amounts of interfering species, was evaluated under optimized experimental conditions described previously. The tolerance limit was defined as the maximum concentration of the interfering substance that caused an error less than ±5% in the determination of both drugs. The presence of Na^+, Cl^- (2000 fold), K^+, NO^{3-}, SO_4^{2-}, HPO_4^{2-} (1000 fold), Zn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{3+}, Na_2EDTA, manitol (500 fold), L-alanine, L-glycine, L-aspartic acid, L-glutamic acid, citric acid (400 fold), lactose, sucrose, starch (300 fold) and L-cysteine (200 fold), had no influence on the peak current of both drugs. Also, nitrite (400 fold) did not interfere with voltammetric response of both drugs although nitrite ions were oxidized at +0.75 V, very close to MOR potential. However, the MWCNTs–Nafion composite film allows facile accumulation of the positively charged MOR molecule, while prevent accumulation of negatively charged oxidizable nitrite ions onto the electrode surface. The experiments displayed that the oxidation peak current of OND and MOR at the MWCNTs–Nafion/GCE did not change after adding 400-fold concentration of ascorbic acid and 300-fold of glucose indicating that the selectivity of the method could be satisfactory for the quantification of both drugs in biological fluids where these endogenous substances are always present. When 200-fold concentration of uric acid was added into the solution containing 1×10^{-6} M OND and MOR, no obvious interference was observed for OND quantification, but the current response of MOR increased significantly due to the oxidation of uric acid at +0.65 V. Selectivity of the developed procedure was also investigated by observing any interference encountered from dopamine. The results showed that dopamine is oxidized at potential +0.49 V, however, in the presence of equal concentration do not cause a competitive adsorption onto the surface of modified electrode since a decrease in the currents of both drugs was not greater than 5%.

3.8. Applications of electrochemical sensor

The applicability of the MWCNTs–Nafion/GCE was examined via quantitation of OND in pharmaceutical dosage form. OND was analyzed in commercial film-coated tablets using the standard addition method in order to eliminate matrix effects. The results obtained using the proposed sensor are in good agreement with the claimed amount (Table 1). The analysis of OND in its pharmaceutical formulation exhibited the mean recovery of 99.7% with the relative standard deviation of 1.3% indicating good accuracy and precision as well as the suitability of the proposed sensor for this purpose. To evaluate possible interactions with excipients, recovery experiments were carried out by spiking the formulation solution samples with known amount of standard OND solution. The mean recovery of 98.1% indicated that excipients have no interference effect on the analysis of OND, therefore a separation step can be avoided.

The film-coated tablets were also analyzed by the reverse-phase HPLC method. The developed HPLC method with UV-detection was validated according to standard procedure [30]. The results obtained

Table 1
Analysis of ondansetron in film-coated tablets by the proposed adsorptive stripping SWV and HPLC methods.

Technique	AdSWV	HPLC
Stated content (mg)	8.00	8.00
Detected content (mg)[a]	7.97	8.02
RSD %	1.25	0.39
Added (mol L^{-1})	5.00×10^{-7}	2.00×10^{-5}
Found (mol L^{-1}) [b]	4.91×10^{-7}	1.97×10^{-5}
Recovery %	98.1	98.5
RSD %	3.46	0.24
F [c]	0.10	–
t [c]	0.37	–

[a] Each value is the mean of five experiments.
[b] Each value is the mean of three experiments.
[c] The theoretical values of F and t-test at 95% confidence limit are 6.39 and 2.31, respectively.

with the proposed sensor were compared to those obtained by the HPLC method (Table 1). Statistical analysis of the results obtained using voltammetric and HPLC procedures showed no significant difference between the performance of the two methods regarding accuracy and precision, as revealed by the student *t*-test and variance ratio *F*-test. However, the electroanalytical method does not require separation, degassing and expensive solvents that are needed for an HPLC procedure.

Finally, the MWCNTs–Nafion/GCE was applied for simultaneous determination of OND and MOR in biological samples. The sensitivity of the proposed method complies with the expected serum concentration level for both drugs after the treatment with the therapeutic daily dose. However, the quantitation of OND and MOR in human serum at the therapeutic concentration range could not be feasible without accumulation of protonated drug molecules on the MWCNTs–Nafion/GCE surface. Furthermore, the MWCNTs–Nafion film contains sulfonate groups and such surface layer prevents accumulation of negatively charged oxidizable substances found in biological fluids onto the electrode surface. The recovery studies of both drugs in serum samples were performed using the standard addition method to nullify any remaining interference. The mean recovery of $98.7 \pm 1.5\%$ for OND and $102.6 \pm 2.7\%$ for MOR was achieved in this type of matrix. From the experimental results, it is obvious that the proposed electrochemical sensor has great potentials for practical analysis of biological samples.

4. Conclusions

The paper describes, for the first time, the oxidative behavior and electrochemical determination of OND. A simple, but powerful, approach was applied for the fabrication of stable CNT-based electrochemical sensor. The MWCNTs–Nafion/GCE exhibited a remarkable enhancement effect on the voltammetric response of OND due to excellent electrocatalytic activity and adsorption capability of the immobilized film toward the drug molecule. This work provides a new and convenient electroanalytical method for direct quantitation of OND in film-coated tablets. In comparison to HPLC method, the proposed sensor possesses numerous advantages such as high sensitivity, rapid response, low cost and simplicity. In addition, the MWCNTs–Nafion/GCE exhibited high selectivity in the voltammetric measurements of OND and co-administrated drug MOR with potential difference of 430 mV. The electrode was successfully applied for the simultaneous determination of both drugs in human serum samples after selective accumulation at the electrode surface.

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3. Voltammetric determination of ropinirole in the presence of levodopa at the surface of a carbon nanotubes based electrochemical sensor in pharmaceuticals and human serum

Journal of Electroanalytical Chemistry 733 (2014) 60–68

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

journal homepage: www.elsevier.com/locate/jelechem

Voltammetric determination of ropinirole in the presence of levodopa at the surface of a carbon nanotubes based electrochemical sensor in pharmaceuticals and human serum

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ARTICLE INFO

Article history:
Received 4 July 2014
Received in revised form 11 September 2014
Accepted 17 September 2014
Available online 28 September 2014

Keywords:
Ropinirole
Levodopa
Carbon nanotubes
Electrochemical sensor
Stripping analysis

ABSTRACT

A nanostructured electrochemical sensor was developed to establish an innovative way to measure ropinirole and levodopa in real pharmaceutical and biological samples, which offers a high selectivity derived from the stable carbon nanotubes-Nafion polymer film. The surface morphology of the modified electrodes was characterized by scanning electron microscopy. The effect of surface modifications, supporting electrolyte, amount of carbon nanotubes suspension, accumulation time and potential were investigated. A sensitive electroanalytical methodology for the simultaneous determination of both drugs co-administrated in advanced Parkinson's disease patients using adsorptive stripping square-wave voltammetry is presented. Under the optimized conditions, ropinirole and levodopa gave a linear response in the range 1×10^{-7}–1×10^{-5} M and 2.5×10^{-7}–1×10^{-5} M with detection limits 1.6×10^{-8} M and 5.2×10^{-8} M, respectively. The method was successfully utilised for their quantification in human serum samples and good recoveries were obtained without interference from endogenous dopamine, uric and ascorbic acid. In addition, the proposed sensor was successfully applied in the independent determination of ropinirole and levodopa content in pharmaceutical formulations, whose accuracy was attested by good agreement of the results with those, obtained using high performance liquid chromatography. The proposed sensor is characterized by high sensitivity and reproducibility, simple fabrication procedure, easy handling, resistance against surface fouling and low cost.

1. Introduction

Parkinson's disease is one of the most common neurodegenerative disorders in humans leading to progressive deterioration of motor function due to loss of dopamine-producing brain cells. Ropinirole (ROP) is a novel nonergoline dopamine agonist indicated for the treatment of early and advanced Parkinson's disease (Scheme 1). It is used as monotherapy in the first stages of the disease [1]. When used as an adjunct to dopamine precursor levodopa (L-dopa) in advanced Parkinson's disease patients with motor fluctuations, ROP reduces daily off time and allows a reduction of L-dopa dose. ROP is also one of the four medications approved by the FDA for the treatment of primary restless legs syndrome affecting up to ten percent of the population [2].

To date, only a few analytical methods have been described for the determination of ROP, such as liquid chromatography-mass spectrometry methods for quantification of ROP in biological fluids [3,4], HPLC with UV detection for drug impurity profiling [5,6] and stability-indicating assays [7], high performance thin layer chromatography [8], capillary zone electrophoresis [9], spectrophotometry [10], spectrofluorimetry [11] and ultra-performance liquid chromatography [12,13]. The official method for quantification of ROP has not yet been approved in European Pharmacopoeia [14]. The literature has only one report for electroanalytical determination of ROP employing glassy carbon electrode (GCE) [15]. However, due to the surface-active properties of drug oxidation product, the adsorption of ROP on the electrode surface could not be used as an effective preconcentration step prior to voltammetric quantification of the drug. Consequently, the obtained concentration range with the GCE was sufficient for measurements of ROP in pharmaceutical formulation, but not in biological samples. Therefore, the development of electrochemical sensors for ROP determination with lower quantification limit without time-consuming surface cleaning as in the case of GCE is of great importance. On the other hand, ROP often coexists with L-dopa in biological fluids as these drugs are co-administrated in advanced Parkinson's disease patients. Surprisingly, up to now, no study has

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http://dx.doi.org/10.1016/j.jelechem.2014.09.020
1572-6657/

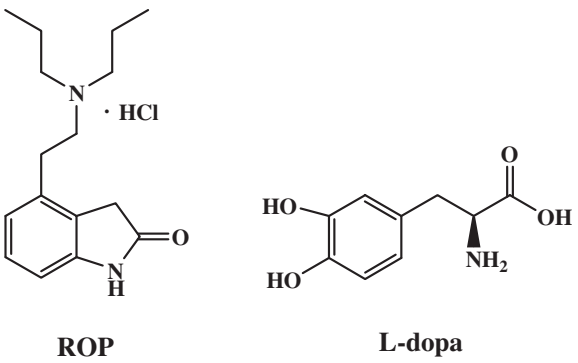
been published in the literature reporting the simultaneous determination of ROP and L-dopa. The monitoring of the therapeutic level concentrations of both drugs in serum samples is a critical subject in clinical medicine to prevent overdose-induced toxicity. The development and application of electrochemical sensors for L-dopa quantification in pharmaceuticals and biological fluids has received considerable interest in recent two years [16–26].

In continuation to our work on analysis of ROP [15], the aim of this study was to construct a simple, stable and sensitive electrochemical sensor for ROP determination. Carbon nanotubes (CNTs) have embarked a new epoch as novel electrode material in pharmaceutical analysis. CNTs can be homogenously dispersed in Nafion (NAF) solution due to hydrophobic side chains [27], while the hydrophilic negatively charged sulfonate group in NAF film enables selective preconcentration of positively charged drug molecules through electrostatic interaction [28,29]. Therefore, the adsorption characteristic of ROP molecule was investigated at the surface of glassy carbon electrode modified with carbon nanotubes and Nafion as cation exchange polymer (CNT-NAF/GCE). The analytical performance of the modified electrode for ROP quantification in the presence of L-dopa was also evaluated. Thus, here we reported for the first time, the development of analytical method for simultaneous determination of ROD and L-dopa. The adsorptive stripping voltammetric method was applied for trace analysis of both drugs in human serum samples. To achieve a better medicinal effect and lower toxicity, it is very important to control the content of ROP and L-dopa in pharmaceutical dosage forms. Therefore, the modified electrode was also used to develop simple, inexpensive, fast and accurate method for direct voltammetric quantification of ROP and L-dopa individually in commercial pharmaceutical formulations. The HPLC was selected as the comparative methods for evaluating the proposed new electroanalytical method.

2. Experimental

2.1. Instrumentation

Voltammetric experiments were carried out using a μ-Autolab potentiostat (Eco Chemie, Utrecht, The Netherlands). The experimental conditions were controlled by General Purpose Electrochemical System (GPES) software, version 4.9. A three-electrode configuration was used with a bare or modified GCE (3-mm diameter, Metrohm, Switzerland) as working electrode, Ag/AgCl (KCl 3 M, Metrohm) reference electrode and a platinum counter electrode. When required, stirring was applied using a computer-controlled stirrer at ca. 300 rpm. All measurements were performed at room temperature (23 ± 2 °C) in a 20 mL electrochemical cell. HPLC analysis were performed using an Agilent 1100 Series LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector, controlled by ChemStation software. The scanning electron microscope employed for surface characterisation of the electrodes was a Jeol JSM-7000F model (Jeol Ltd., Tokyo, Japan) with an operating voltage of 5 or 10 kV.



ROP L-dopa

Scheme 1. Chemical structures of ropinirole (ROP) and levodopa (L-dopa).

2.2. Chemicals

Ropinirole hydrochloride (99.4%), kindly donated by Pliva (Zagreb, Croatia), was used as received without any further purification. L-dopa was purchased from Fluka (Laramie, USA). The purity of L-dopa was high enough to comply with the European Pharmacopoeia. Requip® film-coated tablets (GlaxoSmithKline, London, United Kingdom) containing 2.28 mg of ropinirole hydrochloride, equivalent to 2 mg of ROP, and Madopar® tablets (Hoffmann-La Roche, Basel, Switzerland) containing 100 mg of L-dopa and 25 mg of benserazide, were supplied from local pharmacy. Multi-wall carbon nanotubes (purity more than 98%) with o.d. between 6 and 13 nm and tube length from 2.5 to 20 μm as well as Nafion (5 wt% solution in a mixture of lower aliphatic alcohols and water) were from Sigma–Aldrich (Steinheim, Germany). All solutions were prepared from analytical grade chemicals and ultra-pure water obtained by a Milli-Q system (Millipore, Bradford, USA).

Stock solutions of ROP and L-dopa for voltammetric measurements were prepared in ultra-pure water at a concentration of 1.0×10^{-3} M and stored at 4 °C in a refrigerator. Both ROP and L-dopa standard solutions were prepared by appropriate dilution of these stock solutions with the selected supporting electrolyte just before use. The supporting electrolytes were sulfuric acid, hydrochloric acid, acetic acid and Britton-Robinson buffer adjusted to the desired pH with 0.2 M sodium hydroxide solution.

2.3. Preparation of the modified electrodes

A mass of 50 mg of the multi-wall carbon nanotubes was dispersed in 20 mL of concentrated nitric acid. The mixture was then sonicated for 4 h to generate carboxylic acid-functionalized surface. The suspension was filtered through a 200 nm membrane and washed with double distilled water to reach neutral pH. The solid powders were dried under vacuum at room temperature. Then, 1 mg of the functionalized CNTs was dispersed in 1 mL of 0.5% (m/v) Nafion ethanol solution and sonicated for 30 min to form homogenous suspension. The CNT-NAF/GCE was prepared by casting 5 μL of the immobilizing suspension on the polished GCE surface allowing the solvent to evaporate at room temperature. Finally, the modified electrode was carefully rinsed with purified water and scanned by two successive cyclic voltammetric sweeps between 0 and 1.5 V at 100 mV s^{-1} in a blank solution of 0.1 M H_2SO_4 prior to first electrochemical measurements. The surface areas of modified electrodes were obtained by cyclic voltammetry (CV) using 1.0×10^{-3} M $K_3Fe(CN)_6$ as a probe in 0.1 M KCl electrolyte at different scan rates (v). From the slope of the anodic peak current versus $v^{1/2}$ plot, the surface areas of the GCE and the CNT-NAF/GCE was calculated to be 0.057 and 0.298 cm^2, respectively. For comparison, the carbon nanotubes modified GCE (CNT/GCE) and the Nafion modified GCE (NAF/GCE) were also prepared in the same way as described but without the addition NAF and CNTs, respectively.

2.4. Electrochemical measurements

CV was carried out from 0 to 1.5 V with the scan rate varying from 10 to 500 mV s^{-1}. Square-wave voltammetry (SWV) was used for the determination procedures of ROP and L-dopa. The voltammograms were recorded in the SWV mode from 0.4 to 1.5 V either immediately in quiescent solution or after adsorptive

accumulation for selected time at the predetermined potential in stirred solution. The preconcentration of both analytes was carried out at 0 V with a 240 s accumulation step. After an equilibrium period of 5 s the potential scan was started. Operating instrumental conditions for SWV were: pulse amplitude of 25 mV, frequency of 50 Hz and scan increment of 8 mV. After each measurement, the sensor was transferred to the solution of 0.1 M H_2SO_4 to regenerate the electrode surface by applying single positive-going CV potential scan from 0 to 1.5 V.

2.5. Determination in samples

Ten tablets of each analyzed pharmaceutical formulation were weighed and separately powdered in a mortar. An adequate amount of prepared powders, equivalent to a stock solution of concentration 1.0×10^{-3} M, was weighed and individually dispersed in purified water in a 10.0 mL volumetric flask. The mixtures were sonicated for 10 min to provide complete dissolution of active ingredients and filtered through 0.45 μm Acrodisc GHP filters (Gelman, Ann Arbor, USA). To obtain final concentrations in the range of calibration curve, the sample solutions of both drugs were suitably diluted with supporting electrolyte and subjected to direct SWV measurements as previously mentioned. The content of ROP and L-dopa in formulated products was determined by standard addition method.

The ROP determination by HPLC was carried out using a chromatographic column XBridge C18, 3.0 × 50 mm, particle size 2.5 μm (Waters, Miliford, Mass., USA). The mobile phase composed of acetonitrile, ultra-pure water and ammonium hydroxide (25%) at the ratio of 400:600:0.04 (v/v/v) were filtered through cellulose nitrate filter (0.45 μm, Sartorius, Goettingen, Germany). The chromatography was carried out at constant temperature (25 °C) with a flow-rate of 1 mL/min under isocratic conditions. The standard solutions and the appropriate tablet solutions for HPLC analysis were prepared in mobile phase and filtered through a 0.2 μm Acrodisc GHP filters (Gelman, Ann Arbor, USA). The injection volume was 5 μL. The UV spectra were recorded from 190 to 400 nm and the chromatogram was obtained at 250 nm. The nominal content of ROP tablet amounts were calculated from the corresponding regression equations of previously plotted calibration plots. The official HPLC method described in USP was used for comparative analysis of L-dopa tablets [30].

Serum samples were obtained from healthy volunteers and stored frozen until assay. A 500 μL aliquot of human serum samples was fortified with the appropriate aliquot volume of ROP and L-dopa standard solutions to achieve final concentrations (1.0×10^{-7} and 5×10^{-7} M) that are found after the treatment with therapeutic daily doses of 6 and 100 mg for ROP and L-dopa, respectively [31,32] and mixed with acetonitrile (1.5 mL) to remove serum proteins effectively. The mixture was vortexed for 5 min. After centrifugation of sample for 10 min at 6000 rpm the supernatant was separated. Appropriate volumes of clear supernatant solution were transferred into the volumetric flask and diluted with the supporting electrolyte and analyzed in the voltammetric cell. To reduce the matrix effect the standard addition method was employed in direct analysis of human serum samples.

3. Results and discussion

3.1. Electrochemical behaviour of ROP and L-dopa

The cyclic voltammograms of 5×10^{-5} M ROP solution in 0.1 M H_2SO_4 were recorded at the CNT-NAF/GCE, the CNT/GCE, the NAF/GCE and a bare GCE using a scan rate of 100 mV s^{-1}. A single anodic peak was observed showing that the oxidation of ROP molecule is a totally irreversible process. As seen in Fig. 1, moving from the GCE to CNT-NAF/GCE, the oxidation peak was found to be extremely well-defined with enhanced peak current. ROP is completely in cationic form (p*K*a 9.5) in experimental conditions used in the voltammetric measurements. When the bare GCE surface was coated with 0.5% Nafion film, an oxidation wave (i_p = 26.6 μA) at 1.36 V was increased due to electrostatic attraction of positively charged drug molecules. However, compared with NAF/GCE, the voltammetric response of ROP was remarkable increased at the CNT-NAF/GCE with a current of 91.7 μA and a peak potential of 1.24 V. The oxidation peak potential was shifted to less positive values by 120 mV indicating faster electron transfer at the electrode surface and excellent electrocatalytic activity of immobilized CNTs toward ROP molecule. Therefore, it can be concluded that the synergistic effect of CNTs and NAF combination leads to a considerable improvement in the analytical sensitivity.

To characterize the modified electrode, the surface morphology of the NAF/GCE, the CNT/GCE and the CNT-NAF/GCE was studied using scanning electron microscopy (SEM). The SEM image of the NAF/GCE showed a smooth film of cation-exchange polymer (Fig. 2). On the other hand, the film of the CNT/GCE showed plenty of tubes irregularly placed in thread-like formations revealing a much larger surface area. The SEM image of the CNT-NAF composite film offers a uniform coating with higher accessible surface area forming bundle structures. The CNTs were embedded into the polymer matrix making the composite film surface rough. This unique surface morphology of CNT-NAF/GCE gave a high accessible area improving the preconcentration of ROP molecules through electrostatic interaction with negatively charged sulfonate group in NAF polymer structure. Furthermore, the CNTs were tightly fixed in the NAF matrix onto GCE surface in a reproducible way enhancing the stability of CNT-NAF/GCE.

The influence of the scan rate on the electrochemical response of ROP was investigated by CV at the CNT-NAF/GCE. A plot of the anodic peak current versus the scan rate in the range of 10–200 mV s^{-1} gave the linear relationship i_p (μA) = 0.23 v (mV s^{-1}) + 9.85 (r = 0.996), indicating that the electrooxidation of drug molecule at the modified electrode was a adsorption-controlled process. The oxidation peak potential was shifted to more positive potentials as the scan rate increased from 10 to 500 mV s^{-1}. The dependence of the peak potential is linear with logarithm of the scan rate with a slope of 63.5 mV/decade (inset of Fig. 1), allowing the calculation of αn = 0.93. Using the value of the charge transfer coefficient (α = 0.53) obtained from the difference between the peak

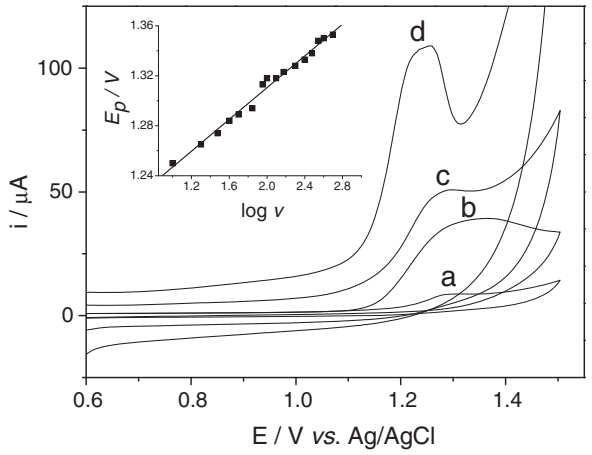


Fig. 1. Cyclic voltammograms of ropinirole (5×10^{-5} M) at a bare GCE (a), the NAF/GCE (b), the CNT/GCE (c) and the CNT-NAF/GCE (d) in 0.1 M H_2SO_4. Scan rate: 100 mV s^{-1}. Inset: plot of peak potential *vs.* logarithm of scan rates from 10 to 500 mV s^{-1} at the CNT-NAF/GCE.

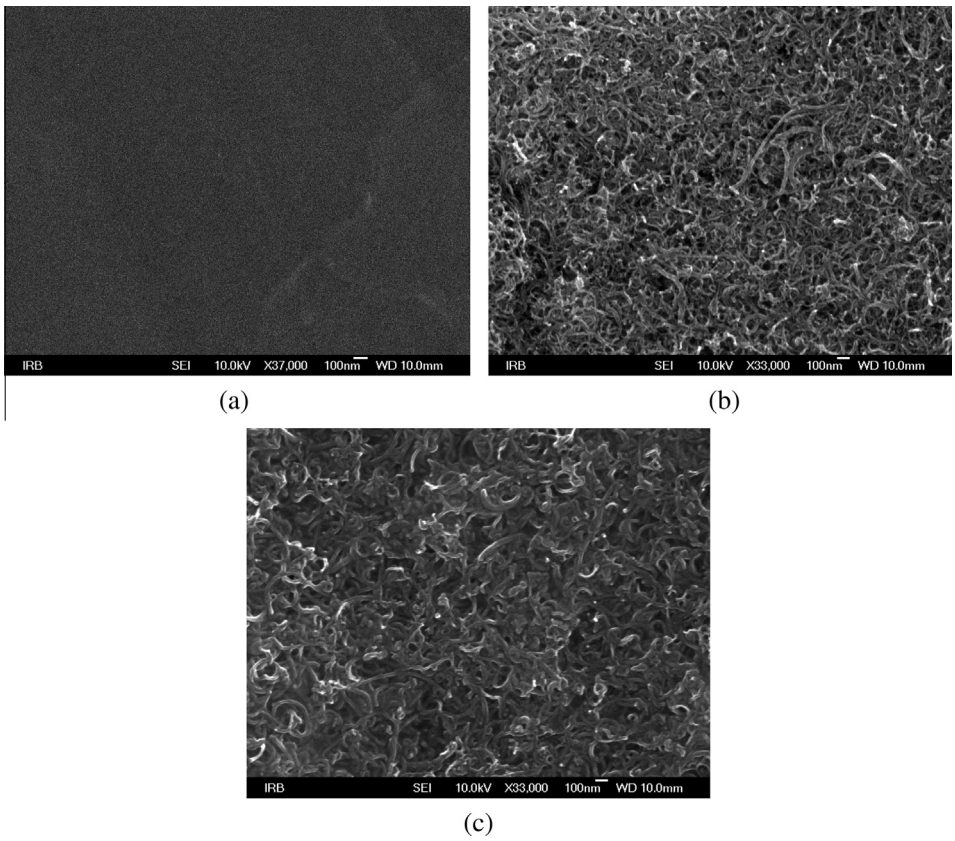


Fig. 2. SEM images of the NAF/GCE (a), the CNT/GCE (b) and the CNT-NAF/GCE (c). Scale bar: 100 nm.

potential (E_p) and the half wave potential ($E_{p/2}$) by the equation $E_p - E_{p/2} = (47.7/\alpha)$ mV, the number of electrons exchanged was calculated to be $n = 1.76$. Our earlier investigation of ROP electrooxidation mechanism at bare GCE over a wide pH range (1.0–12.0) in buffered aqueous media indicated that the voltammetric signal in strong acid media was assigned to an over-all $2e^-/2H^+$ oxidation process involving indol-2-one ring [15]. The oxidation pattern of ROP molecule observed at the CNT-NAF/GCE in 0.1 M H_2SO_4 solution was well-consistent with that obtained at the bare GCE, thus, we can corroborate that the oxidation of ROP at the CNT-NAF composite film occurs at indol-2-one moiety. However, at the surface of the CNT-NAF/GCE not only the sensitivity was increased in comparison with the GCE, but the fouling effect of ROP and its oxidation product was also decreased.

In order to develop electrochemical sensor for simultaneous determination of ROP and L-dopa co-administrated in advanced Parkinson's disease patients, the oxidation of L-dopa was also studied at the CNT-NAF/GCE, the CNT/GCE, the NAF/GCE and a bare GCE using CV. A single reversible oxidation peak at +0.52 V was observed at the CNT-NAF/GCE, which can be assigned, in accordance with previous studies [19], to a $2e^-/2H^+$ oxidation of L-dopa to corresponding quinone form (inset of Fig. 3). The L-dopa molecule has positively charged nitrogen atom (p*K*a 8.7) in 0.1 M H_2SO_4 solution [33] and NAF as ion exchange polymer also attracted cationic form of L-dopa from bulk to electrode surface, thus facilitating the electrode process. The experimental results clearly indicated a higher current response of L-dopa at the CNT-NAF/GCE in comparison with that of using a bare GCE or other two modified electrodes. This remarkable current enhancement can be undoubtedly attributed to the unique structure and properties of CNT-NAF film, including very large specific area, strong adsorptive ability, and subtle electronic properties. The potential

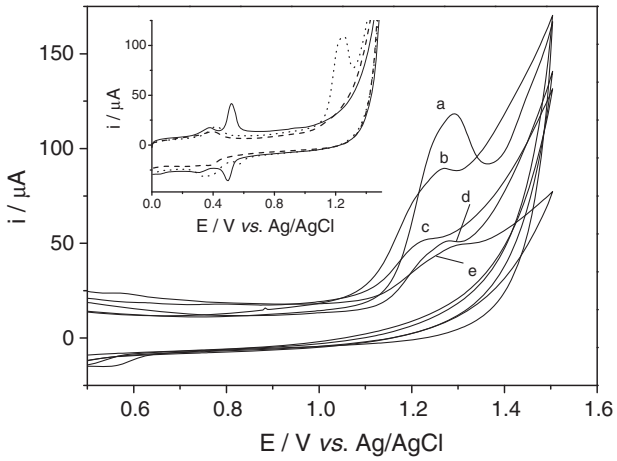


Fig. 3. Effect of supporting electrolyte on ropinirole voltammetric response at the CNT-NAF/GCE: 0.1 M H_2SO_4 (a), 0.01 M H_2SO_4 (b), Britton-Robinson buffer pH 4 (c), 0.01 M HCl (d) and 0.1 M CH_3COOH (e). Scan rate: 100 mV s^{-1}; $c = 5 \times 10^{-5}$ M. Inset: Cyclic voltammograms of levodopa at the CNT-NAF/GCE (solid line) and together with ropinirole (doted line) and blank at CNT-NAF/GCE (dashed line).

difference of 720 mV between the two peak potentials of ROP and L-dopa at the CNT-NAF/GCE is large enough to determine the concentration of both drugs individually and simultaneously.

3.2. Optimisation of experimental conditions

Different supporting electrolytes, including sulfuric acid, hydrochloric acid, acetic acid and BR buffer pH 2.0–6.0, were tested in order to develop a new electroanalytical method for simultaneous

determination of ROP and L-dopa at the CNT-NAF/GCE. The experiments showed that well-defined voltammetric response of ROP was obtained only in strong acid media and beyond pH 2 the peak current started to deteriorate, as shown in Fig. 3. A maximum current intensity was obtained in 0.1 M H_2SO_4 solution and consequently, it was employed for further studies.

Since ROP oxidation was observed at relatively high potential and this drug was found in lower concentration limits then L-dopa in real samples, the effect of modifier amount on the performance of the CNT-NAF/GCE was investigated using SWV in order to obtain the most favourable response of ROP. Therefore, when varying proportions of CNTs dispersed in NAF solution (1–9 μL) were casted onto the GCE surface, 5 μL of homogenous suspension served as the optimum amount for NAF-CNT film coating on the electrode because maximum current of ROP was obtained, and thereafter, the oxidation peak current conversely showed gradual decline. The thickness of the composite film was determined by the volume of NAF-CNT suspension applied on the GCE surface. When the thickness of the film increased, there were more $-SO_3$ sites that conducted ion-exchange with the ROP molecules leading to an increment of accumulation capacities, and similarly, more CNTs dramatically promoted electron transfer reaction. These two aspects resulted in a corresponding increase in the peak current. The SWV response of ROP was increased up to 0.5% of NAF concentration in the immobilizing suspension. Then, the NAF film became thicker and lowered the electrical conductivity of CNTs and, consequently, retarded the electron transfer rate of ROP oxidation as well as the analyte mass transportation through the film resulting in the higher electric resistance.

To obtain a much more sensitive peak currents for analytical studies, the variation of the peak currents recorded at the CNT-NAF/GCE was monitored while changing instrumental SWV parameters, i.e. frequency ($10\ \text{Hz} \leqslant f \leqslant 130\ \text{Hz}$), pulse amplitude ($10\ \text{mV} \leqslant a \leqslant 70\ \text{mV}$) and potential step ($2\ \text{mV} \leqslant \Delta E_s \leqslant 10\ \text{mV}$). The most favourable response was achieved using a combination of a frequency of 50 Hz, pulse amplitude of 25 mV and a potential step of 8 mV. Additionally, according to the square-wave theory [34], the slope of 0.069 V obtained from the E_p versus log f plot was used to calculate the value of αn as 0.86. Taking the value of 0.53 for the charge transfer coefficient, the number of electrons transferred in the oxidation process of ROP was estimated to be 1.71, again a value of approximately 2, which was in agreement with the result obtained by CV experiments confirming the two-electron mechanism of ROP electrooxidation at the CNT-NAF/GCE.

3.3. Adsorption properties of ROP and L-dopa on CNT-NAF/GCE

Since the modification of the electrode surface with CNT-NAF layer led to a strong interfacial accumulation of positively charged drug molecules, possibility of effective analyte preconcentration before voltammetric measurement was examined. To achieve the optimum conditions for the maximum adsorption, the influence of accumulation potential on the stripping peak current was evaluated from −1.0 to 1.0 V. A maximum was observed at 0 V, so this deposition potential was selected in all subsequent studies. Square-wave voltammograms with increasing accumulation times (between 0 s and 360 s) were recorded for solutions containing ROP and L-dopa at concentration level of 2.5×10^{-6} M. Fig. 4 shows square-wave voltammograms for solution without accumulation and after a 240 s accumulation step at the CNT-NAF/GCE. The accumulation time significantly affected the voltammetric response of both drugs at the CNT-NAF/GCE. The peak current of ROP increased up to 360 s, however, the accumulation time of 240 s provided the largest peak current in the linearity range revealing a constant adsorption of drug molecules attracted on the surface of the CNT-NAF/GCE. Further increment of deposition time period showed a deviation of the peak current from linearity suggesting saturation of the surface coverage. On the other hand, the peak current of L-dopa enhanced greatly within 30 s indicating faster adsorption then ROP, as shown in inset of Fig. 4. The voltammetric response of L-dopa increased up to a maximum at 120 s and then remained almost constant. However, the value of 240 s was considered as the optimum for simultaneous quantification of both drugs because the peak current of L-dopa slowly increased up to 240 s which was selected as the optimal value for ROP to achieve the highest possible sensitivity in acceptable analysis time.

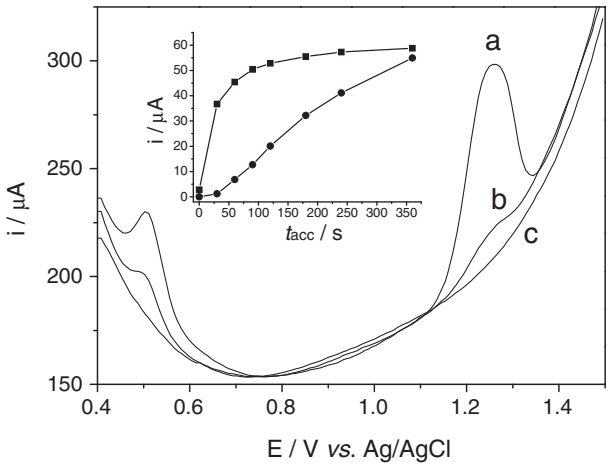


Fig. 4. Square-wave voltammograms of ropinirole (2.5×10^{-6} M) and L-dopa (2.5×10^{-6} M) at the CNT-NAF/GCE in 0.1 M H_2SO_4 after preconcentration time of 240 s (a), without preconcentration (b) and blank solution (c). SWV settings: frequency of 50 Hz, amplitude of 25 mV and potential step of 8 mV, E_{acc} = 0 V. Inset: Effect of the accumulation time on the oxidation peak current for ropinirole (circles) and L-dopa (squares).

3.4. Simultaneous analysis and validation studies

The use of an electrochemical sensor for ROP determination has not been reported in the literature and in addition, this work is the first report for simultaneous determination of ROP and L-dopa used in combination to treat advanced Parkinson's disease. The electrocatalytic peak currents of ROP and L-dopa at 1.24 and 0.52 V, respectively, obtained at proposed sensor were used for determination of both drugs in solution, involving SWV and adsorptive stripping square-wave voltammetry (AdSWV) for drug quantitation at low levels.

When all parameters that affect ROP and L-dopa quantification had been studied, variation of the current response with the drug concentration was performed. In the case of a square-wave scan without any accumulation step, the oxidation peak showed a linear response in the concentration range of 1×10^{-6}–5×10^{-5} M for ROP and 1×10^{-6}–1×10^{-5} M for L-dopa. By using drug accumulation at the CNT-NAF/GCE prior to SWV measurement, higher sensitivities were achieved. Fig. 5 shows the SWV responses from the electrochemical oxidation of ROP and in 0.1 M H_2SO_4 solution after a 240 s preconcentration step. Calibration graph using AdSWV showed two linear segments for ROP detection. The first linear range was from 1×10^{-7} to 1.5×10^{-6} M and the second linear dynamic range was between 1.5×10^{-6} and 1×10^{-5} M (inset of Fig. 5). The break in the calibration curve of ROP adsorptive stripping current reflected the formation of a sub-monolayer in the first range of calibration and formation of a monolayer in the second range. Since the presence of L-dopa in solution may affect the quantification of ROP due to competitive adsorption and therefore,

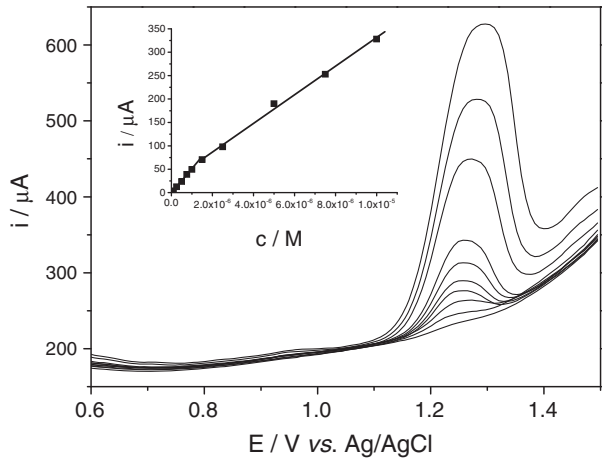


Fig. 5. Adsorptive stripping square-wave voltammograms of ropinirole at the CNT-NAF/GCE recorded in 0.1 M H_2SO_4 at different concentrations (1×10^{-7}–1×10^{-5} M). SWV settings and accumulation conditions same as in Fig. 4. Inset shows a calibration graph for the quantification of ropinirole.

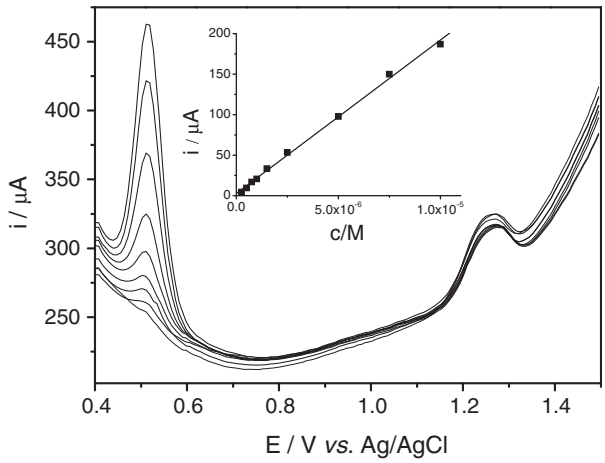


Fig. 6. Adsorptive stripping square-wave voltammograms of the mixture containing levodopa at different concentrations (2.5×10^{-7}–1×10^{-5} M) and ropinirole (1×10^{-6} M) recorded at the CNT-NAF/GCE in 0.1 M H_2SO_4. SWV settings and accumulation conditions same as in Fig. 4. Inset shows a calibration graph for levodopa.

the interference of each analyte in the simultaneous determination of its pairs was performed by changing one analyte concentration and keeping the other unchanged. The variation of adsorptive stripping SWV peak current versus ROP concentration in the presence of L-dopa at the fixed concentration of 2.5×10^{-6} M gave the sensitivities of the CNT-NAF/GCE towards the ROP oxidation of 4.75×10^7 and 2.96×10^7 μA M^{-1} for lower and higher concentration ranges, respectively. The values obtained are very close (relative errors 1.43% and 2.31%) to the values observed in the absence of L-dopa (Table 1), indicating independent adsorption of both drugs in the accumulation step at the proposed electrochemical sensor. Therefore, simultaneous determination of ROP and L-dopa is possible without interferences. Fig. 6 shows the voltammetric responses for the solutions containing various concentrations of L-dopa and constant concentration of ROP (1×10^{-6} M). The relation between the stripping peak current and concentration of L-dopa was found to be linear over the range 2.5×10^{-7}–1×10^{-5} M (inset of Fig. 6). The analytical characteristics and the related validation parameters of developed methods are given in Table 1. The detection and quantification limits of were calculated from the calibration curves as $3s/m$ and $10s/m$, respectively, where s is the standard deviation of the intercept and m is the slope of the calibration curve [35].

For validation of the proposed method, the intra-day precision of the voltammetric response using the CNT-NAF/GCE was evaluated by six replicate measurements of 1.5×10^{-6} M ROP and L-dopa solution yielding unchanged oxidation peak potentials for ROP and L-dopa and the RSD values of 1.4% and 1.7% for ROP (mean i_p = 70.4 μA) and L-dopa peak currents (mean i_p = 33.5 μA), respectively. These results indicated that proposed electrochemical sensor was stable in repeated measurements for selective determination of ROP and L-dopa. The inter-day precision of the electrode response was characterized by three replicate measurements of 2.5×10^{-6} M ROP and L-dopa over three days, each time using a freshly prepared CNT-NAF/GCE and standard solutions. The RSDs values of the anodic stripping currents for ROP (mean i_p = 96.4 μA) and L-dopa (mean i_p = 53.8 μA) were 2.5% and 2.9%, respectively, while the RSDs of peak potentials for both drugs were no greater than 0.7%, indicating excellent fabrication reproducibility.

The proposed electrochemical sensor can be stored in air or kept in distilled water for several weeks without the loss of activity. The stability of the CNT-NAF/GCE was evaluated by measuring the current response of 2.5×10^{-6} M ROP and L-dopa solution over a period of three weeks. The modified electrode was used daily and stored in the air at room temperature. Before measurements, the modified electrode was scanned between 0.5 and 1.4 V in the solution of supporting electrolyte until the SWV response was stable. The experimental results indicated that the CNT-NAF/GCE could retain 97.5% and 98.3% of initial response after two weeks for ROP and L-dopa, respectively, revealing that sensor fabricated possesses long-term stability. Thereafter, the voltammetric signal

Table 1
Analytical parameters for the voltammetric determination of ROP and L-dopa by SWV and AdSWV using CNT-NAF/GCE.

	ROP			L-dopa	
	SWV	AdSWV		SWV	AdSWV
Linearity range (M)	1×10^{-6}–5×10^{-5}	1×10^{-7}–1.5×10^{-6}	1.5×10^{-6}–1×10^{-5}	1×10^{-6}–1×10^{-5}	2.5×10^{-7}–1×10^{-5}
Slope (μA M^{-1})	2.22×10^6	4.81×10^7	3.04×10^7	1.46×10^7	1.89×10^7
Intercept (μA)	0.20	0.28	26.78	−4.45	2.64
$S_{x/y}$ [a]	2.16	1.86	7.40	5.62	3.52
S_b [b]	4.63×10^4	1.62×10^6	1.05×10^6	6.99×10^5	3.55×10^5
S_a [c]	0.19	0.27	1.30	0.79	0.33
Correlation coefficient	0.999	0.998	0.998	0.995	0.999
Detection limit (M)	2.6×10^{-7}	1.6×10^{-8}	1.3×10^{-7}	1.6×10^{-7}	5.2×10^{-8}
Quantitation limit (M)	8.7×10^{-7}	5.5×10^{-8}	4.3×10^{-7}	5.4×10^{-7}	1.7×10^{-7}

[a] Standard deviations of residuals.
[b] Standard deviations of the slope.
[c] Standard deviations of the intercept.

started to decrease indicating the need for re-depositing of a new nanostructured film.

3.5. Interference studies

Prior to the analysis of samples, the selectivity of the proposed electrochemical sensor for quantification of ROP and L-dopa was also evaluated. The addition of possible interference compounds commonly present in the analyzed pharmaceutical dosage forms or biological samples was investigated in a standard solution containing ROP and L-dopa at 1×10^{-6} M concentration. The tolerance limit was taken as the concentration of foreign substances which gave an error less than ±5% in the determination of the drugs. According to the analysis of the obtained voltammetric responses, Na^+, Cl^- (2000-fold), K^+, Zn^{2+}, SO_4^{2-}, NO_3^- (1000-fold), Ca^{2+}, Mg^{2+}, Cu^{2+}, lactate (500-fold), HPO_4^{2-}, citric acid, L-glycine, L-alanine, L-aspartic acid, L-glutamic acid (400-fold), Fe^{3+}, starch (300-fold), lactose, sucrose, L-cysteine (200-fold) did not show interference in the determination of both drugs. Also, the interference of ascorbic acid, glucose and uric acid was examined because theses endogenous substances are always present in complex matrices such as biological fluids. It was observed that the adsorptive stripping peak current of ROP and L-dopa did not change after adding 400-fold of ascorbic acid and 300-fold of glucose. In the presence of uric acid, new well-defined oxidation peak appeared at the potential around +0.69 V. The results showed that 50-fold concentration of uric acid did not cause a positive or a negative error greater than 5% in the determination of L-dopa. However, uric acid did not influence the peak current of ROP even up to 200-fold excess. As expected, the interferences from negatively charged oxidizable ions were eliminated by prevention their accumulation using application of a Nafion-coated membrane. Also, dopamine at the concentration ratio 2:1 (interferent: drug standard solution) did not interfere with ROP voltammetric response, but showed interference with L-dopa quantification due to dopamine oxidation at +0.48 V, very close to L-dopa potential. However, normal plasma levels of endogenous free dopamine (26 pg/mL) [36] did not affect L-dopa voltammetric response at the CNT-NAF/GCE.

3.6. Analytical applications

To evaluate the validity and practical utility of the proposed method for individual quantification of ROP and L-dopa, two commercially available pharmaceutical products containing ROP (Requip, GlaxoSmithKline) as well as L-dopa and benserazide (Madopar, Hoffmann-La Roche) were analyzed using the standard addition method in order to eliminate matrix effects. Due to the high concentration of active ingredients in their dosage forms (2 mg per ROP tablet and 100 mg per L-dopa tablet), no accumulation time was needed and the tablets assay was carried out using direct anodic SWV technique. Results summarized in Table 2 shows that the content of assayed tablets obtained by utilizing the CNT-NAF/GCE are in good agreement with those declared in the label of the pharmaceutical products. The effect of excipients upon the voltammetric response of ROP and L-dopa at the CNT-NAF/GCE was studied by adding different known amounts of standard to the formulation solution samples. The recoveries of 99.2% for ROP and 99.4% for L-dopa were obtained from this type of matrix, indicating adequate accuracy of the proposed analysis procedures. The obtained recoveries revealed that excipients did not interfere with the assay avoiding a separation step, and thus corroborating the suitability of the proposed sensor for this purpose. Also, the data indicate that benserazide present in combined pharmaceutical dosage form with L-dopa had no interference effect on L-dopa determination in real sample using the CNT-NAF/GCE.

The results obtained with the proposed sensor were compared with those obtained by developed reverse-phase HPLC method for ROP and official HPLC method described in USP for L-dopa tablets [30]. Recovery tests were also performed for chromatographic methods. Statistical comparison were performed on data obtained using both voltammetric and HPLC procedures. The results of the student *t*-test and variance ratio *F*-test show that there are no significant differences between the techniques with regard to accuracy and precision (Table 2). Therefore, the modified electrode can be efficiently used for individual determination of ROP and L-dopa in pharmaceutical preparations. The newly developed methods using the CNT-NAF/GCE can be successfully applied in controlling the quality of active pharmaceutical ingredient and dosage forms as a selective method that is very precise, rapid and inexpensive.

To check the analytical applicability of the proposed electrochemical sensor for simultaneous quantification of ROP and L-dopa, the AdSWV method was applied to the determination of both drugs in human serum samples. Pharmacokinetic studies found in the literature suggest that the sensitivity of the proposed sensor complies with the expected serum concentration level in Parkinson patients treated with usual recommended daily dose. The average plasma concentration of L-dopa ranged from 4.4×10^{-6} to 11×10^{-6} M [32]. However, the maximum plasma concentration of ROP at steady state is much lower [31] and the quantitation of ROP in human serum at the therapeutic concentration range could not be feasible without preconcentration step on the modified electrode surface. The recovery studies of both drugs in serum samples were performed using the standard addition method. The mean recovery of 99.6 ± 2.2% for ROP and 98.8 ±

Table 2
Application of the CNT-NAF/GCE for individual determination of ROP and L-dopa in pharmaceutical dosage forms by the proposed SWV.

Samples	ROP		L-dopa	
	SWV	HPLC	SWV	HPLC
Label value (mg)	2	2	100	100
Determined value (mg)[a]	2.02	2.06	95.23	95.04
RSD%	1.16	0.49	1.25	0.06
Bias%	0.95	2.85	−4.77	−4.96
Added (M)	5.00×10^{-6}	5.00×10^{-5}	2.50×10^{-6}	1.00×10^{-3}
Found (M)[a]	4.96×10^{-6}	5.03×10^{-5}	2.48×10^{-6}	1.02×10^{-3}
Recovery%	99.2	100.6	99.4	101.8
RSD%	1.65	0.28	2.23	0.44
Bias%	−0.76	0.64	−0.72	1.80
F[b]	5.41	–	4.21	–
t[b]	1.54	–	1.99	–

[a] $n = 3$.
[b] The theoretical values of *F* and *t*-test at 95% confidence limit are 6.39 and 2.31, respectively.

Table 3
Results on recovery tests for serum samples by proposed AdSWV method for simultaneous determination of ROP and L-dopa using CNT-NAF/GCE.

Samples found/R.S.D., average	Added 10^6 c (M)	Found 10^6 c (M)	Recovery (%)	RSD[a] (%)
ROP	0.10	0.099	98.66	2.47
	0.25	0.247	98.93	2.44
	0.50	0.505	101.07	1.84
L-dopa	10.00	9.873	98.73	2.79
	15.00	14.653	97.69	2.25
	20.00	19.983	99.92	1.86

[a] Average of three measurements.

Table 4
Comparison of various voltammetric methods for the determination of ROP and L-dopa with the present work.

Analyte	Technique	Electrode	Concentration range (M)	LOD (M)	Reference
ROP	DPV	GCE	1×10^{-6}–2×10^{-5}	2.5×10^{-7}	15
	SWV	GCE	5×10^{-7}–2×10^{-5}	1.1×10^{-7}	15
	SWV	CNT-NAF/GCE	1×10^{-6}–5×10^{-5}	2.6×10^{-7}	This work
	AdSWV	CNT-NAF/GCE	1×10^{-7}–1×10^{-5}	1.6×10^{-8}	This work
L-dopa	DPV	Cumarine derivative/TiO_2 NP[a]/IL[b]/CPE[c]	1×10^{-7}–9×10^{-4}	4.1×10^{-8}	16
	AdDPV	$Ni(OH)_2$ NP[a]/MWCNT/GCE	1×10^{-7}–1×10^{-4}	7.6×10^{-8}	17
	AdSWV	Cu(II) complex/Ag NP[a]/CPE[c]	7.8×10^{-9}–9.1×10^{-6}	2.4×10^{-9}	18
	AdDPV	Poly(methyl orange) CPE[c]	2×10^{-5}–8×10^{-4}	3.7×10^{-6}	19
	SWV	Co(III) complex/MWCNT/BPPGE[d]	3×10^{-6}–1×10^{-4}	8.6×10^{-7}	20
	DPV	Quercetin/ MWCNT/GCE	9×10^{-7}–8.5×10^{-5}	3.8×10^{-7}	21
	DPV	Co-porphyrin/TiO_2 NP[a]/CPE[c]	1×10^{-7}–1×10^{-4}	6.9×10^{-8}	22
	DPV	SWCNT/chitosan/IL[b]/GCE	2×10^{-6}–4.5×10^{-4}	1.6×10^{-7}	23
	DPV	Au NP/Nafion/CPE[c]	2×10^{-7}–2×10^{-5}	1.5×10^{-9}	24
			5×10^{-5}–3×10^{-3}	2.8×10^{-6}	24
	DPV	Nanosized mesoporous molecular sieves/CPE[c]	1.3×10^{-7}–1.25×10^{-3}	7.2×10^{-8}	25
	DPV	Co-hexacyanoferrate/large mesopore carbon/GCE	1×10^{-7}–1.9×10^{-3}	1.7×10^{-8}	26
	SWV	CNT-NAF/GCE	1×10^{-6}–1×10^{-5}	1.6×10^{-7}	This work
	AdSWV	CNT-NAF/GCE	2.5×10^{-7}–1×10^{-5}	5.2×10^{-8}	This work

[a] Nanoparticles.
[b] Ionic liquid.
[c] Carbon paste electrode.
[d] Basal plane pyrolytic graphite electrode.

2.3% for L-dopa was achieved (Table 3). Using the proposed electrochemical sensor, no sample pre-treatment was required, other than precipitation of the serum proteins with acetonitrile and a dilution step with the selected supporting electrolyte. The possibility of monitoring ROP and L-dopa concentration expected after therapeutic dose could make the method proposed useful for pharmacokinetic purposes.

3.7. Comparison of analytical methods

The analytical characteristics observed during validation of the proposed methods were compared with those obtained in earlier reported electroanalytical methods for the determination of ROP and L-dopa (Table 4). The electrochemical behaviour of ROP has not been investigated to date at modified electrodes, while many electrochemical sensors have been developed for the L-dopa determination. Very recently, we have developed SWV and differential-pulse voltammetric method (DPV) for ROP determination using a bare GCE [15]. Compared with those methods (slope of the calibration graph was 4.08×10^6 μA M^{-1} for SWV and 1.92×10^5 μA M^{-1} for DPV), the better sensitivity of adsorptive stripping analytical procedure was obtained at the CNT-NAF/GCE for measurement of ROP (Table 1). Also, the LOQ values obtained at proposed sensor using AdSWV are lower than that obtained at the GCE [15]. One of the most important attributes of the CNT-NAF/GCE is its remarkably improved reproducibility without the need for additional and time-consuming mechanical polishing of the electrode surface before each measurement as in the case of GCE.

Additionally, the quantification limits of ROP obtained at developed electrochemical sensor are comparable to some HPLC methods with UV detection [6,7] and are better than that obtained in capillary electrophoresis [9], spectrophotometry [10] and high performance thin layer chromatography [8], but the detection limits are higher than LODs reported for chromatographic methods coupled to mass spectrometry [3,4,13] and spectrofluorimetric method reported previously [11]. However, spectrofluorimetric method additionally require derivatization of ROP with 4-chloro-7-nitrobenzofurazan before detection step, while LC-MS analytical procedures demand expensive and sophisticated equipment that could not be available in many laboratories. In addition, the proposed electroanalytical methods offer several advantages over chromatographic techniques applied only to the quantification of drug, including short analysis time, simplicity of operation and lower running cost.

The comparison between the analytical performance of the CNT-NAF/GCE and other electrochemical sensors described in the literature in recent two years for determination of L-dopa is also presented in Table 4. The proposed electrochemical sensor exhibited wide linear working range and appreciable detection limit compared to previously developed sensors for L-dopa quantification. The LOD obtained with adsorptive accumulation at CNT-NAF/GCE is similar or even better than those found with some other electrochemical sensors described in the literature. However, in comparison with most others it can be easily constructed in a simple procedure and the surface of electrode is also easily renewable. Moreover, the developed methods were applied for both pharmaceutical formulations and biological fluids whereas the reported literature methods [17,19,20,23,24] have been applied either for pharmaceutical or biological samples.

4. Conclusion

The result of this investigation shows that developed electrochemical sensor based on carbon nanotubes embedded into the polymer Nafion matrix is high sensitive and selective analytical tool for voltammetric determination of ROP and L-dopa. For the first time, the analytical method for simultaneous determination of ROP and L-dopa was developed. The large separation of the peak potentials at the CNT-NAF/GCE allows fast simultaneous quantification of trace amounts of both drugs in human serum samples. The very high sensitivity of the modified electrode can be related to very large surface area, low resistance, electrocatalytic effect and strong adsorption ability of the CNTs-NAF nanocomposite. The proposed sensor is also a feasible alternative for direct measurements of ROP and L-Dopa individually in pharmaceutical samples by SWV. The prepared modified electrode shows several advantages such as simple preparation procedure, high sensitivity and stability, ease of the electrode surface regeneration with excellent reproducibility. We believe that the proposed method would be a potential step forward in the development of a reverse-phase HPLC procedure with electrochemical detection for simultaneous trace determination of ROP and L-dopa co-administrated in advanced Parkinson's disease patients.

Conflict of interest

There is no conflict of interest.

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4. Electrochemical sensing of mesalazine and its *N*-acetylated metabolite in biological samples using functionalized carbon nanotube

Talanta 147 (2016) 50–58

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Electrochemical sensing of mesalazine and its *N*-acetylated metabolite in biological samples using functionalized carbon nanotubes

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ARTICLE INFO

Article history:
Received 23 June 2015
Received in revised form
9 September 2015
Accepted 12 September 2015
Available online 16 September 2015

Keywords:
Mesalazine
5-Aminosalicylic acid
Metabolite
Carbon nanotubes
Nafion
Voltammetry

ABSTRACT

A rapid analytical method without the time-consuming separation step was developed to simultaneously determine mesalazine and its *N*-acetylated metabolite. A simply designed electrochemical sensor with functionalized carbon nanotubes in a Nafion matrix was constructed for this purpose. The presence of the nanocomposite modifier on the electrode surface significantly affects the voltammetric response of target analytes. The morphology of the modified surface was investigated by scanning electron microscopy. The effect of modifier amount on the sensor performance was investigated in order to obtain the most favorable response of mesalazine since it was found in lower concentration limits in real samples then its metabolite due to the rapid drug elimination and the slightly slower renal metabolite excretion. Under optimal conditions, the anodic peak currents measured by square-wave voltammetry increased linearly after short accumulation of 30 s in the range of 5.0×10^{-8}–2.5×10^{-6} M and 1.0×10^{-7} – 5.0×10^{-6} M for drug and metabolite, respectively. In addition to stable response, the sensor has excellent performance associated with high sensitivity (2.33×10^{7} and 8.37×10^{6} μA M^{-1} for drug and metabolite, respectively). The synergistic effect of the carbon nanotubes and Nafion polymer film yielded detection limit of 1.2×10^{-8} M for mesalazine and 2.6×10^{-8} M for its metabolite that is comparable to known chromatographic methods. Due to the easy preparation and regeneration, the proposed sensor opens new opportunity for fast, simple and sensitive analysis of drug and its metabolite in human serum samples as well as direct quantification of mesalazine in delayed-release formulations.

1. Introduction

Mesalazine (5-aminosalicylic acid, 5-ASA) is an anti-inflammatory drug widely prescribed for the therapy of ulcerative colitis and Crohn's disease (Scheme 1). It may also provide protection against the development of colorectal cancer in patients suffering from inflammatory bowel diseases [1]. In addition, 5-ASA inhibits cell injury in the inflamed mucosa by scavenging reactive oxygen metabolites, thus suppressing their toxicity. The drug is absorbed quickly from the small intestine when administrated orally and therefore, modified-release dosage forms are designed to deliver drug in the terminal ileum and colon [2]. After absorption, 5-ASA is metabolized by *N*-acetyltransferase to its *N*-acetyled-5-ASA derivative (Ac-5-ASA) in the liver and intestinal mucosa. This compound is the major metabolite present in blood with a half-life of up to 10 h. In plasma, both 5-ASA and Ac-5-ASA are found 40–50% and 80%, respectively bound to proteins.

Various analytical methods were described in the literature for the determination of 5-ASA, such as liquid chromatography coupled to mass spectrometry for drug quantification in plasma samples [3], HPLC with UV detection for stability-indicating assays [4] and analysis in pharmaceutical formulations [5], capillary electrophoresis for drug impurity profiling [6], spectrophotometry [7], fluorescence spectroscopy [8], automated chemiluminescence [9] and ultra-performance liquid chromatography [10]. At present, several HPLC methods with a fluorescence [11–13], electrochemical [14,15] and mass spectrometry detection [16,17] were developed for simultaneous quantification of 5-ASA and its *N*-acetylated metabolite. The satisfactory results were obtained with excellent selectivity and low detection limit. However, the developed chromatographic methods require derivatization of drug and metabolite before detection step, tedious sample preparations, consumption of large solvent volumes and time-consuming procedures. In developed HPLC methods the analytical run time per sample took about 10–40 min.

The electroanalytical methods have proved to be useful for sensitive and selective determination of many pharmaceuticals owing to fast response, low instrument and analysis cost as well as simplicity of samples preparation [18,19]. In spite of that, no attempt has been made to this date to determine 5-ASA and its metabolite by an electroanalytical method. There are only a few

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http://dx.doi.org/10.1016/j.talanta.2015.09.036
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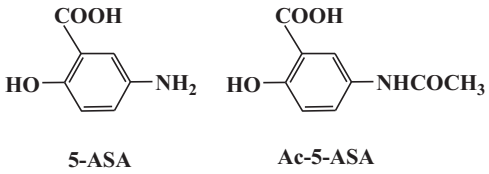

Scheme 1. Chemical structures of mesalazine (5-ASA) and its *N*-acetylated metabolite (Ac-5-ASA).

reports available on the electrochemical determination of 5-ASA. The sonolinear sweep voltammetric method was developed for determination of drug in tissue culture medium [20]. 5-ASA was quantified using glassy carbon electrode (GCE) [21] and pencil graphite electrode [22], however both methods were applied only to analysis of pharmaceutical formulations. The abrasive stripping voltammetry was used for identification of 5-ASA in commercial dosage forms [23], but proposed method was not quantitative. The amperometric catalase-peroxidase based biosensor was developed for measurement of 5-ASA concentration by indirect method monitoring dissolved oxygen level [24]. The high cost of enzymes used in the kit limits widespread application of the method for routine purpose. The oxidative behavior of 5-ASA was studied on the surface of GCE modified with nanoporous film and subsequently, electrochemically deposited polypyrrole using 1,5-naphtalenedisulfonic acid as dopant [25], however the developed method was designed only for quantitation of 5-ASA. Due to prolonged therapy and maintenance of remission in inflammatory bowel diseases, as well as the need for clinical and bioequivalence studies after the administration of newly developed delayed-release formulations, a simple, inexpensive and highly sensitive electroanalytical method is in great demand for simultaneous determination of 5-ASA and its metabolite.

Carbon based electrodes are commonly used in electroanalytical chemistry because of their low cost and wide availability. On the other hand, carbon nanotubes (CNTs) have attracted scientific interests in recent years owing to unusual mechanical strength and electrical conductivity, unique structure with huge surface area and excellent electrocatalytic activity [26]. Theirs exceptional properties offered versatile platform to employ them as the active material for the preparation of various composite electrode materials. CNTs-based sensors generally have higher sensitivities at low concentration levels or in the complex matrix, lower detection limits and faster electron transfer kinetics than traditional carbon electrodes. However, many factors need to be investigated in order to create an optimal CNTs-based sensor.

In the present study, the composite material based on the coupling of multi-walled CNTs and Nafion as cation exchange polymer was used to develop a fast and simple method for simultaneous measurement of 5-ASA and its metabolite without a separation step. The developed electrochemical sensor was successfully applied for simultaneous determination of 5-ASA and Ac-5-ASA metabolite at trace levels in serum samples using adsorptive stripping square-wave voltammetry (SWV). In addition, the proposed sensor was used to develop inexpensive and rapid method for direct measurement of active ingredient in different 5-ASA delayed-release formulations.

2. Experimental

2.1. Apparatus

Voltammetric measurements were performed using a μ-Autolab potentiostat (Eco Chemie, Utrecht, The Netherlands) controlled by GPES 4.9 software. A three-electrode cell system was used. The multi-walled CNTs and Nafion polymer modified GCE (CNTs-N/GCE) and a bare GCE (3-mm diameter, Metrohm, Switzerland) were used as a working electrode, a platinum wire and an Ag/AgCl/3 M KCl (Metrohm) were used as the counter and reference electrodes, respectively. In preliminary measurements, a bare gold electrode (2-mm diameter, Metrohm, Switzerland) and gold electrode modified in the same way as GCE were also employed as the working electrode. Prior to modification, the working electrode was polished with aqueous slurry of 0.05 μm alumina powder on a smooth polishing cloth, thoroughly rinsed with water and then ultrasonically cleaned in water for 30 s. Finally, the electrode was washed with purified water and dried. All electrochemical experiments were carried out at room temperature (23 ± 1 °C). When required, stirring was applied using a computer-controlled stirrer at ca. 300 rpm.

Scanning electron microscopy (SEM) measurement was peformed on a Jeol JSM-7000F microscope (Jeol Ltd., Tokyo, Japan).

2.2. Chemicals

5-ASA supplied by Merck (Darmstadt, Germany) was used without further purification. Ac-5-ASA metabolite, the multi-walled CNTs (> 98%, O.D. 6–13 nm, length 2.5–20 μm) and Nafion (5 wt% solution in a mixture of lower aliphatic alcohols and water) were obtained from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical grade quality. Ultra pure water used for the preparation of standard solutions and buffers was obtained by a Milli-Q system (Millipore, Bradford, USA). Salofak® (Dr. Falk Pharma, Germany) and Pentase® (Ferring GmbH, Germany) delay-released tablets containing 500 mg of 5-ASA were supplied from local pharmacy.

Stock solution of 5-ASA (1×10^{-3} M) was prepared by dissolving appropriate amount of the compound in purified water with addition of a drop of 1 M HCl to minimize the risk of 5-ASA oxidation [21] and stored in the dark under refrigeration. Stock solution of Ac-5-ASA (1×10^{-3} M) was prepared by dissolving its adequate amount in methanol. Standard solutions were prepared daily by diluting the stock solutions with a supporting electrolyte just before use. Britton–Robinson (BR) buffer solutions (0.04 M in each of acetic, phosphoric and boric acids) adjusted to the desired pH with addition of a 0.2 M NaOH were used as supporting electrolytes.

2.3. Preparation of CNTs-N/GCE

To generate carboxylic acid-functionalized surface the multi-walled CNTs (50 mg) were added to plentiful concentrated nitric acid and then sonicated for about 4 h. The suspension was filtered and washed with redistilled water to neutral. The solid powders were dried under vacuum at room temperature. One milligram of acid-treated CNTs was dispersed in 0.3% Nafion ethanol solution to give a 1.0 mg mL^{-1} black suspension with the aid of ultrasonic agitation. The nanocomposite film modified electrode was fabricated by dropping 3 μL of black suspension on cleaned working electrode surface and evaporating the solvent at room temperature. To obtain a stable cyclic voltammogram as well as strength CNTs adhesion to the electrode surface, the modified electrode was scanned prior to first measurement by two successive cyclic voltammetric sweeps between 0 and 1.5 V at 100 mV s^{-1} in a blank solution of BR buffer pH 2.0. The surface area of modified electrode was obtained by cyclic voltammetry (CV) using 1.0×10^{-3} M $K_3Fe(CN)_6$ in 0.1 M KCl electrolyte at different scan rates (ν) between −0.2 and 0.7 V. From the slope of the anodic peak current versus $\nu^{1/2}$ relation, the surface area of the CNTs-N/GCE was calculated to be 0.156 cm^2, which was about three times greater than the surface of the bare GCE. For comparison, the carbon nanotubes modified

GCE (CNTs/GCE) and the Nafion modified GCE (NGCE) were also prepared in the same way as described but without the addition Nafion and CNTs, respectively.

2.4. Electrochemical measurement procedures

The oxidative behavior of 5-ASA and Ac-5-ASA was investigated by CV in the range from 0 V to 1.0 V with the scan rate varying from 10 to 500 mV s^{-1}. Before the square-wave voltammetric determination of drug and its metabolite in the adsorptive stripping procedures, the CNTs-N/GCE was immersed in the sample solution with 30 s accumulation at 0.1 V under stirring condition. After 5 s equilibrium period, the voltammogram was recorded by applying a SWV potential scan from 0.3 to 1.0 V. The CNTs-N/GCE was applied for direct SWV measurement of 5-ASA in delay-released dosage forms. Operating instrumental conditions for SWV were: pulse amplitude of 50 mV, frequency of 70 Hz and scan increment of 2 mV. The analytes adsorbed on the modified electrode surface after measurement were readily removed by applying single positive-going CV potential scan from 0 to 1.5 V in blank supporting electrolyte.

2.5. Determination in samples

Serum samples were obtained from healthy volunteers and stored frozen until assay. Samples were fortified with appropriate aliquot volume of 5-ASA and Ac-5-ASA standard solutions to achieve final concentrations (2.5×10^{-7} and 1.0×10^{-6} M) that are found in serum after treatment with recommended daily dose of 2 g [16,27]. To remove serum proteins a 500 μL aliquot of serum sample containing drug and its metabolite was mixed with acetonitrile (1:1). After vortexing for 60 s, the mixture was centrifuged for 6 min at 6000 rpm. Appropriate volumes of clear supernatant were transferred into the volumetric flask and diluted with supporting electrolyte before voltammetric measurements. Analysis of human serum samples were performed using the standard addition method to reduce the matrix effect by adding three successive aliquots of standard solutions.

To prepare the solutions of 5-ASA delay-released dosage forms, 10 tablets were weighted and crushed to a fine powder. A quantity of finely ground material equivalent to 1.5 mg of 5-ASA was transferred into a calibrated 10.0 mL flask and dispersed in water. The content of the flask was sonicated for 10 min to provide complete dissolution of active ingredient and then completed to the volume with water. Tablet solution was then filtered through 0.45 μm Acrodisc GHP filters (Gelman, Ann Arbor, USA). An aliquot of filtrate was transferred into a calibrated flask and diluted with water to yield a final drug concentration of 1.0×10^{-4} M. A series of dilutions were made with supporting electrolyte to cover the working concentration range. Solutions were subjected to direct SWV measurements and the content of 5-ASA in the commercial pharmaceutical products was determined by standard addition method. For recovery studies, aliquots of the 5-ASA standard solutions were added to real samples prepared from tablets.

3. Results and discussion

3.1. Electrochemical behavior of 5-ASA and its metabolite on developed sensor

To improve the sensor response we examined in preliminary measurements the influence of the working electrode material, glassy carbon and gold, on oxidation peak heights of 5-ASA and its metabolite. As sensitivity did not improve when using gold electrode (the inset of Fig. 1), the GCE were used in subsequent

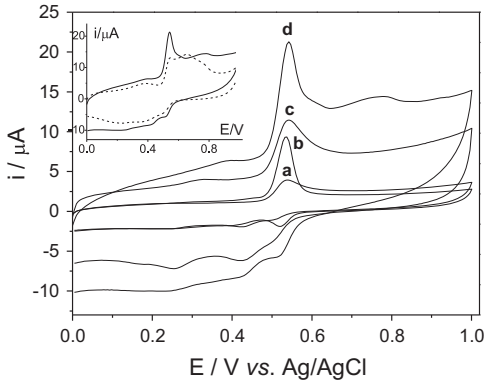

Fig. 1. Cyclic voltammograms of 5-ASA (5×10^{-5} M) at a bare GCE (a), the NGCE (b), the CNTs/GCE (c) and the CNTs-N/GCE (d) in BR buffer pH 2.0. Inset: comparison of gold electrode (dashed line) and GCE (solid line) modified with CNTs/Nafion composite film in the same conditions. Scan rate: 100 mV s^{-1}.

experiments due to their significantly lower cost. The cyclic voltammograms of 5×10^{-5} M 5-ASA solution in BR buffer pH 2.0 obtained at the bare GCE, the NGCE, the CNTs/GCE and the CNTs-N/GCE using a scan rate of 100 mV s^{-1} are presented in Fig. 1. As can be seen, 5-ASA shows one well-defined voltammetric oxidation peak in aqueous solutions and moving from the bare GCE to CNTs-N/GCE it becomes well-defined with enhanced peak current. Due to the presence of the primary aromatic amino group (pK_a=6), carboxylic group (pK_a=3) and phenolic group (pK_a=13.9) in the molecule [28], 5-ASA was completely in cationic form in experimental conditions used in the voltammetric measurements. Nafion as a cation-exchanger attracted positively charged drug molecules through electrostatic interaction from the bulk solution to enhance the anodic signal (i_p=7.25 μA). However, the voltammetric response of 5-ASA was remarkable increased at the CNTs-N/GCE with a current of 13.9 μA at peak potential of 0.54 V compared with that of using a bare GCE (i_p=2.85 μA). The synergistic effect of CNTs and cation-exchange polymer combination leads to a considerable improvement in the analytical sensitivity. To obtain information regarding the adsorptive behavior of 5-ASA at the CNTs-N/GCE, the influence of the scan rate on the electrochemical response of drug molecule was investigated by CV. The oxidation peak current of 5-ASA increased with scan rate increasing and a linear relationship was observed in the range 10–500 mV s^{-1} following the relation i_p (μA)=0.071 ν (mV s^{-1})+5.14. The oxidation peak current was significantly suppressed in second cyclic scan. Those results indicated that the electrode process of 5-ASA at the developed electrochemical sensor is controlled by an adsorption step.

5-ASA showed at slow scan rates an irreversible oxidation wave, while in the reverse scan a single reduction wave was observed at much more negative potential (0.43 V) than would be expected for reversible process. This indicated that the initial oxidation product underwent a chemical reaction to yield a second product that was reduced at more negative potentials. With scan rate increase the oxidation of 5-ASA became quasi-reversible. Furthermore, the electrochemical behavior of 5-ASA at developed sensor was studied over pH range of 2.0–5.0 in buffered aqueous media. A linear shift of the peak potential towards less positive values was observed while increasing the pH. The slope value obtained was 65.0 mV pH^{-1}. The oxidation pattern of 5-ASA molecule observed at the CNT-N/GCE was well-consistent with our previous studies at the GCE [21], thus, we can corroborate that the oxidation of drug at the nanocomposite film involves the initial $2e^-/2H^+$ oxidation of 5-ASA to the quinone imine, followed by hydrolysis to produce the corresponding quinones.

In this paper, the redox behavior of Ac-5-ASA was studied for

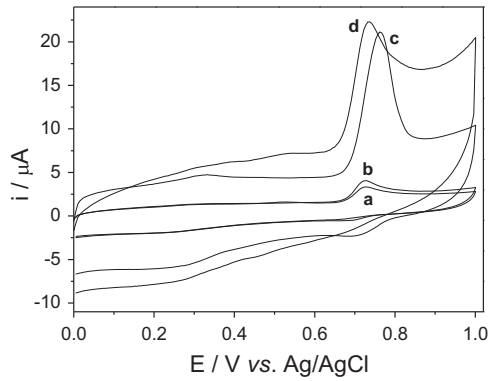

Fig. 2. Cyclic voltammograms of Ac-5-ASA (5×10^{-5} M) at a bare GCE (a), the NGCE (b), the CNTs/GCE (c) and the CNTs-N/GCE (d) in BR buffer pH 2.0. Scan rate: 100 mV s^{-1}.

the first time. The cyclic voltammograms of Ac-5-ASA in BR buffer pH 2.0 obtained at the bare GCE, the NGCE, the CNTs/GCE and the CNTs-N/GCE using a scan rate of 100 mV s^{-1} are depicted in Fig. 2. When the bare GCE surface was coated with Nafion film, the voltammetric response of metabolite did not change significantly. The metabolite molecule has a pK_a of 2.7 and 12.9 [28]. Therefore, 83.4% of the metabolite was in unionised form under used voltammetric condition and cannot not be attracted through electrostatic interaction with cation-exchange polymer. Upon modification with multi-walled CNTs, remarkable enhancement effect to the current response of metabolite was observed due to very larger surface area of the modified electrode. However, the CNTs were tightly fixed in the Nafion matrix onto GCE surface in a reproducible way enhancing the stability of proposed electrochemical sensor and improving the measurement precision. Hence, the CNTs-N/GCE exhibited convenient electroanalytical performance for simultaneous measuring of 5-ASA and Ac-5-ASA. The potential difference of 200 mV between the two peak potentials at the CNTs-N/GCE is large enough to determine the concentration of drug and its metabolite simultaneously.

A well-defined anodic current response of Ac-5-ASA was observed at 0.74 V at the CNTs-N/GCE. On the reverse potential scan, there was no corresponding reduction peak, suggesting that the electrode reaction is irreversible. The electrochemical oxidation of Ac-5-ASA was studied using CNTs-N/GCE in aqueous solutions with different pH values in the range of 2.0–5.0. The peak potentials were shifted to less positive values with increasing pH and a linear relationship was observed following the equations: E_p (V)= 0.86 − 0.057 pH (r=0.999). The linear dependence implies that the protonation reaction occurred in the overall electrode process. A slope of −0.059 V pH^{-1} is characteristic for the oxidation potential as a function of the pH in the $2e^-/2H^+$ process corresponding to the oxidation of the hydroxyl group in metabolite molecule to the corresponding ketone. The cyclic voltammetric behavior was further examined as a function of the scan rate in the range of 10–500 mV s^{-1}. The signal for Ac-5-ASA was linearly dependent on the scan rate with relation i_p (μA)=0.048 ν (mV s^{-1})+2.21 and together with the observed positive potential shift of the oxidation signals with increasing the scan rate indicate that the electrode process is an adsorption-controlled irreversible oxidation.

The surface morphology of the CNTs-N/GCE was characterized by SEM. As shown in Fig. 3a, the nanocomposite film deposited formed unique bundle structures. The SEM image of composite film (Fig. 3b) showed a uniform coating of CNTs well dispersed in polymer matrix making the surface rough with a high accessible area improving the preconcentration of 5-ASA molecules through electrostatic interaction with negatively charged sulfonate group in Nafion polymer structure as well as metabolite molecules caused by the increase in working electrode efficient area due to CNTs incorporation. On the other hand, the large surface area of the CNTs/GCE had the tubes irregularly placed in thread-like formations.

3.2. Optimization studies

To get more insights into the electroanalytical performance of the CNTs-N/GCE and in addition, to optimize its performance with respect to nanocomposite film deposition, SWV was used for further electrochemical measurement of 5-ASA and its metabolite. The mean steady state plasma levels of unchanged 5-ASA are rather lower (range 0.02–1.2 μg mL^{-1}) whereas those of Ac-5-ASA are always higher (range 0.1–2.9 μg mL^{-1}). This is due to the rapid elimination of 5-ASA ($t_{1/2}$=0.4–2.4 h) and the slightly slower renal excretion of the Ac-5-ASA ($t_{1/2}$=6–9 h) [27]. Since the drug was found in lower concentration limits then its metabolite in real samples, the effect of modifier amount on the performance of the CNTs-N/GCE was investigated in order to obtain the most favorable response of 5-ASA.

Firstly, when varying different amounts of multi-walled CNTs dispersed in Nafion ethanol solution, the concentration of 1 mg mL^{-1} was selected as optimum. The SWV response of 5-ASA was increased up to 0.5% of Nafion concentration in the immobilizing suspension due to more – SO_3^- sites in polymer film that conducted ion-exchange with drug molecules leading to an increment of accumulation capacities. At higher concentrations, the polymer film became thicker and lowered the electrical conductivity of CNTs. Furthermore, the Nafion polymer retarded the electron transfer rate of Ac-5-ASA oxidation as well as its mass transportation through the film resulting in the lower current response. The SWV response of both analytes in simultaneous analysis reached the optimal value when the composite film was uniformly coated on the electrode surface using 0.3% Nafion concentration for preparation of homogenous CNTs suspension. The thickness of the composite film was determined by the volume of

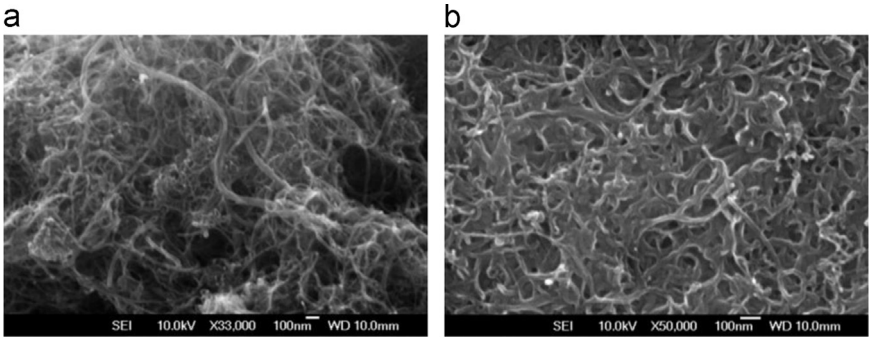

Fig. 3. The surface image of the CNTs-N/GCE obtained by scanning electron microscopy (a) and same surface at high magnification (b). Scale bar: 100 nm.

CNTs-Nafion suspension coated onto the GCE surface. When the volume of suspension increased up to 3 μL, the oxidation peak current enhanced notably for both analytes due to increment of effective surface area and more CNTs dramatically promoted electron transfer reaction, but with further increase the oxidation peak currents showed gradual decline owing to the higher electric resistance.

Additionally, various types of the supporting electrolytes, including 0.01 and 0.1 M sulfuric acid, 0.01 M hydrochloric acid, acetate buffer pH 3.5 and BR buffer pH 2.0–5.0, were tested in order to obtain better sensitivity for simultaneous determination of drug and its metabolite. A maximum current intensity and lower background current was achieved in BR buffer solution at pH 2.0 and consequently, it was employed for further studies.

To obtain much more sensitive peak currents for analytical studies, the variation of the current response recorded at the CNTs-N/GCE was monitored while changing instrumental SWV parameters, i.e. frequency ($20\ \text{Hz} \le f \le 110\ \text{Hz}$), pulse amplitude ($10\ \text{mV} \le a \le 60\ \text{mV}$) and potential step ($2\ \text{mV} \le \Delta E_s \le 10\ \text{mV}$). The most favorable responses were achieved using a combination of a frequency of 70 Hz, pulse amplitude of 50 mV and a potential step of 2 mV. Compared to other voltammetric techniques, the SWV offers several advantages such as a high speed in analysis, a wide dynamic concentration range, low consumption of electroactive compounds and reduced problems with electrode surface blocking, a high signal-to-noise ratio and a low detection limit because of its efficient discrimination of capacitance current [29].

To obtain lower detection limits the possibility of 5-ASA and its metabolite preconcentration before SWV measurement was investigated because the modification of the electrode surface led to a strong interfacial accumulation of both analytes. To achieve the optimum conditions for the maximum adsorption, the influence of accumulation potential (between −1.0 and 0.9 V) and deposition time (between 10 s and 120 s) on the voltammetric responses of both analytes was evaluated in solution containing 5-ASA and Ac-5-ASA at concentration level of 1×10^{-6} M. A maximum was observed at 0.1 V, so this accumulation potential was selected in all subsequent studies. The deposition time significantly affected the voltammetric responses of both analytes at the CNTs-N/GCE. The peak current of 5-ASA enhanced greatly within 30 s due to electrostatic interactions of positively charged drug molecules with negatively charged sulfonate groups in Nafion polymer structure (Fig. 4). The voltammetric response increased up to 60 s and then became constant at higher accumulation times. On the other hand, the peak current of Ac-5-ASA increased slowly up to 120 s revealing a constant adsorption of metabolite molecules attracted on the large surface of the CNTs-N/GCE. The increment of accumulation time period above 60 s showed a deviation of the metabolite

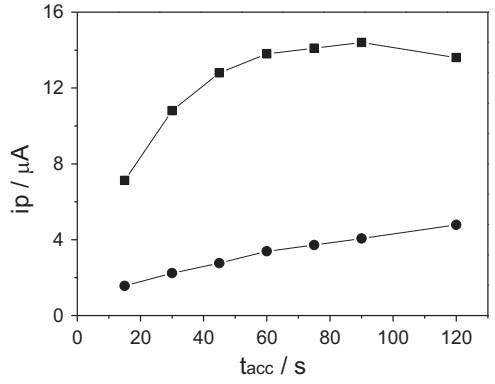

Fig. 4. Effect of the accumulation time on the oxidation peak current for 5-ASA (squares) and its metabolite (circles). SWV settings: frequency of 15 Hz, amplitude of 25 mV and potential step of 4 mV, $c = 1 \times 10^{-6}$ M.

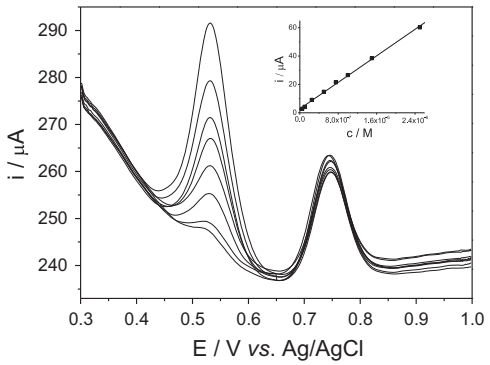

Fig. 5. Square-wave voltammograms of 5-ASA at the CNTs-N/GCE recorded in BR buffer pH 2.0 at different concentrations (5.0×10^{-8}–2.5×10^{-6} M) in the presence of its metabolite (2.5×10^{-6} M). t_{acc}=30 s, SWV settings same as in Fig. 4. Inset shows a calibration graph for the quantification of 5-ASA.

peak current from linearity, thus indicating surface adsorption saturation. The value of 30 s was considered as optimal value to achieve the highest possible sensitivity of both analytes in a more short analysis time.

3.3. Simultaneous determination of 5-ASA and its metabolite

The peak currents of 5-ASA and Ac-5-ASA at 0.53 and 0.74 V, respectively, obtained at proposed sensor were used for simultaneous determination of drug and its metabolite involving adsorptive stripping SWV. The SWV measurement in the potential range from 0.3 to 1.0 V takes only 5 s using the pulse frequency of 70 Hz with 2 mV potential step. Since the presence of metabolite in solution may affect the quantification of drug, and vise versa, the interference of each analyte in the simultaneous determination of its pairs was performed by changing one analyte concentration and keeping the other unchanged. Fig. 5 shows the SWV responses obtained for solutions of increasing drug concentration in the presence of metabolite at the fixed concentration of 2.5×10^{-6} M. The relation between the stripping peak current and concentration of 5-ASA was found to be linear over the range 5.0×10^{-8}–2.5×10^{-6} M (the inset of Fig. 5). The variation of adsorptive stripping SWV peak current versus 5-ASA concentration in the absence of metabolite gave the sensitivities of the CNTs-N/GCE towards drug oxidation of 2.33×10^{7} μA M^{-1}. The value obtained is very close (relative error is 1.1%) to the values observed in the presence of metabolite (Table 1), indicating independent adsorption of both analytes in the accumulation step at the surface of the proposed electrochemical sensor. Fig. 6 shows the voltammetric

Table 1
Validation data for determination of 5-ASA and its *N*-acetylated metabolite using developed electrochemical sensor.

Parameter	Adsorptive stripping SWV		SWV
	5-ASA	Ac-5-ASA	5-ASA
Linearity range (M)	5.0×10^{-8}–2.5×10^{-6}	1.0×10^{-7}–5.0×10^{-6}	5.0×10^{-7}–1.0×10^{-5}
Slope (μA M^{-1})	2.33×10^{7}	8.37×10^{6}	1.75×10^{6}
Intercept (μA)	2.79	−0.15	−0.56
$S_{x/y}$	0.964	0.662	0.386
SD of slope (S_b)	4.37×10^{5}	1.61×10^{5}	4.57×10^{4}
SD of intercept (S_a)	0.100	0.072	0.063
Correlation coefficient	0.999	0.999	0.999
Limit of detection (M)	1.2×10^{-8}	2.6×10^{-8}	1.1×10^{-7}
Limit of quantitation (M)	4.2×10^{-8}	8.7×10^{-8}	3.6×10^{-7}

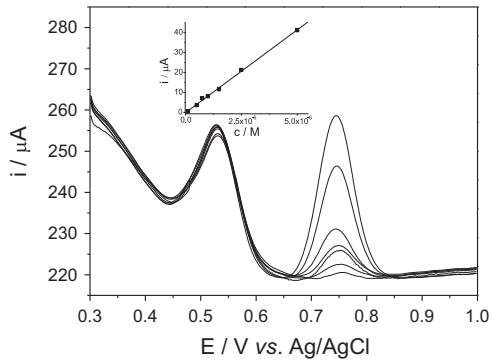

Fig. 6. Square-wave voltammograms of Ac-5-ASA at the CNTs-N/GCE recorded in BR buffer pH 2.0 at different concentrations (1.0×10^{-7}–5.0×10^{-6} M) in the presence of 5-ASA (1.0×10^{-6} M). t_{acc}=30 s, SWV settings same as in Fig. 4. Inset shows a calibration graph for the quantification of As-5-ASA.

responses for the solutions containing various concentrations of metabolite and constant concentration of 5-ASA (1×10^{-6} M). An excellent linear response in the concentration range of 1.0×10^{-7}–5.0×10^{-6} M was obtained for Ac-5-ASA (the inset of Fig. 6). The response of CNTs-N/GCE was also followed for successive increase of the metabolite concentrations in the absence of drug. The sensor revealed the sensitivity of 8.37×10^{6} μA M^{-1} with relative error of 0.7% in comparison to the slope observed in the absence of 5-ASA confirming independent preconcentration and oxidation of both analytes. Therefore, simultaneous determination of drug and its metabolite is possible without interferences. Since drug monitoring plays important role in drug quality control and has a great impact on public health, it is very important to quantify of active ingredient in pharmaceutical dosage forms. Due to the high concentration of 5-ASA in its dosage forms (500 mg per tablet), the variation of the current response at the CNTs-N/GCE with the drug concentration was monitored by direct SWV measurements. In the case of a square-wave scan without any accumulation step, the oxidation peak showed a linear response in the concentration range of 5.0×10^{-7}–1.0×10^{-5} M. The analytical characteristics and the related validation parameters of developed methods are given in Table 1. The detection and quantification limits were calculated from the calibration curves as 3*s*/*m* and 10*s*/*m*, respectively, where *s* is the standard deviation of the intercept and *m* is the slope of the calibration curve [30].

3.4. Selectivity, reproducibility and stability of developed sensor

The selectivity of the CNTs-N/GCE was investigated in solutions containing 5-ASA and Ac-5-ASA at 2.5×10^{-6} M concentration. The influence of potential interfering species, commonly present in biological samples or co-formulated with active ingredient, on the voltammetric responses of both analytes was examined. The tolerance limit was defined as the maximum concentration of the interfering substance that caused an error less than ±5% in the voltammetric responses. The experiments displayed that the oxidation peak current of 5-ASA and its metabolite at the CNTs-N/GCE did not change after adding 300-fold of glucose and 500-fold concentration of ascorbic acid indicating that the selectivity of the method may be adequate for the quantification in biological fluids where these endogenous substances are always present. In the presence of uric acid, new well-defined oxidation peak appeared at the potential around +0.66 V. However, uric acid did not influence on the peak current of 5-ASA and its metabolite up to 5-fold excess. According to the analysis of the obtained voltammetric responses, Na^{+}, Cl^{-} (2000-fold), SO_4^{2-} (1000-fold), K^{+}, NO_3^{-}, HPO_4^{2-} (500-fold), Zn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, lactate (50-fold), Fe^{3+} (10-fold), L-glycine, L-alanine, L-aspartic acid, L-glutamic acid (500-fold) and L-cysteine (200-fold) did not show interference in the determination of both analytes. The presence of citric acid, starch, lactose, sucrose (500-fold) had no influence on the peak current of 5-ASA.

The repeatability of the voltammetric response was evaluated by six replicate measurements of 1.0×10^{-6} M 5-ASA and Ac-5-ASA solution yielding unchanged oxidation peak potentials for both analytes and the RSD values of 1.7% and 1.3% for the peak currents of 5-ASA (mean i_p=26.6 μA) and Ac-5-ASA (mean i_p=8.1 μA), respectively. These results indicated that proposed electrochemical sensor was stable in repeated measurements for selective determination of 5-ASA and its metabolite. The reproducibility of the electrode response was characterized by three replicate measurements of 1.0×10^{-6} M 5-ASA and Ac-5-ASA over three days, each time using a freshly prepared the CNTs-N/GCE and standard solutions. The RSDs values of the anodic stripping currents for 5-ASA and As-5-ASA were 2.6% and 2.8%, respectively, while the RSDs of peak potentials for both drugs were no greater than 0.9%, indicating excellent fabrication reproducibility.

The proposed electrochemical sensor can be stored in air at room temperature or kept in distilled water for several weeks without the loss of activity. The stability of the CNTs-N/GCE was checked by measuring the voltammetric response in 2.5×10^{-6} M of 5-ASA and Ac-5-ASA solution over a period of four weeks. Before measurements, the modified electrode was scanned by CV between 0 and 1.5 V in the solution of blank supporting electrolyte until the response was stable. The experimental results indicated that the peak potential for both analytes was unchanged and the current response of drug and its metabolite decreased after two weeks by about 3.2% and 3.5%, respectively. Repeating the experiments after longer time, it was found that the current responses decreased about 20% in four weeks for both analytes. The experiments revealed that the prepared multi-walled CNTs-Nafion composite film has good stability and durability due to high mechanical strength of the CNTs incorporated into polymer matrix and strong adherence to electrode surface enabling long-term electrochemical sensing applications.

3.5. Analytical applications

The CNTs-N/GCE was applied for simultaneous determination of 5-ASA and its metabolite in human serum samples (Fig. 7). The expected serum concentration level for drug and its metabolite after treatment with the therapeutic daily dose recommended for ulcerative colitis and Crohn's disease are within the linear concentration range of the newly developed method using proposed electrochemical sensor. The quantitation of drug and metabolite in

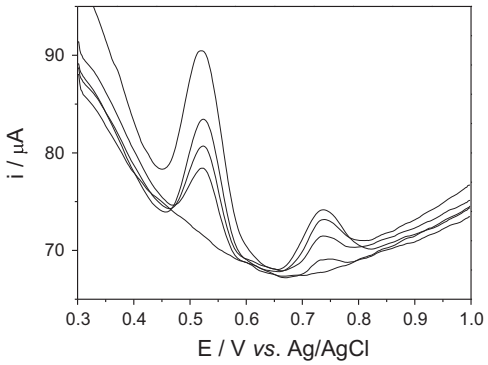

Fig. 7. Square-wave voltammograms of 5-ASA and its metabolite at the CNTs-N/GCE recorded in spiked human serum for increasing concentrations together with corresponding background recordings. t_{acc}=30 s, SWV settings same as in Fig. 4.

Table 2
Quantification of 5-ASA and its *N*-acetylated metabolite in serum samples by adsorptive stripping SWV.

Samples	Added 10^6 c (M)	Found 10^6 c (M)	Recovery (%)	RSD[a] (%)
5-ASA	0.25	0.253	101.28	2.65
	0.50	0.501	100.27	2.96
	0.75	0.745	99.33	2.28
	1.00	0.995	99.47	2.70
Ac-5-ASA	1.00	0.989	98.91	3.34
	2.00	1.964	98.22	3.11
	3.00	2.988	99.61	2.82
	4.00	4.019	100.47	2.06

[a] Average of three measurements.

human serum at the therapeutic concentration range is not feasible without analyte accumulation on the CNTs-N/GCE surface. On the other hand, the Nafion-coated membrane contains sulfonate groups and such surface layer prevents accumulation of negatively charged oxidizable ions found in biological fluids onto the electrode surface. The recovery studies of both analytes in serum samples were performed using the standard addition method to nullify any remaining interference. The mean recovery of $100.1 \pm 2.7\%$ for 5-ASA and $99.3 \pm 2.8\%$ for Ac-5ASA was achieved from this type of matrix (Table 2). From the experimental results, it is obvious that the proposed electrochemical sensor has great potentials for practical analysis of biological samples and bioequivalence studies when a new drug formulation is prepared. The knowledge of the pharmacokinetic profile of 5-ASA from the different controlled-released formulations is important for the effective therapy of inflammatory bowel diseases.

The determination of 5-ASA content in pharmaceutical formulations was carried out using the direct SWV measurements. To eliminate matrix effects 5-ASA was quantified in delay-released tablets using the standard addition method. The analysis of 5-ASA in its pharmaceutical formulations exhibited mean recoveries of 100.7% and 99.8% with relative standard deviations of 0.8% and 0.7% for Salofak® and Pentase®, respectively, indicating adequate precision and accuracy of the SWV method as well as the suitability of the developed sensor for this purpose. The effect of excipients on the voltammetric response of 5-ASA was studied by adding of known amount of drug standard solution (2.5×10^{-7} M) to the different pre-analyzed formulation solution samples. The recoveries in the range of 98.4–101.6% indicated that excipients did not interfere with assay of active ingredient. The results obtained with the described method were compared with those obtained by the reported HPLC methods [4,5]. No significant differences were found between the performances of those methods regarding the accuracy and precision. Moreover, the electroanalytical method does not require separation, sophisticated instrument and expensive solvents that are needed for HPLC procedures and only 5 s is necessary for direct SWV measurement.

3.6. Comparison with other analytical methods

The analytical characteristics of the CNTs-N/GCE were compared with those obtained by HPLC methods for the simultaneous determination of 5-ASA and its metabolite (Table 3.). The detection limits of both analytes at the CNTs-N/GCE are comparable to chromatographic methods reported previously, moreover, the achieved values are better than those reported for HPLC methods with fluorescence [12] and electrochemical detection [15]. Compared with HPLC method coupled to mass spectrometry [17], the LODs obtained with CNTs-N/GCE for both examined analytes were in a higher concentration range. However, the LOQ values obtained at the CNTs-N/GCE as 6.3 and 16.9 ng/mL for 5-ASA and Ac-5-ASA, respectively, are adequate to quantify concentration levels of 5-ASA and its main metabolite generally found in human plasma samples collected during clinical and pharmacokinetic studies in which delay-released formulations are administered at therapeutic dosage. In comparison with the previously developed chromatographic methods, the present electroanalytical method offers undoubted advantages including short analysis time, simplicity of operation and lower running cost. The HPLC methods require a high percentage of organic solvent making them less environmental friendly. Additionally, HPLC method with fluorescence detection require derivatization reaction of analytes before detection step, while LC–MS analytical procedures demand expensive and sophisticated equipment that is not available in many laboratories.

The electrochemical behavior of Ac-5-ASA has not been investigated to date, while only few electroanalytical methods were developed for the determination of 5-ASA. The comparison between the analytical performance of the CNT-N/GCE and other electrochemical sensors described earlier in the literature for the quantification of drug is also presented in Table 4. The LOQ value for 5-ASA obtained in this work is almost two orders of magnitude better than that obtained at bare GCE [21]. However, the most important advantage of the CNT-N/GCE is its remarkable improvement in the reproducibility, with no requirement for tedious mechanical maintenance by polishing the substrate surface before each measurement as in the case of GCE. Also compared with other methods, the linearity range obtained at proposed electrochemical sensor for 5-ASA was in the lower concentration range. The CNT-N/GCE with adsorptive accumulation of 30 s exhibited a similar linear range as GCE modified with nanoporous film and electrochemically deposited polypyrrole after 90 s accumulation step (slope of the calibration plot was 1.36×10^5 μA M^{-1}) [25], but the improved sensitivity was obtained in the case of CNT-N/GCE (slope of the calibration plot was 2.33×10^7). In relation to other electroanalytical methods for determination of 5-ASA, the LOD obtained with direct SWV measurement at CNT-N/GCE is similar,

Table 3
Comparison of HPLC methods for the simultaneous determination of 5-ASA and its *N*-acetylated metabolite with the present work.

	5-ASA		Ac-5-ASA		
Detection	Concentration range	LOD	Concentration range	LOD	Reference
Fluorescence	5–10,000 ng/mL	5 ng/mL	5–10,000 ng/mL	5 ng/mL	[11]
Fluorescence	100–8000 ng/mL	20 ng/mL	100–8000 ng/mL	20 ng/mL	[12]
Fluorescence	3.2×10^{-7}–4.9×10^{-6} M	5.0×10^{-8} M	1.3×10^{-7}–4.9×10^{-6} M	2.0×10^{-8} M	[13]
Electrochemical	1–300 ng/mL	1 ng/mL	10–1000 ng/mL	1 ng/mL	[14]
Electrochemical	50–3200 ng/mL	5 ng/mL	50–3200 ng/mL	10 ng/mL	[15]
Mass spectrometry	50–4000 ng/mL	15 ng/mL	50–4000 ng/mL	15 ng/mL	[16]
Mass spectrometry	2–1500 ng/mL	1 pg/mL	10–2000 ng/mL	2 pg/mL	[17]
CNTs-N/GCE	5.0×10^{-8}–2.5×10^{-6} M (7.5–375 ng/mL)	1.2×10^{-8} M (1.8 ng/mL)	1.0×10^{-7}–5.0×10^{-6} M (19.5–975 ng/mL)	2.6×10^{-8} M (5.1 ng/mL)	This work

Table 4
Comparison of the analytical parameters obtained using different electrodes and the CNTs-N/GCE for 5-ASA determination.

Technique	Electrode	Concentration range (M)	LOD (M)	Reference
Sono LSV[a]	GCE	1.0×10^{-6}–5.7×10^{-5}	3.0×10^{-7}	[20]
DPV[b]	GCE	2.0×10^{-6}–1.0×10^{-4}	8.2×10^{-7}	[21]
SWV	Pencil graphite electrodes	9.8×10^{-7}–7.3×10^{-5}	2.1×10^{-8}	[22]
Amperometry	Catalase-peroxidase based biosensor	2.0×10^{-5}–6.0×10^{-4}	–	[23]
AdLSV[c]	CNT/PPY doped by 1,5-naphthalenedisulfonic acid	1.0×10^{-8}– 1.0×10^{-6}	3.0×10^{-9}	[24]
SWV	CNTs-N/GCE	5.0×10^{-7}–1.0×10^{-5}	1.1×10^{-7}	This work
AdSWV[d]	CNTs-N/GCE	5.0×10^{-8}–2.5×10^{-6}	1.2×10^{-8}	This work

[a] Sonoelectrochemically enhanced linear sweep voltammetry.
[b] Differential pulse voltammetry.
[c] Adsorptive stripping linear sweep voltammetry.
[d] Adsorptive stripping square-wave voltammetry.

or even better using adsorptive accumulation of drug molecules on the porous thin film at its surface, than those found with other electrodes. The lower LOD value showed only the GCE modified using layer by layer procedure with nanofilm deposition and subsequently electropolimerization of pyrrole in the presence of aromatic anion dopant [25], however, the CNT-N/GCE can be easier fabricated in a simple and fast procedure and the surface of electrode is also easily renewable.

4. Conclusions

In the present work, we have developed for the first time analytical method for simultaneous determination of 5-ASA and its metabolite without the need for prior time-consuming separation step using electrochemical sensor with functionalized CNTs. A simple approach was applied for the modification of electrode surface. The presence of the nanocomposite modifier on the surface of the GCE significantly affects the response sensitivity of target analytes. Additionally, the proposed electrode showed long-term stability and very easy surface regeneration. The obtained results reveal that the CNT-N/GCE in combination with the proposed pulse voltammetric method is reliable and extremely selective tool with short response time for sensing conveniently low concentration levels of 5-ASA and its metabolite in biological samples. The reported results also imply a promising application of the CNT-N/GCE for rapid and direct measurement of 5-ASA in pharmaceutical samples providing significant advantage over previously developed methods.

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5. Development of electrochemical platform based on carbon nanotubes decorated with zirconium oxide nanoparticles for determination of nebivolol

Int. J. Electrochem. Sci., 12 **(2017)** 9675 – 9688, doi: 10.20964/2017.10.10

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Development of Electrochemical Platform Based on Carbon Nanotubes Decorated with Zirconium Oxide Nanoparticles for Determination of Nebivolol

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Received: 23 June 2017 / *Accepted:* 19 July 2017 / *Published:* 12 September 2017

Electrochemical sensor based on carbon nanotubes decorated with zirconium oxide nanoparticles was developed for the determination of nebivolol at the potential of +1.05 V (*vs.* Ag/AgCl). Nanomaterials were dispersed in an anionic polymer and deposited by simple one-step casting method on glassy carbon electrode. Scanning electron microscopy and energy dispersive X-ray spectroscopy were used to study the surface morphology and structure characterization of deposited film. The sensor has a strongly improved sensitivity (9.29×10^{-6} μA M^{-1}). The electrocatalytical peak current of nebivolol shows a linear response from 100 nM to 10 mM with detection limit of 12 nM. Low-cost analysis of drug in serum samples was carried out after adsorption of nebivolol at the nanocomposite using differential pulse voltammetry. In comparison with HPLC method, the electrochemical method ensures faster and simpler quantification of nebivolol in pharmaceutical samples.

Keywords: Nebivolol, Zirconium oxide nanoparticles, Carbon nanotubes, Differential-pulse voltammetry, Nafion

1. INTRODUCTION

Nebivolol (NBV) is a recently approved beta(1)-selective blocker drug for the treatment of hypertension. According to the World Health Organization, hypertension is one of the leading causes of premature death worldwide. Hypertension increases the risk for a variety of cardiovascular diseases, including stroke, coronary artery disease, heart failure, atrial fibrillation and peripheral vascular disease [1]. NBV has additional mechanism besides beta-blocking effects that contribute to its unique pharmacologic profile. It has an endothelium dependent vasodilator property mediated via L-arginine-nitric oxide pathway [2]. Nitric oxide also inhibits platelet and leucocyte adhesion to vascular

endothelium, prevents smooth muscle hyperplasia following vascular injury and scavenging superoxide anion activity.

The development of sensitive analytical methods for determination of NBV is highly required due to the important role of the drug in treatment of chronic diseases. In addition, an official method for its quantification in bulk form and pharmaceutical formulations has not been approved in any pharmacopoeia. Several analytical methods were reported for drug quantification in biological samples and pharmaceuticals including LC-MS methods [3,4], HPLC methods with UV detection [5-7], high performance thin layer chromatography [8], spectrophotometry [9] and spectrofluorimetry [10] Among these methods, electrochemical techniques usually provide greater sensitivity, in addition to its other outstanding features such as rapid response, low cost, easy operation, short analysis time and excellent potential for miniaturization and portable equipment construction [11, 12].

At present, in the literature only three methods were published on the voltammetric behaviour of NBV. The poorly defined voltammetric response was obtained on an electrode modified with undoped silver oxide nanoparticles using two-electrode system [13]. The proposed method was not fully validated and it was not applied to real samples of bulk drug or its pharmaceutical dosage forms. We used unmodified boron-doped diamond electrode for rapid square-wave voltammetric determination of NBV [14], but the working range of the method is suitable for quantification of NBV in its pharmaceutical dosage forms. Very recently, graphene nanocomposite modified electrode was employed for determination of NBV at relatively high potential of 1.33 V versus Ag/AgCl [15].

One of the most widely used nanostructure materials for electrode surface modification is carbon nanotubes (CNTs) [16]. The CNTs are very attractive in the field of sensor development due to their advantageous properties for electroanalysis, such as enhanced electronic properties, high electrical conductivity, excellent chemical stability and high surface-to-volume ratio [17]. Therefore, the CNTs-based sensors generally have higher sensitivities and lower detection limits than traditional carbon electrodes [18]. Also, CNTs have the ability to support metal nanoparticles (NPs). The combination of metal NPs with CNTs can provide a promising way for fabrication of novel sensors with significantly improved performance [19]. Metal exhibits very important physical, optical and electrocatalytic properties that can be improved by mixing with CNTs without losing any of the electronic properties of CNTs. There are several methods of preparing metal NPs-CNT nanocomposites including electrochemical deposition method, electroless deposition, dispersion of metal NPs on the functionalized CNTs and physical methods. For successful external decoration of CNTs, metal NPs could be obtained from bulk metals or the interaction of CNTs with already prepared suspensions of metal NPs [20, 21].

In recent years, the use of zirconium dioxide nanoparticles (ZrO_2 NPs) is rapidly growing in biomedical fields. They are widely used as drug delivery carriers for controlled release of medicines, as gene delivery vehicles with target specificity for some tissues, biocompatible matrix for protein immobilization as well as in orthopedics for improving the properties of traditional bone cements [22]. ZrO_2 NPs have also been employed for construction of various electrochemical sensors and biosensors due to their large surface area, chemical inertness, thermal stability, mechanical strength and lack of toxicity [23-25]. ZrO_2 is naturally available, environmentally friendly and neutral bioceramic material with good potential to be extensively used in pharmaceutical and medical applications.

In this paper, glassy carbon electrode modified with carbon nanotubes and ZrO_2 nanoparticles (ZrONP-CNT/GCE), obtained by simple one-step modification, was used to provide sensitive method for the voltammetric determination of NBV. Nafion is a useful material to enhance the adsorption of the drug molecules onto the electrode [26, 27] and therefore, the nanomaterials were dispersed in a Nafion matrix to ensure high sensitivity. The developed adsorptive stripping differential-pulse voltammetric (AdDPV) method was used for NBV analysis in serum samples. The modified electrode showed excellent performance for direct measurements of NBV in pharmaceutical dosage forms with good accuracy. The voltammetric data were estimated with those obtained by validated HPLC method.

2. EXPERIMENTAL

2.1 Chemicals

Nebivolol hydrochloride was obtained by the Agency for Medicinal Products and Medical Devices (Zagreb, Croatia). Nibel® tablets (Belupo, Croatia) containing nebivolol hydrochloride, equivalent to 5 mg of NBV, were supplied from a local pharmacy. ZrO_2 nanoparticles (purity 99.95%, crystal phase monoclinic), average particle size 20 nm, were purchased from US Research Nanomaterials, Inc. (Houston, USA, http://www.us-nano.com). The multi-walled CNTs (>98%, O.D. 6-13 nm, length 2.5-20 μm), Nafion (5 wt % solution in a mixture of lower aliphatic alcohols and water) and propranolol hydrochloride were obtained from Sigma-Aldrich (Steinheim, Germany). Bisoprolol fumarate, carvedilol and atenolol were supplied from Pliva (Zagreb, Croatia). Hydrochlorothiazide was purchased from Fluka (Laramie, USA).

Stock solutions of NBV (1.0×10^{-3} M) for voltammetric measurements were prepared in ultra-pure water and stored at 4 °C. The standard solutions were prepared by appropriate dilution of these stock solutions with the selected electrolyte just before use. The supporting electrolytes were 0.5, 0.1 and 0.01 M H_2SO_4, 0.1 and 0.01 M HCl and Britton-Robinson buffer adjusted to the desired pH with 0.2 M sodium hydroxide solution.

2.2 Equipment and Conditions

Voltammetric measurements were taken at room temperature using a computer-controlled μ-Autolab potentiostat (Eco Chemie, Utrecht, The Netherlands) with GPES 4.9 software. A three-electrode configuration was used with a bare or modified GCE (3-mm diameter, Metrohm, Switzerland) as working electrode, Ag/AgCl (KCl 3M, Metrohm) reference electrode and a platinum counter electrode. Cyclic voltammetry (CV) was carried out from 0.5 to 1.5 V with the scan rate from 10 to 350 mV s^{-1}. Differential pulse voltammetry (DPV) was used for determination of NBV in the potential range from 0.6 to 1.4 V. The accumulation of NBV was carried out at 0 V with a 240 s step using a computer-controlled stirrer. The pulse amplitude of 50 mV, pulse width of 75 ms and scan rate of 20 mV s^{-1} were used. After each measurement, the modified electrode was refreshed in blank electrolyte by one CV scan from 0.5 to 1.4 V until the peak of NBV disappeared completely.

The structure and morphology of nanocomposite was characterized by a field emission scanning electron microscope Jeol JSM-7000F coupled with an energy dispersive X-ray spectroscopy (Jeol Ltd., Tokyo, Japan). HPLC analysis were performed using an Agilent 1100 Series LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector, controlled by ChemStation software.

2.3 Preparation of the ZrONP-CNT/GCE

Before modification, the working electrode was polished with 0.05 μm alumina slurry, rinsed with water and then cleaned ultrasonically for 30 s in water and dried. The suspension (1 mg/mL) was prepared by dispersing the ZrO_2 NPs and functionalized CNTs in 0.5% Nafion ethanol solution to obtain mixture containing a ratio in weight of 1:1 (ZrO_2 NPs-CNTs) under ultrasonic stirring for 2 h prior to use. The carboxylic acid functionalized CNTs were prepared according to the previously described method [28]. The GCE was modified by casting 5 μL of suspension on an electrode surface and dried at room temperature. To obtain a stable cyclic voltammogram and strength adhesion to the electrode surface, the ZrONP-CNT/GCE was scanned prior to the first measurement by two successive cyclic voltammetric sweeps between 0 and 1.5 V at 100 mV s^{-1} in a blank solution of 0.1 M H_2SO_4. To measure the electroactive surface area of the ZrONP-CNT/GCE, the cyclic voltammograms of 1.0×10^{-3} M $K_3Fe(CN)_6$ as redox probe were recorded. According to the Randles-Sevcik equation the electroactive area was calculated to be 0.311 cm^2, nearly 5.5 times greater than GCE [16]. For comparison of electrochemical performance, the ZrO_2 modified GCE (ZrONP/GCE), the carbon nanotubes modified GCE (CNT/GCE) and the Nafion modified GCE (Naf/GCE) were also prepared in the same way as described but without the addition of CNTs, the ZrO_2 NPs and both nanomaterials, respectively.

2.4 Application of the ZrONP-CNT/GCE

To prepare the sample of NBV dosage forms, a quantity of finely ground tablets equivalent to 4.4 mg of NBV was dispersed into a calibrated 10.0 mL flask. The mixture was sonicated for 10 min. The mixture was filtered through 0.45 μm Acrodisc GHP filters (Gelman, Ann Arbor, USA) and was diluted with supporting electrolyte to prepare the solutions in certain concentrations. Solutions were analysed by direct DPV measurements. The content of NBV in the commercial pharmaceutical products was determined by standard addition method. For recovery studies, aliquots of the NBV standard solutions were added to samples prepared from tablets. The NBV determination by HPLC was carried out according to procedure described earlier [14].

Serum samples were collected from healthy volunteers and stored at -20°C until assay. Samples were fortified with aliquot volume of NBV standard solution to achieve concentrations (1.0×10^{-7} M) found in serum after treatment with recommended dose of 10 mg [29]. A 500 μL aliquot of serum sample containing drug was vortexed with acetonitrile (1:1) for 60 s and than centrifuged for 6 min at 6000 rpm. The suitable volumes of supernatant were transferred into the volumetric flask and diluted

with supporting electrolyte before AdDPV measurements. Analysis of human serum samples were performed using the standard addition method.

3. RESULTS AND DISCUSSION

3.1 Morphological characterization of ZrO_2 NPs-CNTs nanocomposite

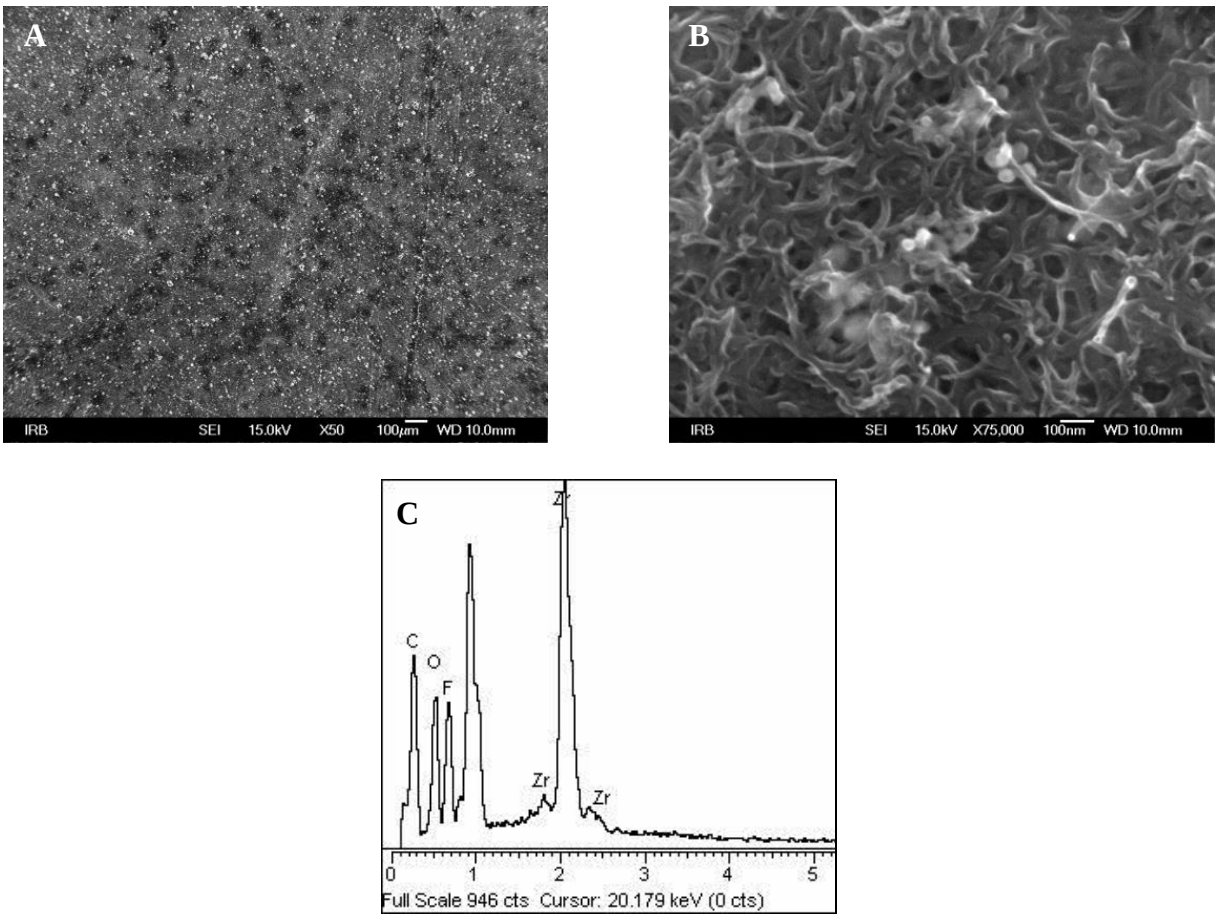

Figure 1. SEM images of the ZrONP-CNT/GCE with different magnifications (A, scale at 100 μm and B, scale at 100 nm) and its corresponding EDS spectra (C).

The surface morphology of the ZrONP-CNT/GCE was examined by a field emission scanning electron microscopy (FE-SEM). As shown in Fig. 1a, low-magnification FE-SEM image indicates that homogeneous film is very uniformly deposited onto the whole surface. The compact coating reveals ZrO_2 NPs observed as white dots well dispersed in CNTs-Nafion matrix. Generally, coating was well adherent to the substrate. The Fig. 1b displays the same surface at high magnification. The unique structure of CNTs with tubes placed in the bundle reveals a large surface area of the coating with ZrO_2 NPs entrapped into the matrix. The ZrO_2 NPs are in uniform spherical shape with average diameter of about 20 nm.

The structure characterization of the ZrO_2 NPs-CNTs nanocomposite was examined using the energy dispersive X-ray spectroscopic (EDS) pattern (Fig. 1c). EDS analysis shows presence of zirconium at 2.10 keV and oxygen at 0.54 keV. The EDS pattern presents carbon peak at 0.26 keV attributed to the carbon nanotubes. The oxygen signal detected also originates from carboxyl and hydroxyl groups introduced onto the CNT surfaces in performed functionalisation procedure [16]. The fluor detected at 0.66 keV is present from Nafion. The peak corresponding to the copper appears at 0.90 keV and comes from the supporting copper grid. The composition of nanomaterial on electrode surface was calculated using the EDS measurement and presented 34.86% of carbon, 18.10% of oxygen, 16.95% of fluor and 30.12% of zirconium.

3.2 Electrochemical properties of NBV on ZrONP-CNT/GCE

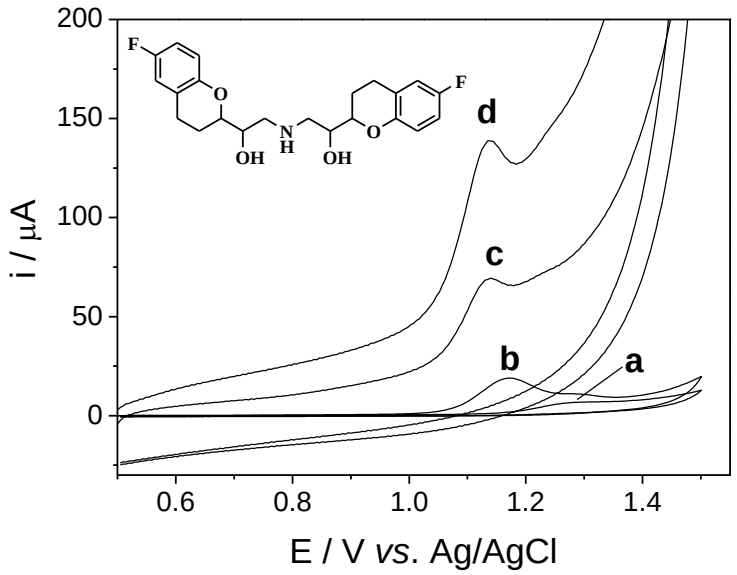

Figure 2. Cyclic voltammograms of NBV (1.0×10^{-4} M) in 0.1 M H_2SO_4 recorded on different electrode surfaces: bare GCE (a), ZrO/GCE (b), CNT/GCE (c) and ZrONP-CNT/GCE (d). Scan rate: 100 mVs^{-1}. Inset: Chemical structure of NBV.

As can be seen in Fig 2, NBV exhibits a weak irreversible voltammetric response (5.6 μA) at 1.28 V using a bare GCE. When the GCE surface is modified with ZrO_2 NPs in Nafion matrix, the response is increased with a current of 18.1 μA. The oxidation peak potential is shifted to less positive values by 110 mV. To enhance sensitivity towards drug molecule, the GCE functionalized with CNTs was also tested under the same condition [16]. After modification of electrode surface, the oxidation peak current intensity on the CNT/GCE is about 7.8-fold higher than that on the bare GCE. Finally, the ZrONP-CNT/GCE reveals a remarkable increase of the voltammetric response of NBV obtained from the combined effects of CNTs and ZrO_2 NPs. Compared with the response obtained at bare GCE, the oxidation peak current is increased up to 88.7 μA at the ZrONP-CNT/GCE with a well-defined response at 1.13 V shifted by 152 mV to less positive potential. This finding is due to increased

electroactive surface area of the ZrO_2 NPs-CNTs nanocomposite as well as the the electronic conductivity and electrocatalytic activity of CNTs modifying layer [16]. Nafion polymer was used to fix the nanomaterials tightly onto GCE [30]. Additionally, the sulfonate groups in Nafion have ability to attract positively charged NBV prior to quantification in order to increase sensitivity.

The effect of scan rate on the oxidative peak currents at the ZrONP-CNT/GCE was also evaluated. In the range of 10 - 350 mV s^{-1} a linear relationship between the peak current and the scan rate was obtained. This result indicates adsorption-controlled process at the ZrONP-CNT/GCE. The dependence of the peak potential is linear with logarithm of the scan rate with a slope of 82.6 mV/decade, indicating αn = 0.72. Using the calculated value of the charge transfer coefficient (0.40), the number of electrons exchanged was found to be n=1.8.

It is known that alcohols are oxidised in many cases in $2e^-/2H^+$ process to ketones as stable intermediates [31]. The voltammetric response observed at ZrONP-CNT/GCE corresponds to the oxidation of one of the secondary hydroxyl groups to the corresponding C=O group in drug molecule (inset of Fig. 2). The same mechanism was proposed for electrooxidation of NBV at boron-doped diamond electrode [14] and graphene nanocomposite modified electrode [15] as well as for other beta-blocker drugs such as propranolol [32] and atenolol whose oxidation product was isolated and characterized by ^{1}H NMR [33].

3.3 Optimisation of ZrONP-CNT/GCE preparation

The relationship between voltammetric signal of NBV at the ZrONP-CNT/GCE and different concentration ratios of ZrO_2 NPs : CNTs (1:1, 2:1) and Nafion concentration (0.3-0.7% v/v) in suspension for surface modification was studied by DPV. The most favorable peak current was achieved at concentration ratio of 1:1 (ZrO_2 NPs : CNTs) with the ZrO_2 NPs concentration of 1 mg mL^{-1} in 0.5% Nafion ethanol solution. The peak current decreased with the increase of ZrO_2 NPs applied on the electrode surface over the concentration of 1 mg mL^{-1}. The decrease of peak current at higher amount of ZrO_2 NPs may be due to lower adhesion between the nanocomposite film and the electrode surface as well as the agglomeration of the nanoparticles [34]. The 0.5% Nafion concentration was the optimized loading of nanomaterials. The film became thicker at higher concentrations hindering the conductivity of nanomaterials doped in polymer.

To obtain a much more sensitive peak current for analytical studies, the variation of NBV current response was examined at the ZrONP-CNT/GCE in different supporting electrolytes, such as sulfuric acid, hydrochloric acid and Britton-Robinson buffer (pH 2-6) [28]. The comparison of voltammograms indicates that NBV exhibits higher peak current at lower pH values. The NBV (pKa=8.9) is completely protonated on the nitrogen atom in acidic media and therefore, more effectively attracted to the modified electrode surface. Analysing the current response, 0.1 M sulfuric acid was chosen as the optimum supporting electrolyte for subsequent analytical measurements.

3.4 Adsorption properties of NBV and analytical performance

Since CV detected some adsorption of NBV at the ZrONP-CNT/GCE, the possibility of analyte preconcentration at electrode surface before DPV measurements was investigated. The differential pulse voltammograms without accumulation and after a 240 s accumulation step at the ZrONP-CNT/GCE are shown in Fig. 3. The improved sensitivity is achieved due to adsorption of NBV through electrostatic interaction with sulfonate group in Nafion matrix [30]. To maximise the DPV signal, the influence of accumulation potential and preconcentration time was estimated in solution containing NBV at 5×10^{-7} M concentration. A maximum enhancement of the voltammetric response was observed at accumulation potential of 0 V. The peak current magnified linearly up to 300 s preconcentration time (inset of Fig. 3), however to achieve the highest sensitivity in shorter analysis time, the value of 240 s was taken as the optimum for NBV quantification.

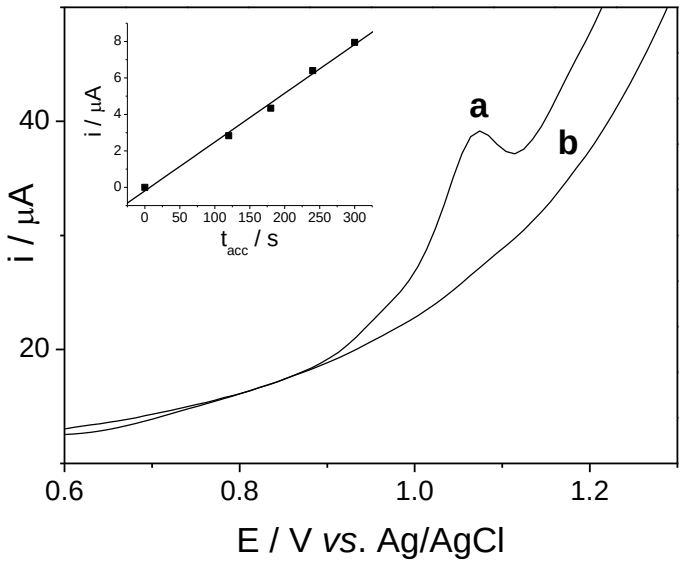

Figure 3. Differential-pulse voltammograms of NBV (5.0×10^{-7} M) at the ZrONP-CNT/GCE in 0.1 M H_2SO_4 after preconcentration time of 240 s (a) and without preconcentration (b). DPV settings: pulse amplitude of 50 mV, pulse width of 75 ms and scan rate of 20 mV s^{-1}, E_{acc} = 0 V. Inset: Influence of the preconcentration time on the NBV oxidation peak current.

Table 1. Analytical parameters for the calibration curves of nebivolol determination using ZrONP-CNT/GCE

Parameter	**DPV**	**AdDPV**
Linearity range (M)	$1.5 \times 10^{-6} – 1.0 \times 10^{-4}$	$1.0 \times 10^{-7} – 6.0 \times 10^{-6}$
Slope (μA M^{-1})	4.36×10^{5}	9.29×10^{6}
Intercept (μA)	-0.37	0.79
$S_{x/y}$	16.21	16.85
SD of slope (S_b)	1.39×10^{4}	2.01×10^{5}
SD of intercept (S_a)	0.072	0.058
Correlation coefficient	0.996	0.998
Limit of detection (M)	5.5×10^{-7}	1.8×10^{-8}
Limit of quantitation (M)	1.6×10^{-6}	6.2×10^{-8}

The electrocatalytic peak current of NBV obtained at 1.05 V using the ZrONP-CNT/GCE was employed for determination of drug in aqueous solution. In the case of a DPV scan without preconcentration, the oxidation peak showed a linear response in the concentration range of 1.5×10^{-6} – 1.0×10^{-4} M. By using drug accumulation at the ZrONP-CNT/GCE prior to DPV measurement (Fig. 4), the current response after 240 s accumulation showed linear relationship in the range of 1.0×10^{-7} – 6.0×10^{-6} M (inset of Fig. 4). The analytical parameters are given in Table 1. The detection limit (LOD) and the quantification limit (LOQ) were estimated as LOD = 3s/b and LOQ = 10s/b, where s is the standard deviation of the intercept and b is the slope of the calibration curve [35].

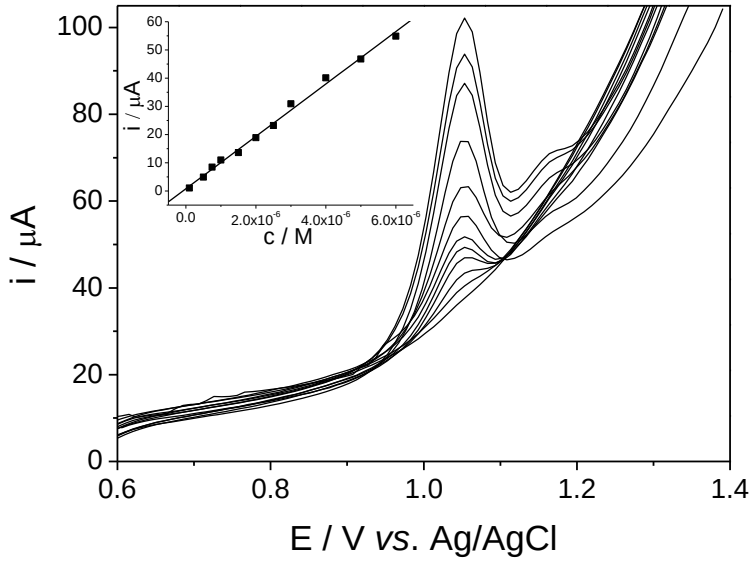

Figure 4. Adsorptive stripping differential-pulse voltammograms of NBV at ZrONP-CNT/GCE in 0.1 M H_2SO_4 recorded at different concentrations (1×10^{-7} – 6×10^{-6} M) after preconcentration time of 240 s with corresponding background recording. DPV settings same as in Fig. 3. Inset: calibration graph for NBV quantification.

3.5 Selectivity, precision and stability of ZrONP-CNT/GCE

The effect of different possible interfering species in the analysis of NBV was studied in the mixed solutions of these species with 2.5×10^{-6} M NBV using AdDPV method. The tolerance limit was calculated as molar ratio of NBV/interference which gave an error less than ±5% in the determination of the drug [36]. It was found that some ions such as Na^+, K^+, Cl^-, HPO_4^{2-} had no influence on NBV response at concentration of about 1000-fold of NBV. The experiments also revealed that NBV response did not change after adding 500-fold of ascorbic acid, lactose and citric acid as well as 400-fold of glucose. In the presence of uric acid, new well-defined oxidation peak was registered at the potential +0.49 V. However, the results showed that 100-fold concentration of uric acid did not cause an increase in the NBV current as well as dopamine present in equal concentration. However, the interference effect was observed with equal concentrations of other beta-blockers like atenolol, carvedilol, propranolol and bisoprolol. In the presence of propranolol and carvedilol, new

oxidation peaks were found in the voltammograms (+0.75 V for propranolol and +0.96 for carvedilol) with a significant influence on the distortion of the NBV response (-10.1% and -38.9%, respectively). On the other hand, atenolol and bisoprolol did not display well-developed voltammetric response in investigated potential range; however, these substances caused a positive error (+9.3% and +25.4%, respectively) in the NBV current. Furthermore, the interference studies showed that the equal concentration of hydrochlorothiazide caused an increase of 46.4% in the NBV current response. Hydrochlorothiazide is used for the treatment of hypertension, separately or together with NBV in a combined pharmaceutical formulation. Its oxidation potential (+1.02 V) is very close to that of NBV, resulting in overlapping voltammetric responses (Fig. 5).

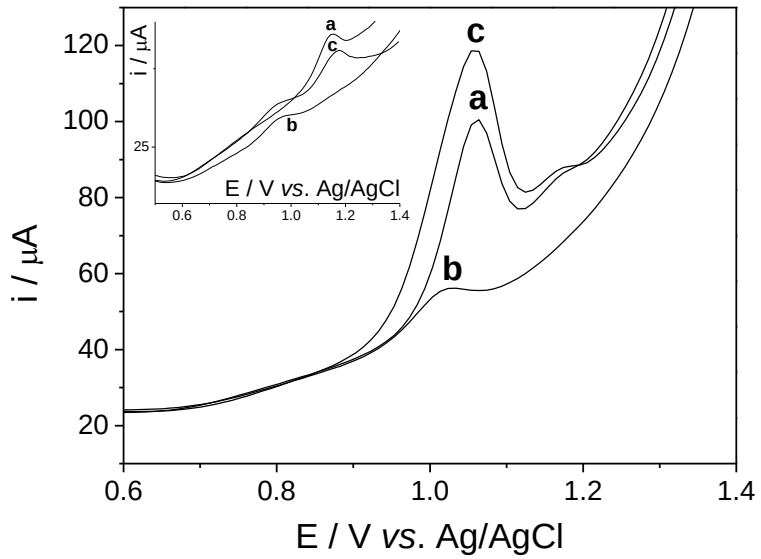

Figure 5. Comparison of voltammetric responses of NBV (line a), hydrochlorothiazide (line b) and their mixture (1:1) (line c) recorded at ZrONP-CNT/GCE in 0.1 M H_2SO_4. DPV settings same as in Fig. 3. Inset: voltammetric responses recorded in BR buffer pH 4.

To obtain the differentiation of the responses, the influence of supporting electrolyte pH value (0.1 M sulfuric acid and BR buffer pH 4) on peak potentials was studied on a mixture containing both drugs. With the increase of pH, the peak potential of hydrochlorothiazide was shifted to less positive value (inset of Fig. 5). Unfortunately, the potential difference obtained at the ZrONP-CNT/GCE was not large enough to overcome this problem. Therefore, the ZrONP-CNT/GCE cannot be applied to simultaneous analysis of NBV and hydrochlorothiazide as well as studied beta-blockers.

For method validation, the precisions of the electrode response were evaluated by adsorptive stripping DPV measurements of 2.5×10^{-6} M NBV solutions. Unchanged oxidation peak potential and the RSD value of 1.1% for peak current (mean i_p = 23.2 μA) were estimated for intra-day measurements with the same modified electrode. The inter-day precision of the electrode response was tested by three replicate measurements over three days [16], each time using a freshly prepared ZrONP-CNT/GCE. The RSD value of the peak current was 2.8%, while the RSD of peak potential for was 0.4%, indicating excellent fabrication reproducibility.

The stability of the ZrONP-CNT/GCE was examined by recording the voltammetric response over the course of two weeks. The modified electrode was kept at room temperature between experiments. The peak potential was unchanged, while the drug current response decreased after a 10-day use by 4.1%. After this period the signal decreased progressively. To explore a reusable application of electrochemical sensor the renewability of the ZrONP-CNT/GCE was examined considering the adsorption of NBV or its oxidation product on the modified surface. A simple electrochemical approach to clean the used sensor was used by running CV between 0.5 and 1.4 V in blank supporting electrolyte. The voltammetric responses obtained at regenerated ZrONP-CNT/GCE after CV scan were compared with the ones obtained during its first-time use. The regenerated sensor exhibited similar responses (recovery of 99.1±2.5% based on three measurements) indicating that the adsorbed molecules were successfully removed through the cleaning process.

3.6 Analytical applications

The ZrONP-CNT/GCE in combination with the DPV was applied for direct measurement of NBV in pharmaceutical formulations using the standard addition method in order to eliminate matrix effects. The assay showed that drug content of pharmaceutical product is in good agreement with the declared value (Table 2).

Table 2. Analysis of NBV in pharmaceutical dosage forms by the proposed DPV method at ZrONP-CNT/GCE and HPLC method

	DPV	HPLC
Label value (mg)	5	5
Determined value (mg)[a]	4.92	4.91
Recovery %	98.3	98.2
RSD %	1.15	0.64
Bias %	-1.60	-1.80
Added (M)	1.00×10^{-5}	3.00×10^{-5}
Found (M) [a]	0.98×10^{-5}	2.96×10^{-5}
Recovery %	98.1	98.7
RSD %	1.28	0.87
Bias %	-2.00	-1.33
F [b]	3.21	-
t [b]	0.85	-

[a] n=5

[b] The theoretical values of F and t-test at 95% confidence limit are 6.39 and 2.31, respectively.

The recovery study was carried out by adding known concentration of standard to the formulation solution to define the accuracy of the new method using ZrONP-CNT/GCE. In addition, the results obtained with the voltammetric method were compared with those obtained by developed

reverse-phase HPLC method [14] for NBV (Table 2). The results of the student *t*-test and variance ratio *F*-test show that there are no significant differences between the methods regarding the accuracy and precision. This finding revealed that the ZrONP-CNT/GCE can be used as a reliable and inexpensive electrochemical platform for quantification of NBV in pharmaceutical preparation. Moreover, the method developed at the ZrONP-CNT/GCE is faster and simpler in comparison with HPLC (the retention time of NBV was 17.6 min) [14].

The serum concentration of NBV administered in therapy of hypertension and cardiovascular diseases, the leading cause of the death in the world, can be monitored only after preconcentration of the drug on the modified electrode surface. To check the potential of ZrONP-CNT/GCE for use in analysis of NBV at trace level, the AdDPV method was applied to drug determination in human serum. NBV is on the list of prohibited substances in certain sports according to list of several anti-doping agencies. Therefore, the ZrONP-CNT/GCE was also tested for screening of drug intake or abuse. The standard addition method was used for the recovery studies in serum samples. The results are presented in Table 3. From mean recovery of 100.8±1.7%, it is obvious that the ZrONP-CNT/GCE has potential to determine of NBV in serum samples.

Table 3. Quantification of NBV in serum samples at ZrONP-CNT/GCE by adsorptive stripping DPV

Added 10^7 c (M)	Found 10^6 c (M)	Recovery (%)	RSD[a] (%)
1.50	1.51	100.67	1.99
2.50	2.52	100.80	2.21
5.00	5.09	101.87	2.16
7.50	7.49	99.87	0.61

[a] average of three measurements

3.7. Comparison of analytical methods

The ZrONP-CNT/GCE was compared with other electrochemical sensors developed for the determination of NBV (Table 4). The higher sensitivity was obtained at the ZrONP-CNT/GCE for measurement of NBV and wider linear range was achieved allowing the use of method for both applications, pharmaceutical products and biological samples. The LOD value obtained at the ZrONP-CNT/GCE using adsorptive stripping DPV is in lower concentration range than that obtained at other electrodes, except for the electrode modified with undoped silver oxide nanoparticles [12]. However, it is unclear whether the current response at this electrode has changed with concentration, and the method was not fully validated. The method developed using unmodified boron-doped diamond electrode is faster but obtained sensitivity is lower compared to the ZrONP-CNT/GCE.

In relation to the other developed analytical methods for determination of NBV, the linearity obtained at the ZrONP-CNT/GCE is in a lower concentration range compared to the previously reported HPLC methods with UV detection [6,9]. The LOQ value obtained by AdDPV is better than those found with HPLC using UV detector [6,9], high performance thin layer chromatography [8],

spectrophotometry [9] and comparable with spectrofluorimetry [10]. The LOD value is higher than LODs reported for LC-MS method [4,5], but this technique is not widely available.

Table 4. Comparison of various voltammetric methods for the determination of NBV with the present work

Technique	Electrode	Concentration range (M)	Sensitivity ($\mu A\ M^{-1}$)	LOD (M)	Reference
I-V[a]	Undoped AgO NPs/GCE	$5.5 \times 10^{-9} - 9.9 \times 10^{-5}$	3.48×10^{3}	0.9×10^{-9}	12
SWV[b]	BDDE	$2.5 \times 10^{-7} - 1.5 \times 10^{-5}$	3.46×10^{5}	3.2×10^{-8}	13
AdDPV	Graphene-Nafion/GCE	$5.0 \times 10^{-7} - 2.4 \times 10^{-5}$	4.52×10^{5}	4.6×10^{-8}	14
DPV	ZrONP-CNT/GCE	$1.5 \times 10^{-6} - 1.0 \times 10^{-4}$	4.36×10^{5}	5.5×10^{-7}	This work
AdDPV	ZrONP-CNT/GCE	$1.0 \times 10^{-7} - 6.0 \times 10^{-6}$	9.29×10^{6}	1.8×10^{-8}	This work

[a] I-V measurement in two electrode system

[b] Square-wave voltammetry

[c] Boron-doped diamond electrode

4. CONCLUSION

A simple modification of a GCE with ZrO_2 NPs and carboxylic acid functionalized multi-walled CNTs was examined and elaborated. Nanocomposite film displayed high electrocatalytic activity to oxidation of NBV. DPV and AdDPV were used to investigate the sensitivity of ZrONP-CNT/GCE to drug molecule oxidized at 1.03 V. The modified electrode showed a wide linear range for NBV with high sensitivity. The ZrONP-CNT/GCE is able to detect NBV in serum samples. This modified electrode can be used with short response time for direct measurements of NBV in pharmaceutical dosage forms with satisfactory results in comparison with HPLC method.

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6. GENERAL DISCUSSION

Due to strict and highly demanding requirements regarding the quality of pharmaceuticals, imposed by the pharmacopoeias, pharmaceutical industry and quality control laboratories, there is an ever-present challenge of developing reliable, more robust and selective methodologies with short analysis times, minimizing the influence of excipient substances and matrix effects and also allowing low LOD and LOQ to be obtained as newly developed drugs are often more physiologically active and therefore can be administrated in smaller amounts [9,117]. Due to their considerable advantages, electroanalytical techniques have been proved to meet all the above-mentioned requirements. Voltammetric techniques are the most frequently used electroanalytical techniques in pharmaceutical analysis. Since the development of electrochemical sensors is currently one of the most active areas of electroanalytical research [9], the main focus is finding a novel material in fabrication of sensors in order to make the developed methods more selective and sensitive. Seeing that the recent developments in nanotechnology have provided excellent opportunities in design and construction of novel electrochemical detection tools [5], we have chosen carbon nanotubes (CNTs) as the basis of electrochemical sensors developed in this doctoral thesis.

The fact that there are two groups of CNTs, SWCNTs and MWCNTs, raised a challenging question: which type is right for targeted analytical investigations? Although there are many examples in the literature describing benefits of both types of nanotubes in pharmaceutical analysis, in the end, we have chosen to use the MWCNTs in our research. Moreover, the low production yield of SWCNTs compared to that of MWCNTs makes it rather challenging to obtain in large quantities and at affordable prices [81]. Bulk synthesis of MWCNTs is easier than that of SWCNTs and MWCNTs can be produced without a catalyst because their purity level is much higher [118]. The synthesis of SWCNTs requires higher quality control process than MWCNTs and hence it is more costly to produce large amounts of SWCNTs than MWCNTs [113], which results in their higher price. The commercial cost of SWCNTs is much higher, but with lower purity. Also, MWCNTs are achiral and therefore always regarded as metallic conductors, a highly attractive property for an electrode, whereas SWCNTs' conductivity depends on their chirality and diameter [80].

The aim of our research was to develop new electroanalytical methods for direct quantification of selected pharmaceutically active substances (active pharmaceutical ingredients, API) in their dosage forms using novel electrochemical sensors with MWCNTs. Furthermore, those same sensors were also applied for the simultaneous determination of selected pharmaceuticals in trace amounts with other co-administered drugs in therapy or their

metabolites in very complex media, such as biological fluids, using adsorptive stripping voltammetry. The selection of pharmaceuticals to be investigated followed few criteria. Although derivatisation can be used to introduce an electroactive group into the structure of compound in order to determine it electrochemically [7], instead we have chosen to use drugs that are electroactive by its nature. Fortunately, it was shown that most of the drugs are electrochemically active [42]. By avoiding the derivatisation step, the developed methods are cheaper and simpler. In contrast, spectrophotometric and chromatographic techniques often aquire some form of derivatisation step prior to analysis [27]. Another criterion that was considered was the lack of available analytical methods described in the literature for the selected drugs, with emphasis on the lack of electroanalytical methods. The choice of drugs depended also on their wide use in clinical medicine. Selected drugs are often prescribed by physicians, very often as the first line of therapy. Despite the extensive use in approved indications, some of the selected drugs are also being widely studied in numerous new indications and therefore the development of new reliable methods for evaluation of the quality of their pharmaceutical products or determination drug concentrations in biological fluids is highly required.

The first drug that was investigated as a part of this doctoral thesis was the antiemetic drug ondansetron (OND), a selective 5-HT_3 antagonist. We started our research with this particular drug because its redox behaviour and electrochemical determination in pharmaceutical formulations or human fluids have not been reported in the literature at all. It was investigated electrochemically for the first time as a part of our research. Ondansetron often coexists with morphine (MOR) in biological fluids as these drugs are co-administered for control of pain, nausea and vomiting in patients undergoing surgery or chemotherapy. Furhermore, in recent years ondansetron has been successfully used in prevention of morphine-induced pruritus and postoperative nausea and vomiting in patients receiving neuraxial injections of morphine as postoperative analgesia [119,120]. Despite of that, there is no electroanalytical method for the simultaneous determination of OND and MOR in biological fluids, which is why we applied the proposed electrochemical sensor for their simultaneous quantification in human serum samples. Up to now, only one analytical method has been reported in the literature regarding their simultaneous determination in injections and not in biological fluids. Two analytes were separated within 20 minutes by HPLC method with UV detection [121].

Ropinirole (ROP) is a novel nonergoline dopamine agonist indicated for the treatment of early and advanced Parkinson's disease. The only report for electroanalytical determination of ROP found in the literature up to that time was published by our group [122] at the Department of Pharmaceutical Analysis of Faculty of Pharmacy and Biochemistry. However, employment of the bare GCE for ROP determination had its drawbacks. Therefore, one of the goals of this doctoral thesis was to construct for the first time an electrochemical sensor for ROP determination with the aim of improving some deficiencies of previously developed methods on the bare GCE. Not only that the electrochemical behaviour of ROP at modified electrodes has not been investigated up to that time, but also the official method for quantification of ROP in the Ph. Eur. has not yet been approved at the time of our research of ROP. The monograph of ROP was reported for the first time in the Ph. Eur. just recently with its edition 8.7 that was implented on 1 April, 2016, while during our research of ROP, the 7th edition was in use [44]. After publishing our work on ROP, the awareness of practical utility of electroanalytical methods and the importance of development of new sensors for ROP determination has been recognised by other researchers [123–125]. ROP often coexists with L-dopa in biological fluids as these drugs are co-administered in advanced Parkinson's disease patients but surprisingly, up to now, no study has been published in the literature reporting the simultaneous determination of ROP and L-dopa.

Mesalazine (5-aminosalicylic acid, 5-ASA) is an anti-inflammatory drug widely prescribed for the therapy of chronic inflammatory bowel diseases (IBDs), such as ulcerative colitis and Crohn's disease. Although it has been used in therapy for some time now and there are few reports [126–131] available on the electrochemical determination of 5-ASA, still no attempt has been made to determine 5-ASA and its major *N*-acetylated metabolite (Ac-5-ASA) by an electrochemical method. The electrochemical behaviour of Ac-5-ASA was investigated for the first time as a part of this doctoral research. The knowledge of pharmacokinetics and metabolism of 5-ASA from mesalazine-containing drugs is mandatory when new drug formulations are developed for the treatment of IBDs, and therefore validated analytical methods are needed for bioequivalence studies [132]. Development of the method for simultaneous determination of the drug and its metabolite can be quite a challenge due to high selectivity of the method required in order to simultaneously determine structurally very similar compounds. Furthermore, we have employed proposed sensor for fast and reliable determination of 5-ASA content in two different delayed-release formulations which enabled

us to compare the qualities of two pharmaceutical products with the same active ingredient present on our market.

Nebivolol is a third-generation, long-acting and highly selective beta(1) blocker (β_1-blocker) with a pharmacological profile that differs from those of other drugs in its class. In addition to cardioselectivity mediated via β_1 receptor blockade, NBV induces nitric oxide-mediated vasodilation by stimulating endothelial nitric oxide synthase. Its unique pharmacological profile, based on the highest cardioselectivity and additional vasodilatory effect, provides NBV the highest efficacy and tolerability for the treatment of hypertension and chronic heart failure among others of its class [133]. Due to its important role in treatment of chronic cardiovascular diseases and the fact that an official method for its quantification in bulk form and pharmaceutical formulations has not been approved in any pharmacopoeia, we have recognized the need to develop new sensitive analytical method for NBV determination. NBV has already been investigated [134] at bare boron-doped electrode (BDDE) as well as other β_1-blockers [104], by our group at the Department of Pharmaceutical Analysis. Studies have been shown that metal NPs supported on the MWCNTs may provide much improved electrocatalytic activity [135]. Therefore, in order to take full advantage of the two kinds of nanomaterials, our idea was to construct a simple and sensitive electrochemical sensor based on both NPs and MWCNTs, so that the individual properties of each material can be integrated and the interactions between the two components may bring out novel properties. No electrochemical studies on NBV determination using this kind of nanocomposite film were reported in scientific literature. However, during the preparation of the manuscript, a paper dealing with the electrochemical determination of NBV appeared, but it is based on oxidation of drug at GCE modified with graphene as another type of nanomaterial [136].

Once obtained, MWCNTs were purified and activated before use. The most common pretreatment step with nanotube modified electrodes is to purify the tubes in strong acid which gives an improvement in the electron transfer rate. Solutions of sulfuric, nitric and hydrochloric acids, either concentrated or diluted, alone or mixed have been used at room temperature or under refluxing, with or without sonication for different times [92]. Sonication of dispersion of MWCNTs in concentrated HNO_3 for 4 hours showed to be suitable protocol for activation of nanotubes for our purposes.

To take advantage of the remarkable properties of MWCNTs in electrochemical sensing applications, the MWCNTs should be properly functionalized and uniformly

immobilized at electrode surface. For that purpose, we chose Nafion as the dispersing agent. Not only did Nafion homogenously disperse MWCNTs, fixing them onto the electrode surface tightly and enhancing the stability of proposed electrochemical sensor, but it also enhanced the electrochemical response together with MWCNTs thanks to their synergistic effect. The investigated drugs are all weak basic compounds that were completely protonated in the experimental conditions under which voltammetric measurements were performed and Nafion as a cation-exchanger attracted them from the bulk solution. The electrode that was employed in performed voltammetric measurements was GCE, which is not untypical for this type of investigation. Selected drugs (except for 5-ASA) were oxidized at relatively high potential values on the bare GCE, and MWCNTs, due to their electrocatalytic activity, made that oxidations more facile, and thus placing them outside the solvent window. Modification of GCE with either MWCNTs or Nafion led to advantageous electroanalytical performance, while a remarkable enhancement effect on electrochemical responses of all investigated drugs was obtained from the combined contribution of MWCNTs and Nafion. The analytical performance of the modified electrodes, besides for the drugs in our primary focus, was also evaluated for the co-administered drugs and metabolite. The results also showed a considerable improvement of their voltammetric responses. The differences between the two peak potentials were large enough for the development of simultaneous methods in all cases, even for the structurally very similar 5-ASA and its metabolite. Furthermore, implementing the ZrO_2 NPs for NBV determination enabled even more enhanced voltammetric response obtained from the combined effects of ZrO_2, MWCNTs and Nafion.

During the preliminary investigations and before constructing electrochemical sensors based on MWCNTs, different modifiers and different types of electrodes were studied to find the most favorable electrochemical response. Since the redox behaviour of OND was studied for the first time as a part of this doctoral thesis, the investigation of the electrochemical oxidation of drug molecule was started at a bare GCE. The preliminary results indicated that the voltammetric response of OND at bare GCE was not satisfactory for the analytical purposes because of slow heterogeneous electron transfer, electrode fouling problems, unstable analytical signal and reduced sensitivity due to the fact that adsorption of drug molecule could not be purposely used as a preconcentration step. In light of these findings, it was a challenge to find a new electrode material for determination of OND using voltammetric technique.

One common approach for incorporating a modifier onto the solid surface is to cover it with an appropriate polymer film [76]. Polymer-modified electrodes are often prepared by casting a solution containing the dissolved polymer onto the surface and allowing the solvent to evaporate or via electropolymerization in the presence of the dissolved monomer. Polymer-modified electrodes prepared by electropolymerization have received extensive interest in the detection of analytes, not only due to their high sensitivity and selectivity, but also because of the film homogeneity attained in electrochemical deposition, its chemical stability and strong adherence to the electrode surface [137]. So, for the initial modification of GCE for the investigation of OND behaviour, 2-hydroxy-5-[(4-sulfophenyl)azo]benzoic acid (SPAB) was chosen. The electrochemical properties of SPAB were investigated at GCE [138,139] and hanging mercury drop electrode (HMDE) [140,141] in previously published papers. Getting the insight into the electrochemical behaviour of SPAB enabled the application of electrooxidation of its monomer to generate polymer layer on GCE with incorporated polyanions that can show cation selectivity and adsorption of positively charged drug molecules [137]. The electrochemical sensor based on SPAB was developed for the first time to determine analytes by voltammetry at the Department of Pharmaceutical Analysis by our group. Based on excellent experience with poly(SPAB) as a novel sensor for sensitive and selective determination of antipsychotic drug quetiapine, we tested it in preliminary studies of OND using experimental conditions (Supplemental data 1, Figure 1) that were previously selected as optimum (SPAB, c = 1 x 10^{-3} M in Britton-Robinson (BR) buffer pH 4, between -0.1 and 1.6 V, v = 100 mV/s, 10 cycles).

Because of the presence of the charged carboxylic and sulfonate groups in the structure of SPAB, the adsorption of positively charged OND molecules onto the modified electrode due to the electrostatic interactions was expected. Nevertheless, the electrode modified with SPAB did not give much better results than that of bare GCE as it was the case for quetiapine. The peak potential of OND was shifted to less positive values by 20 mV, indicating weak electrocatalytic activity of immobilized film towards OND, but there was clearly no enhanced effect of SPAB on peak current (Supplemental data 1, Figure 2). The adsorption of drug molecules could not be obtained on GCE modified with poly(SPAB) under such experimental conditions, and therefore higher sensitivity could not be achieved which was needed for the application of the sensor to determine trace amounts of OND found in biological fluids after the therapeutic doses. It is possible that some steric factors limit the good electrostatic interaction between positively charged nitrogen atom in OND structure and

negatively charged carboxylic and sulfonate groups in SPAB structure. On the other hand, functional groups of SPAB may not be charged enough under the acidic conditions of the experiments; however, a maximum current intensity was obtained in 0.1 M H_2SO_4 during the preliminary investigation on bare GCE. Therefore, it was employed as optimum supporting electrolyte for further studies.

SPAB belongs to the same class of compounds as two azosalicylic acids, sulfasalazine and olsalazine, which are employed in clinical praxis as precursor of 5-ASA [137]. SPAB was also proposed as potential novel prodrug of 5-ASA by the earlier investigations [138]. 5-ASA is identified as an active component in the therapy of IBDs and a novel sensor has been proposed for its determination as a part of this doctoral thesis. However, due to its similarity with SPAB, which was applied as a modifier, we investigated the possible use of 5-ASA for synthesis of polymer film for sensitive determination of ROP. Also, Eriksson et al. (142) proposed possible way of 5-ASA polymerization during its electrooxidation on GCE and Mu (143) performed the copolymerization of aniline and 5-ASA by CV to introduce pH functional groups (-OH and –COOH) with 5-ASA in order to improve the pH dependance of redox activity of polyaniline, which is also used for electrochemical sensing due to its conductivity and redox activity. The assumption was that 5-ASA could attract positively charged ROP molecules from the bulk solution by its negatively charged carboxylic groups. Voltammetric response of ROP turned out to be much better on GCE modified with poly(5-ASA) than that obtained at the bare GCE, so we proceeded with the optimization of 5-ASA polymer film in order to get the most favorable response for ROP (Supplemental data 2, Figure 3).

Parameters that could affect electropolymerization of 5-ASA and therefore the voltammetric response of ROP on GCE modified with poly(5-ASA) were optimized: potential window, number of cycles, scan rate, as well as concentration and pH of 5-ASA solution. The efficiency of the film growth was the highest when an electrode was cycled in the modification solution between -0.2 and +0.9 V. The wider potential window resulted in lower peak currents of 5-ASA and the preparation of the polymer film was longer. Also, peak current for ROP was sharper and higher when shorter potential range of cyclization was applied. When 10 cycles with v = 100 mV/s were used for the deposition of the polymer film, voltammetric responses of ROP were sharper and higher in comparison with 20 cycles with v = 200 mV/s. Poly(5-ASA) was prepared in BR buffer. In order to find optimum conditions for its electropolymerization, the pH value of BR buffer was varied between 2 and 6. Although

the pH had great effect on 5-ASA oxidation, it did not seem to affect the voltammetric response of ROP that much. BR buffer pH 2.0 was finally chosen as optimum because it resulted in the highest 5-ASA peak current and therefore the highest film growth. On the other hand, concentration of 5-ASA solution appeared to be the critical parameter for ROP voltammetric response. Its peak current significantly increased with the increase of 5-ASA concentration and the value of 1 x 10^{-3} M was selected as optimum (Supplemental data 2, Figures 1 and 2).

Although the sensor with poly(5-ASA) looked very promising compared to SPAB that has similar structure, we were not able to achieve the accumulation of ROP on that film. It might have been that carboxylic groups where not charged enough to concentrate ROP molecules by electrostatic interaction. Since one of the imperatives of our research was to develop methods that would be sensitive enough to be able to quantify very low concentrations of drugs in biofluids, GCE modified with poly(5-ASA) proved to be unsatisfactory for this purposes. However, some other type of investigation, such as the determination of drug content in pharmaceutical dosage forms where such high sensitivities are not necessary, could possibly benefit from this type of sensor. In order to achieve accumulation we tried to construct an electrochemical sensor based on both 5-ASA and MWCNTs. Instead on bare GCE, electropolymerization of 5-ASA was performed on the GCE surface on which was previously casted a suspension of MWCNTs in DMF, but we did not achieve satisfactory results with that modification either. Later, we found in the literature a publication concerning a novel solid-phase extractor based on MWCNTs with 5-ASA for preconcentration of Pb^{2+} [144]. To construct such an extractor, the covalent bond was established between carboxylic groups of functionalized MWCNTs and amino group of 5-ASA. In our electroanalytical studies we did not use those covalent types of MWCNTs functionalisation, but rather non-covalent, which are simpler. Future investigations could perhaps try to employ such covalent functionalisation with 5-ASA for electrochemical sensing.

BDDE have found many applications as electrode material in electroanalysis due to their outstanding properties, such as very wide electrochemical potential window, low background current [145], exceptional chemical inertness and mechanical strength, negligible adsorption of organic compounds [146], and thus reduced fouling [147], extreme hardness and applications in aggressive media such as strong acids [42]. These properties make BDDE particularly suitable for electrochemical studies of analytes with a high oxidation potential

[148] and ROP is one of them. Furthermore, previous work on ROP by our group at the bare GCE [122] revealed fouling effect of ROP oxidation product. The sp^3 surface of BDDE is less vulnerable to surface oxidation and fouling than sp^2, such as GCE [78], which is why we investigated the electrochemical behaviour of ROP on modified and bare BDDE as well. Modification of BDDE with MWCNTs and Nafion led to similar results as observed on GCE in terms of synergistic effect from MWCNTs and Nafion (Supplemental data 2, Figure 5). When compared to MWCNTs/Nafion-GCE (Supplemental data 2, Figure 6), MWCNTs/Nafion-BDDE did not provide much better results (BDDE: i_p = 128 μA at 1.26 V vs. GCE: i_p = 110 μA at 1.24 V for c (ROP) = 5 x 10^{-5} M). However, it is more expensive and more difficult to maintain as it cannot be cleaned by severe polishing because of its surface doping [77]. It is usually pretreated cathodically [148,149] or anodically [77], which can last even for a few minutes. Also, it has been reported that repeated electrochemical cleaning of the BDDE surface can decrease the electrode activation [77]. The fouling effect of ROP and its oxidation product was decreased on MWCNTs/Nafion-GCE anyway, and so it was not necessary to use expensive BDDE for such a small progress in peak current.

To improve the sensor response we examined in preliminary measurements, once again, the influence of the working electrode material, this time glassy carbon and gold (Au), on oxidation peak height of 5-ASA. Au is together with platinum (Pt) the most used solid metal electrode material [11]. They are commonly considered to be inert [76], but their inertness should be considered as relative [42] because under certain electrochemical conditions these electrodes are highly active [76]. At certain applied potentials, oxides are formed by reaction between the electrooxidised metal and oxygen and electrodes' otherwise highly active surface can be covered by a surface oxide film over a broad range of positive potentials. This will influence the electrode oxidation processes since the electrochemical charge transfer reactions will occur at the surface oxide rather than the metal surface [42]. Such films can also strongly alter the kinetics of the electrode reaction, leading to irreproducible data [11]. Since the Au electrode in aqueous electrolyte at potentials higher than +0.8 V is covered by an Au oxide film, making its use in the positive potential range reduced [42], this type od metal electrode could only be applied in our research for 5-ASA as its oxidation occurs below +0.8 V. For other investigated drugs oxidation happens at potentials higher than +1.0 V. As sensitivity did not improve when using gold electrode (Supplement data 3, Figures 1-4), the GCE was used in subsequent experiments due to its significantly lower cost.

For fabrication of novel nanosensor based on both metal nanoparticles and carbon nanotubes, we investigated NPs that were not so commonly used for sensing of pharmaceuticals. Noble metal nanoparticles (NPs) such as gold, platinum and silver were mostly used in this area [5], but a wide variety of metal NPs, which are not typically used materials such as those mentioned above, have been studied in electroanalysis [109]. Bismuth NPs represent innovative material in sensing applications. They have attracted growing attention recently [150] and so we tried them in combination with carbon nanotubes that have already proven to be an excellent tool for sensing. However, the results obtained from combining those two modifiers were unexpected. The peak potential of NBV shifted to less positive values, while the peak current was higher at the electrode modified only with nanotubes. There was no synergistic effect observed from those two types of nanomaterials (Supplemental data 4, Figures 1 and 2). Lee et al. [151] reported the formation of agglomerates with petal shape, observed by SEM, emerging from well-connected spherical NPs with Nafion, which existed at the beginning of the measurement process. It is believed that they decreased due to chemical reaction of Bi with ions in electrolyte solution and Bi oxidation. X-ray diffraction (XRD) patterns confirmed that most of the Bi oxidized to BiOCl at pH 3.0 and the agglomerates observed by SEM are thought to be BiOCl. Thus, it is suggested that the pH of the solution significantly influences the phase stability of bismuth and that the phase transition of pure bismuth into the other forms leads to a considerable decrease in sensitivity of the nano-bismuth fixed electrode. Our preliminary study on NBV oxidation has shown that the best voltammetric response was obtained in very acidic media due to NBV protonation and interaction with Nafion, which is why it was not feasible to apply this nanosensor with Bi NPs for NBV determination in this selected conditions. On the other hand, ZrO_2 NPs proved to be a useful material for enhancing sensitivity together with MWCNTs and Nafion. Since only few papers on its use in sensing of pharmaceuticals have been reported [152–154], it is considered as a very attractive and innovative nanomaterial in this field. Although some sensors based on both ZrO_2 NPs and MWCNTs have been reported, the sensor that would combine ZrO_2 NPs and MWCNTs together with Nafion, to our knowledge, has not yet been reported in pharmaceutical and biomedical field.

Although various modifiers and types of electrodes were explored in our search for a novel electrochemical sensor, those based on MWCNTs dispersed in Nafion, decorated with ZrO_2 NPs in case of NBV, proved to give the most favorable voltammetric responses for all drugs investigated. Therefore, the next step was optimisation of all experimental conditions

and parameters that could affect the electroanalytical performance of that sensor. The surface morphology and structure characterization of the modified electrodes was examined by scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDXS) and CV. Since the modification of the electrode with nanomaterials and Nafion led to such great improvement in electrochemical responses of all drugs, it was necessary to optimize the amounts of modifiers in suspension. An increase of the amounts of nanomaterials in polymer film was expected to result in enhanced peak currents due to their unique properties; however, too high MWCNTs or ZrO_2 NPs concentrations led to large background currents and lower stability of the nanocomposite film due to lower adhesion on the electrode surface as well as the agglomeration of nanomaterials. The optimum concentration ratios for our investigations appeared to be 1:1 (MWCNTs : Nafion) and 1:1:1 (MWCNTs : ZrO_2 : Nafion) for NBV sensing. Nafion concentrations were also studied in the range of 0.1-0.7 %. The optimum value was 0.5 % for all selected pharmaceuticals, except for the Ac-5-ASA. The metabolite could not have been effectively attracted by the cation-exchange polymer, which is why 0.3 % Nafion concentration was chosen as optimal value for simultaneous analysis of 5-ASA and its acetylated metabolite. Finally, the volume of suspension, which determines the thickness of the cast film, was varied between 1 and 9 μL. The maximum responses were achieved when electrode surface was coated with 5 μL, except for simultaneous analysis of 5-ASA and Ac-5-ASA where 3 μL were casted. The smaller amount of Nafion used in this method (0.3 %) resulted in less viscosity of the suspension and therefore the better adhesion at the electrode surface and better stability of the immobilised film were obtained with smaller suspension volume.

The choice of the supporting electrolyte is very important in method development. If no information is found in the literature for the given electroactive compound or related compounds, a number of various supporting electrolytes must be used to find one in which the best developed and easy measurable waves are obtained [42]. First, to obtain better sensitivity for analytical studies, the electrochemical oxidation of selected drugs was examined using CV in different supporting electrolytes, including BR buffer and acetate buffer with different pH values, as well as the sulfuric acid, hydrochloric acid and acetic acid in different concentrations. For analyses of electroactive organic components that are most frequently present in drugs, the supporting electrolytes are usually buffers, solutions of a strong acid or of a strong base [31]. The experiments have shown that well-defined voltammetric response of selected drugs was obtained only at lower pH values. A 0.1 M H_2SO_4 was chosen as the

optimum electrolyte for sensitive determination of NBV, as well as for selective simultaneous determination of OND with MOR and ROP with L-dopa. On the other hand, in BR buffer solution pH 2.0 a maximum current intensity together with lower background current was achieved for simultaneous determination of 5-ASA and its metabolite. These supporting electrolytes were employed in further studies. As was already mentioned, all of these drugs are weak basic compounds and in this kind of acidic media they were completely protonated and thus effectively attracted by Nafion to the electrode surface.

For evaluating the redox reaction character of selected drugs on modified electrodes, the CV measurements with different scan rates (v) were performed and the effects of scan rate change on peak potentials and peak currents were evaluated. For all drugs peak currents linearly increased with the scan rate, and a plot of anodic peak current versus the scan rate gave the linear relationship, indicating that the eletrooxidation of drug molecules at modified electrodes was an adsorption-controlled process. Therefore, to obtain the highest sensitivity of the method, the adsorption of drug molecules was used in subsequent analytical measurements as an effective accumulation step prior to the voltammetric quantification of drugs. In cases of all drugs, peak potentials shifted positively when increasing scan rate and dependance of the peak potential was linear with logaritm of scan rate, as expected for an irreversible electrode process. This established relation allowed, using the appropriate equations, to calculate the number of electrons exchanged during the oxidation process on modified electrodes. The slopes of the plots of peak potentials versus pH demonstrated that equal number of protons participated in redox processes of ROP, 5-ASA and NBV. As for OND, the peak potential remained almost constant with the pH change, indicating that protons did not participate in its oxidation process.

To obtain a much more sensitive peak currents for analytical studies, the variation of the peak currents recorded at the modified electrodes was monitered while changing instrumental SWV parameters for OND, ROP and 5-ASA and DPV parameters for NBV determination. The pulse amplitude and the potential scan rate were optimized to acquire voltammograms of relatively high sensitivity and well-shaped waves with a relatively narrow peak width. Selection of these parameters is very important for sensitivity, resolution and analysis time [9]. Because of its speed, SWV was employed in most cases for sensitive and fast determinations in our research. The effective scan rate is given by $f\,\Delta E$ [11]. For example, the optimisation of SWV parameters for OND determination resulted in f = 75 Hz and ΔE = 8 mV, which gave the value of effective scan rate of 600 mV/s. Since the SWV voltammograms

were recorded in potential scan from 0.7 to 1.4 V, it took only 1.17 seconds to obtain the entire voltammogram for OND determination. Analysis time is, therefore, drastically reduced by the careful choice of SWV parameters.

CV studies revealed surface-controlled processes at the modified electrodes; therefore, the possibility of effective analyte preconcentration before voltammetric measurement was examined. To achieve the optimum conditions for the maximum adsorption, the influence of accumulation potential and deposition time on voltammetric responses of investigated drugs were evaluated. Our observations of the influence of accumulation potential on the stripping peak currents were in accordance with the Langmuir isotherm in voltammetric studies, which means that the peak currents increased, for such electroactive molecules oxidized at positive potentials, as the accumulation potential shifted to more negative values. As it is common, accumulation potentials were not close to the oxidation potentials of investigated drugs [49]. The accumulation time significantly affected voltammetric responses of all selected drugs with enhanced increase of their oxidation peak currents with the increase of t_{acc} until surface adsorption saturation occured. However, the adsorption kinetics appeared to be quite different for simultaneously determined drugs. Although, MOR showed much faster adsorption at the modified electrode surface than OND, as well as L-dopa opposed to ROP, a little longer t_{acc} was chosen as optimum in order to achieve the highest possible sensitivity in the linearity range for quantification of the targeted drugs of this research in biological fluids (OND and ROP) in acceptable analysis time. As for 5-ASA and its metabolite, an extremely short t_{acc} allowed the needed sensitivity to measure the expected serum concentrations after treatment with the therapeutic daily doses recommended for IBDs.

Every analytical procedure needs to be validated to be regarded as suitable for intended purpose [155]. After all the experimental conditions and instrumental parameters that could affect voltammetric response of our investigated drugs were optimized, and before the applicability of proposed sensors on pharmaceutical dosage forms and human serum samples was examined, the developed methods were validated according to ICH guidelines using those optimized conditions. The validation parameters that were checked during the development of new methods using proposed sensors were selectivity, linear range, LOD and LOQ, precision as inter-day and intra-day precision, accuracy and stability. The linearity range was examined with pulse techniques, DPV for NBV and SWV for all other selected drugs, both with and without accumulation step. Obtained linear responses without preconcentration step allowed direct determination of these drugs in their dosage forms due to

high concentrations of API in tablets. Although electrochemical methods are generally considered to be very sensitive [156], the quantification in human serum samples where these drugs are basically found in trace amounts after therapeutic doses could not be feasible without accumulation step prior to SWV or DPV. So, linearity was also checked under optimum accumulation conditions, and, thus higher sensitivities were achieved.

By changing one analyte concentration and keeping the other unchanged, obtained sensitivities (slopes of the calibration curves) were compared to the values observed in the absence of co-administered drugs or metabolite. Results indicated independent adsorption of examined analytes in the accumulation step at the proposed electrochemical sensor. Therefore, simultaneous determination of selected drugs or drug with its metabolite was possible without inteferences. There are several different approaches for LOD and LOQ determination recommended by the official guidelines and pharmacopoeias. The most widely used approach in electroanalytical chemistry and also in our investigations, estimates the LOD and LOQ values from the standard deviation of the intercept and the slope of the related calibration curves [42]. LOD and LOQ values obtained with proposed sensors were comparable or even better than in previously reported chromatographic methods which are less environmentally friendly, more expensive and demand sophisticated equipment.

ICH Q2 defines that precision of the method can be considered at three levels: repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (ruggedness, USP). Reproducibility is used especially for collaborative studies. It is employed for the standardization of an electroanalytical method, for instance, for inclusion of procedures in pharmacopoeias [42], but it is not normally expected as the mandatory validation parameter if intermediate precision is performed [156]. The intra-day precision of voltammetric responses using proposed sensors was evaluated by six replicate measurements yielding unchanged oxidation peak potentials for all investigated drugs and relative standard deviations (RSD) for stripping peak currents, all below 2 %. The inter-day precision was characterized by three replicate measurements over three days, each time using freshly prepared sensor and standard solutions. The RSD values for stripping currents were not greater then 3 % and were also less then 1 % for peak potentials. For evaluation of precisions of the electrode response, adsorptive stripping technique was employed, that was later used for trace amount assay in biological fluids. Since the precision criterion for trace amount assay in electroanalytical methods should be $\leq$ 5 % [156], we can conclude that proposed sensors showed excellent precision and also great fabrication reproducibility. The

stability of proposed sensors was checked over a period of four weeks by evaluating the analytes oxidation peak potential and peak currents.

Accuracy of the method is usually expressed as recovery and rarely as bias or relative bias and it can be obtained in different ways. It is not uncommon for the electroanalytical assay of pharmaceuticals that accuracy is determined by spiking a blank matrix with known concentrations of compounds [42], for example when measuring a drug active compound in a complex and/or unknown matrix media such as biological samples or pharmaceutical dosage forms. Also, in order to eliminate interferences such as excipients in tablets or endogenous substances in serum samples, standard addition methods can be used for quantification and recovery studies.

Prior to the analysis of samples, the selectivity of the proposed electrochemical sensors for quantification of selected drugs and metabolite was also evaluated. Their fixed amounts in standard solutions were spiked with various excess amounts of interfering species that are likely to be found in biological samples or are co-formulated with the API. Many inorganic electrolytes, such as Na^+, Cl^-, K^+, NO_3^-, SO_4^{2-}, HPO_4^{2-}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+} and Fe^{3+} did not intefere even when they were added in amounts much larger than usual physiological levels. L-alanine, L-glycine, L-aspartic acid, L-glutamic acid, L-cysteine, lactose, sucrose, glucose, starch, citric acid, Na_2EDTA and manitol also did not cause an error more then ± 5 % in the voltammetric responses of investigated analytes.

Exploring the influence of dopamine (DA), ascorbic acid (AA), uric acid (UA) and nitrite ions (NO_2^-) was particularly interesting for quantification of our analytes in serum samples as these biomolecules regularly coexist in many biological matrices and their simultaneous determination is one of the most important points in sensor fabrication due to their very close oxidation potentials at bare electrodes [157]. At bare GCE they can usually be seen as one overlapped and broad oxidation peak, but modifications with different MWCNTs-based sensors allowed separation of their peaks [158,159]. Unfortunately, the separated peaks due to their potential values may intefere with the voltammetric determinations of some of our analytes. However, Nafion with its negatively charged sulfonate groups prevented the accumulation of negatively charged oxidizable NO_2^-, while allowing facile accumulation of the positively charged analytes (Supplemental data 1, Figure 4). Ascorbic acid (pK_a = 4,17) is neutral [160] under the experimental conditions so it was not rejected by Nafion, but its oxidation potential on electrodes modified with MWCNTs can be seen at very low oxidation

potentials, outside our potential window. DA is positively charged (pK_a = 8.89) under experimental conditions [160] conducted in our measurements and was attracted by Nafion and therefore could have been showing possible competitive adsorption on this type of electrode surface. However, DA showed only interference with L-dopa (Supplemental data 2, Figure 7 a) and b)), which is actually used as DA precursor in the treatment of Parkinson's disease, due to their very similar structure and very close peak potentials (+0.48 V for DA vs +0.52 V for L-dopa). However, normal plasma levels of endogenous free DA (26 pg/mL) did not affect L-dopa voltammetric response at the proposed sensor. UA, although without charge [160] under optimized experimental conditions (pK_a = 5.4), influenced on peak currents of MOR (Supplemental data 1, Figure 3) and L-dopa (Supplemental data 2, Figure 8), as well as 5-ASA and its metabolite (Supplemental data 3, Figure 5) due to closeness of their potentials. As it was the case with DA, no interferences were seen on real samples. For NBV, additional inteferences studies were performed and the inteference effect was observed with equal concentrations of other β-blockers, like atenolol, carvedilol, propranolol and bisoprolol (Supplemental data 4, Figures 2-5), as well as with hydrochlorothiazide that can be combined with NBV for the treatment of hypertension.

To evaluate the validity and practical utility of newly developed methods on proposed sensors, analytes were determined in tablets obtained from local pharmacy and in human serum samples obtained from healthy volunteers. Direct measurements with pulse techniques (DPV for NBV and SWV for others) were employed for the determination of drug contents in dosage forms, except for OND due to unstable analytical signal without preconcentration step. Otherwise, due to high concentration of API in tablets, no accumulation step was needed. Voltammograms were obtained by SWV within few seconds and such fast quantification in pharmaceutical formulations, without complicated sample preparation and with higher or at least of the same order sensitivity and selectivity, cannot be usually achieved with commonly used analytical methods for determination of drug content, such as chromatographic, spectroscopic and capillary electrophoretic methods. For NBV determination DPV is employed instead of SWV because of the much lower background current and significantly lower overpotential of NBV achieved in this way. It has been reported in the literature that SWV can lead to drawbacks with kinetically slow processes because of its high speed, when DPV is to be preferred [42].

The results obtained with the proposed sensors were in good agreement with the claimed amount. Mean recoveries were 98.3 – 101.0 % with RSD values 0.7 – 1.3 %,

depending on the analyte, indicating good accuracy and precision as well as the suitability of the proposed sensor for this purpose. Only for L-dopa determination recoveries obtained by both SWV (95.23 %) and HPLC (95.04 %) were slightly outside of that narrow range, but were still more than acceptable. Similar recoveries were obtained with both techniques which is why it cannot be prescribed to the technique used for measurements but were probably caused by the nature of sample as L-dopa is present with another component - benzerazid in combined pharmaceutical dosage form. Also, the recoveries in the range of 98.1 – 99.4 % indicated that excipients did not interfere with assay of active ingredient in any case. The results obtained with newly developed methods were compared with those obtained by HPLC methods reported in literature (5-ASA), official HPLC methods described in USP (L-dopa), previously developed HPLC methods by our group (ROP, NBV) or HPLC methods developed as a part of this doctoral thesis (OND). Recovery tests were also performed for chromatographic methods. Statistical analysis of the results obtained using voltammetric and HPLC procedures showed no significant difference between the performance of the two methods regarding accuracy and precision, as revealed by the student *t*-test and variance ratio *F*-test.

MWCNTs/Nafion sensor was also applied for the first time for simultaneous determination of OND and MOR, ROP and L-dopa, 5-ASA and its metabolite Ac-5-ASA in human serum samples by AdSWV. ZrO_2-MWCNTs/Nafion sensor was applied for the determination of NBV in serum by AdDPV. The expected serum concentrations with the therapeutic daily doses were within linear concentration ranges of the newly developed method using proposed electrochemical sensors after the accumulation step in duration of 360 s for OND and MOR, 240 s for ROP and L-dopa, 30 s for 5-ASA and metabolite and 240 s for NBV. The analysis time was still very short, especially when it was compared to other methods and there was no need for expensive and complicated extraction procedures. The recovery studies in serum samples were performed using the standard addition method to nullify any remaining interference. In this type of complex matrix, such as biological fluids, excellent recoveries were obtained in the range 98.7 – 102.6 % with RSD 1.5 – 2.8 %.

7. CONCLUSIONS

In present doctoral thesis new electroanalytical methods using novel MWCNTs/Nafion or ZrO_2-MWCNTs/Nafion sensors were developed, validated and applied in pharmaceutical dosage forms and human serum samples for selected pharmaceuticals.

- The oxidative behaviour and electrochemical determination of OND were studied for the first time. MWCNTs/Nafion-GCE exhibited high selectivity in the voltammetric measurements of OND and co-administered drug MOR with potential difference of 430 mV. It was succesfully applied for the simultaneous determination of both drugs in human serum samples by AdSWV after short accumulation at the electrode surface. A newly developed voltammetric method was applied for quantification of OND in film-coated tablets.
- The use of an electrochemical sensor for ROP determination has been reported for the first time by our work. The proposed nanostructured sensor was utilised for direct measurements of ROP, as well as co-administered drug L-dopa, individually in pharmaceutical formulations by SWV. In addition, the analytical method for their simultaneous determination was developed for the first time. The large separation of the peak potentials of 720 mV at the MWCNTs/Nafion sensor allowed fast simultaneous quantification of trace amounts of both drugs in human serum samples.
- For the first time, an electroanalytical method was developed for selective simultaneous determination of 5-ASA and its major metabolite, Ac-5-ASA without the need for prior time-consuming separation step. The potential difference of 200 mV between the peaks at the MWCNTs/Nafion sensor was large enough to determine structurally very similar analytes in very low concentrations by AdSWV. Proposed sensor was also used for direct measurements of 5-ASA contents by SWV in two pharmaceutical formulations available on our market.
- Proposed sensor with MWCNTs in Nafion matrix was decorated with ZrO_2 NPs in order to improve its performance and electrochemical sensing of NBV by utilising all of the exceptional properties of the two nanomaterials. ZrO_2-MWCNTs/Nafion sensor was applied for fast quantification of NBV in tablets by DPV and also for very sensitive analysis of NBV in serum samples by AdDPV.

Development of new electroanalytical methods in the present doctoral thesis represent an important scientific contribution of our research in the field of pharmaceutical analysis, especially for the drugs characterised with the lack of analytical methods for their qualitative and quantitative examination. It is a significant progress for drugs that have not yet been investigated electrochemically, particularly using novel electrochemical sensors.

A considerable enhancement effect on voltammetric responses of selected drugs was observed due to synergy of remarkable properties of nanomaterials and cation-exchange polymer that were used to construct a promising platform for electrochemical sensing. Enhanced peak currents and lower potential values were obtained due to larger electroactive surface area and excellent electrocatalytic activity of nanomaterials together with ion-exchange capacity and improved accumulation capability provided by Nafion matrix. Electrochemical sensors proposed in the present doctoral thesis, therefore, offer improved sensitivity and selectivity for electroanalytical methods, which is with their low cost and short analysis time very appealing in pharmaceutical analysis.

The preparation of sensors was quite simple; obtained by dispersing nanomaterials in polymer and depositing of the homogenous dispersion by one-step casting method on GCE. Additionally, the proposed sensor has shown long-term stability due to high mechanical strength of MWCNTs and also very easy surface regeneration by applying a single SWV or CV potential scan in blank supporting electrolyte, thus avoiding tedious polishing as in the case of bare GCE before every measurement.

The modern electroanalytical methods based on such modified electrodes have great potential for practical application in complex media such as real pharmaceutical and biological samples, not only for fundamental research in laboratories, but also for routine analyses. Generally, the separation and extraction procedures are not necessary, as compared to the chromatographic methods that usually require some type of sample pretreatment, such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE) prior to the analysis of biological samples.

Novel sensors proposed in this doctoral thesis can be used for evaluation of the quality of pharmaceutical products in order to achieve better medicinal effect and lower toxicity. They can also be successfully applied for monitoring of the therapeutic levels of drug concentrations in biological samples in patients in clinical medicine to prevent overdose toxicity or for pharmacokinetic purposes.

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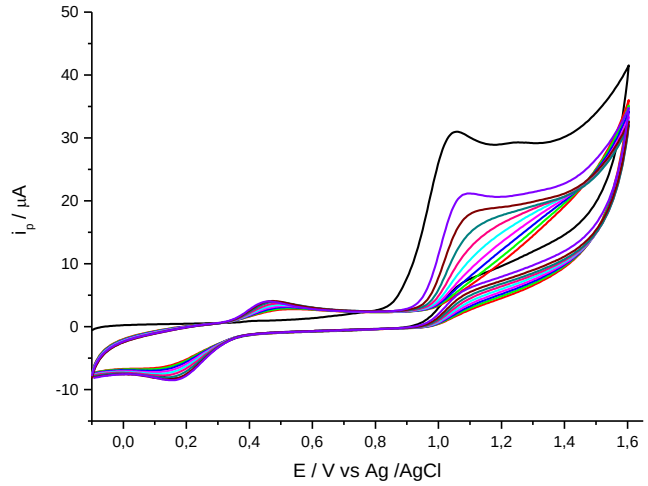
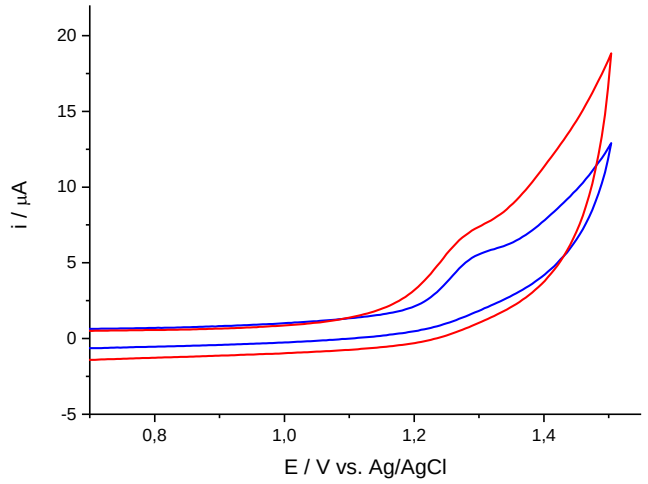
9. SUPPLEMENTAL DATA

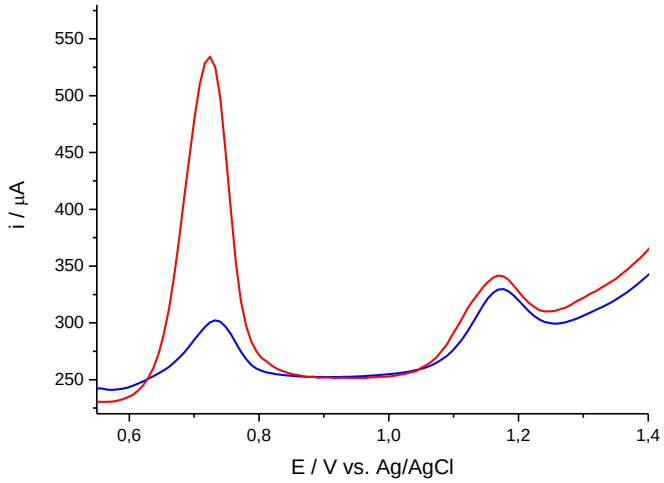
9.1. **Supplemental data 1:** Multi-walled carbon nanotubes/Nafion composite film modified electrode as a sensor for simultaneous determination of ondansetron and morphine



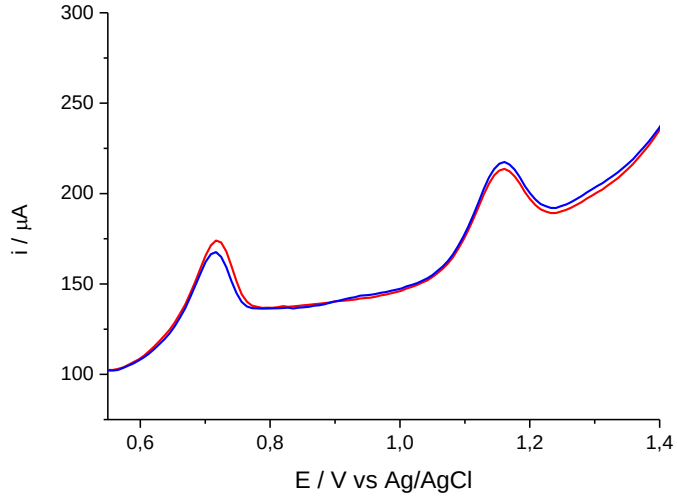
Supplementary Figure 1. Electrochemical polymerization of 2-hydroxy-5- [(4-sulfophenyl)azo]benzoic acid on GCE surface for ten cycles in Britton-Robinson buffer solution (pH 4) containing 1 x 10^{-3} M SPAB (scan rate 100 mVs^{-1})



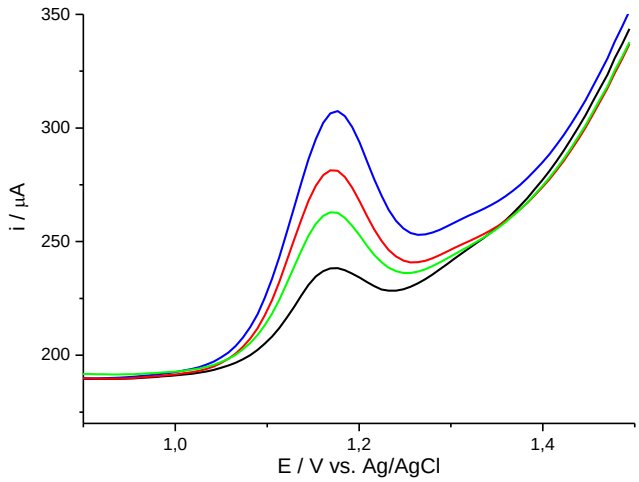
Supplementary Figure 2. Cyclic voltammograms of OND (1 x 10^{-4} M) at a bare GCE (blue) and the poly(SPAB)-GCE (red) in 0.1 M H_2SO_4



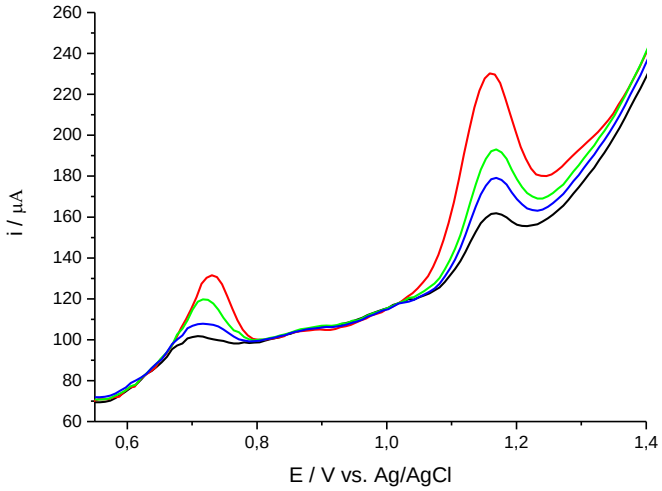
Supplementary Figure 3. Square-wave voltammograms of OND and MOR (1 x 10^{-6} M) at the MWCNTs/Nafion-GCE with 200-fold uric acid (red) and without uric acid (blue). SWV settings: f = 75 Hz, E_{sw} = 25 mV and ΔE = 8 mV (E_{acc} = -0.5 V, t_{acc} = 360 s)



Supplementary Figure 4. Square-wave voltammograms of OND and MOR (1 x 10^{-6} M) at the MWCNTs/Nafion-GCE with 400-fold nitrite ions (red) and without nitrite ions (blue). SWV settings: f = 75 Hz, E_{sw} = 25 mV and ΔE = 8 mV (E_{acc} = -0.5 V, t_{acc} = 360 s)

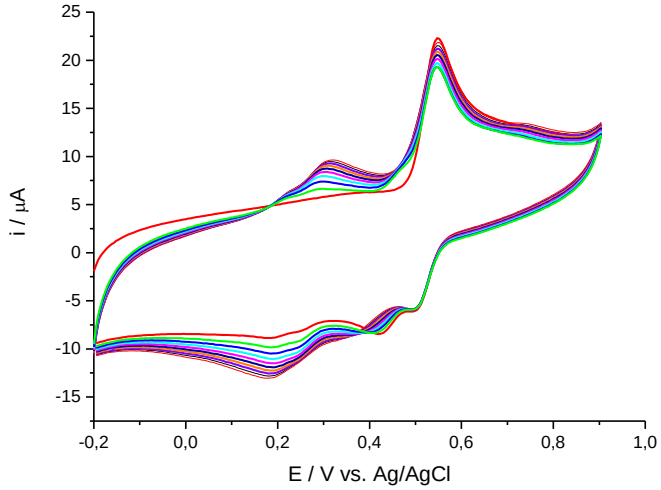


Supplementary Figure 5. Square-wave voltammograms of OND at the MWCNTs/Nafion-GCE recorded in tablets for increasing concentrations (1.0 x 10^{-6} M – 2.5 x 10^{-6} M). SWV settings: f = 75 Hz, E_{sw} = 25 mV and ΔE = 8 mV (E_{acc} = -0.5 V, t_{acc} = 360 s)

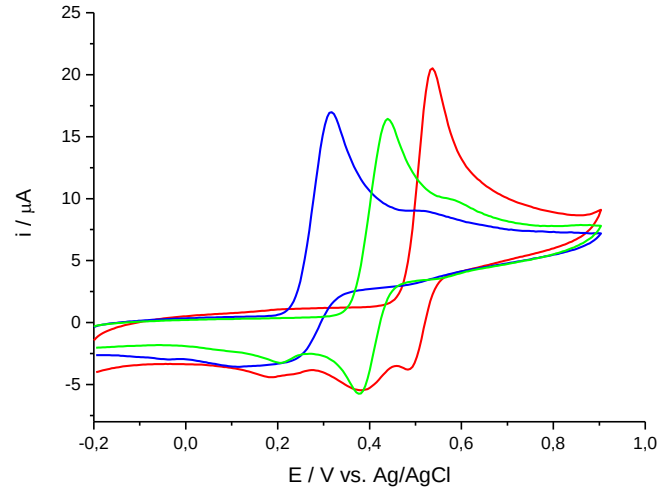


Supplementary Figure 6. Square-wave voltammograms of OND and MOR at the MWCNTs/Nafion-GCE recorded in spiked human serum for increasing concentrations (5.0 x 10^{-7} M – 2.5 x 10^{-6} M for OND and 1.0 x 10^{-7} M – 1.0 x 10^{-6} M for MOR). SWV settings: f = 75 Hz, E_{sw} = 25 mV and ΔE = 8 mV (E_{acc} = -0.5 V, t_{acc} = 360 s)

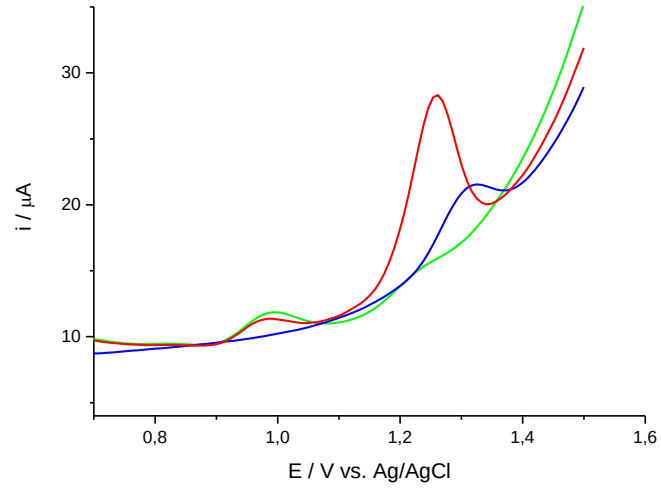
9.2. Supplemental data 2: Voltammetric determination of ropinirole in the presence of levodopa at the surface of a carbon nanotubes based electrochemical sensor in pharmaceuticals and human serum



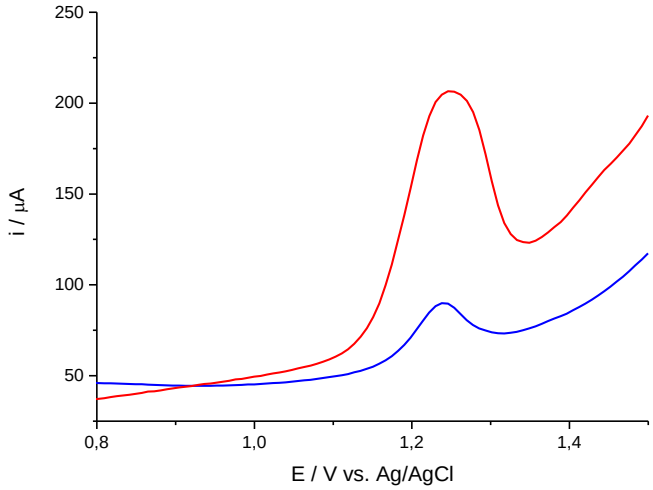
Supplementary Figure 1. Electrochemical polymerization of 5-ASA on GCE surface for ten cycles in BR buffer solution (pH 2) containing 1 x 10^{-3} M 5-ASA (scan rate 100 mVs^{-1})



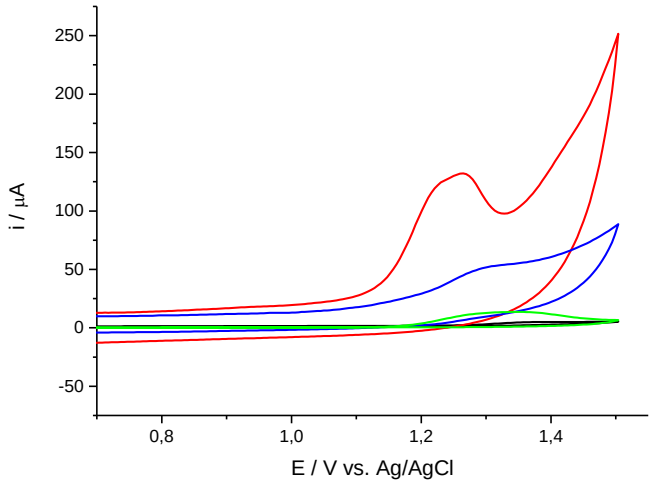
Supplementary Figure 2. The effect of pH value of BR buffer solution on 5-ASA (5 x 10^{-4} M) electropolymerization (first cycle): pH 2 (red), pH 4 (green) and pH 6 (blue)



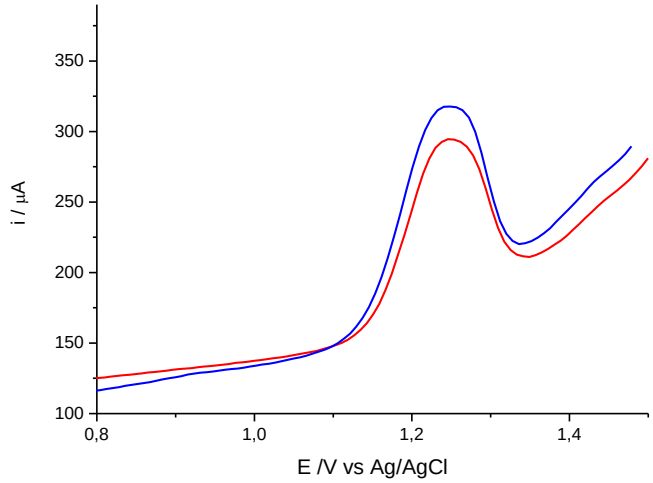
Supplementary Figure 3. Square-wave voltammograms of ROP (5 x 10^{-5} M) at a bare GCE (blue) and at poly(5-ASA)-GCE (red) together with corresponding background recording in BR buffer pH 2 (green)



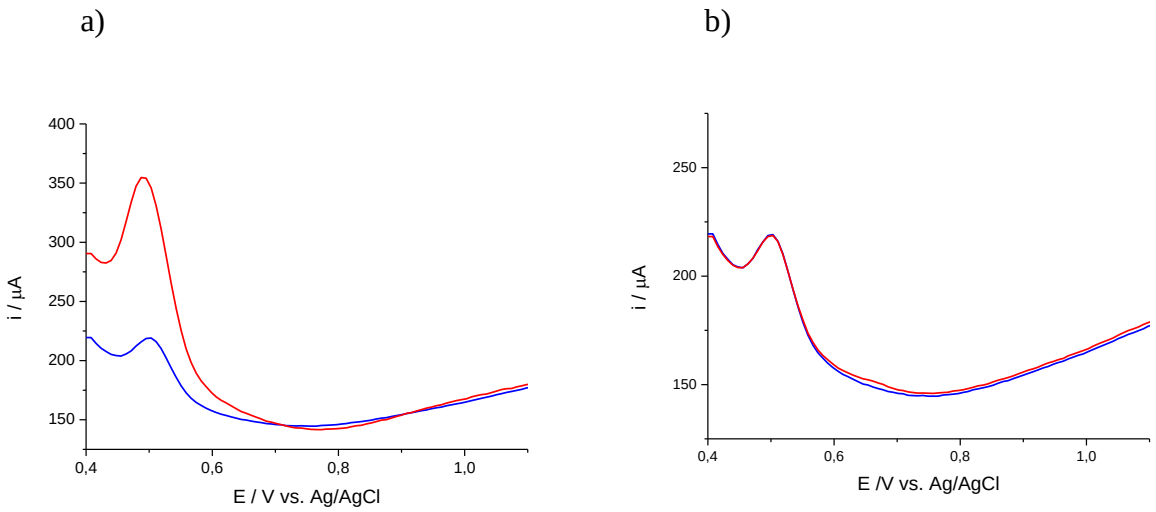
Supplementary Figure 4. Square-wave voltammograms of ROP (5 x 10^{-5} M) in 0.1 M H_2SO_4 at poly(5-ASA)-GCE (blue) and at the MWCNTs/Nafion-GCE (red)



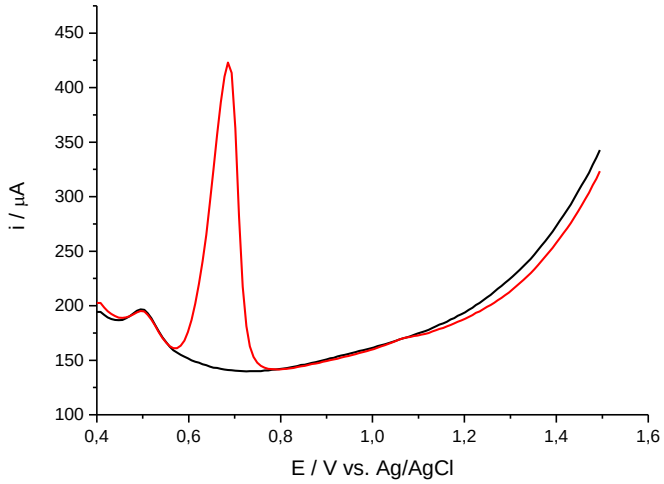
Supplementary Figure 5. Cyclic voltammograms of ROP (5 x 10^{-5} M) at a bare BDDE (black), the Nafion-BDDE (green), the MWCNTs-BDDE (blue) and MWCNTs/Nafion-BDDE (red). Scan rate: 100 mVs^{-1}



Supplementary Figure 6. Square-wave voltammograms of ROP (5 x 10^{-5} M) at the MWCNTs/Nafion-BDDE (blue) and the MWCNTs/Nafion-GCE (red)

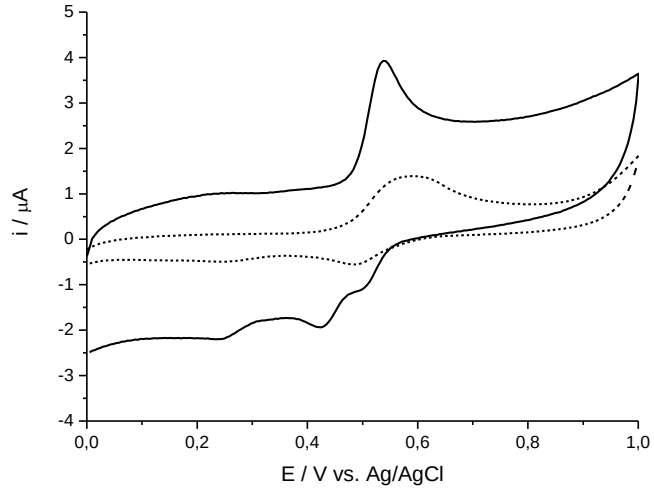


Supplementary Figure 7. Square-wave voltammograms of L-dopa (1 x 10^{-6} M) at the MWCNTs/Nafion-GCE: (**a**) with equal DA concentration (red) and without DA (blue). (**b**) with plasma concentration (26 pg/mL) of DA (red) and without DA (blue). SWV settings: f = 50 Hz, E_{sw} = 25 mV and ΔE = 8 mV (E_{acc} = 0 V, t_{acc} = 240 s)

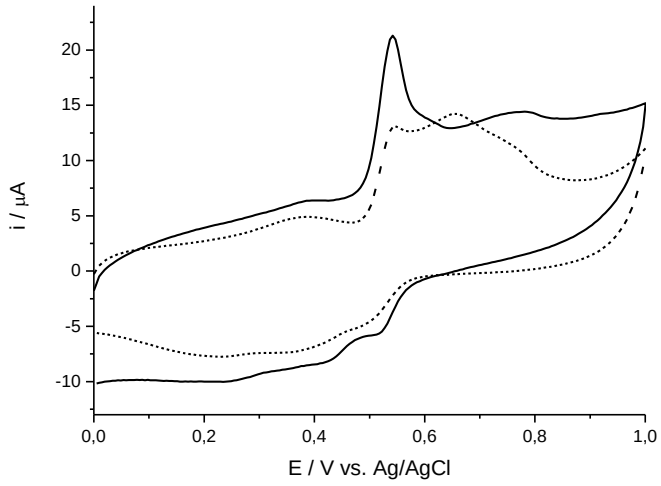


Supplementary Figure 8. Square-wave voltammograms of L-dopa (1 x 10^{-6} M) at the MWCNTs/Nafion-GCE with 50-fold uric acid (red) and without uric acid (black). SWV settings: f = 50 Hz, E_{sw} = 25 mV and ΔE = 8 mV (E_{acc} = 0 V, t_{acc} = 240 s)

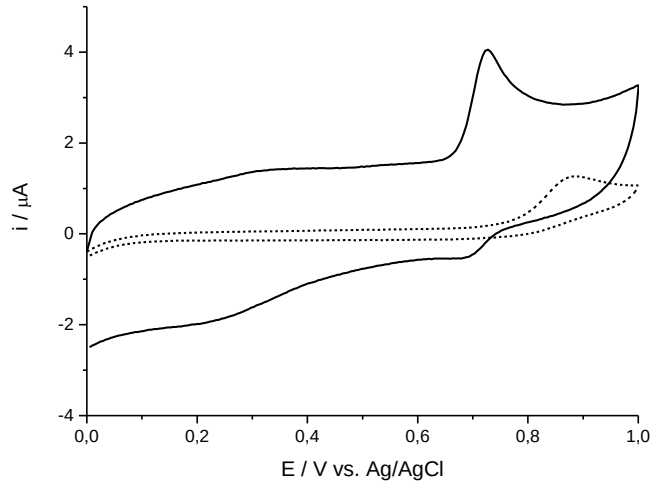
9.3. Supplemental data 3: Electrochemical sensing of mesalazine and its N-acetylated metabolite in biological samples using functionalized carbon nanotubes



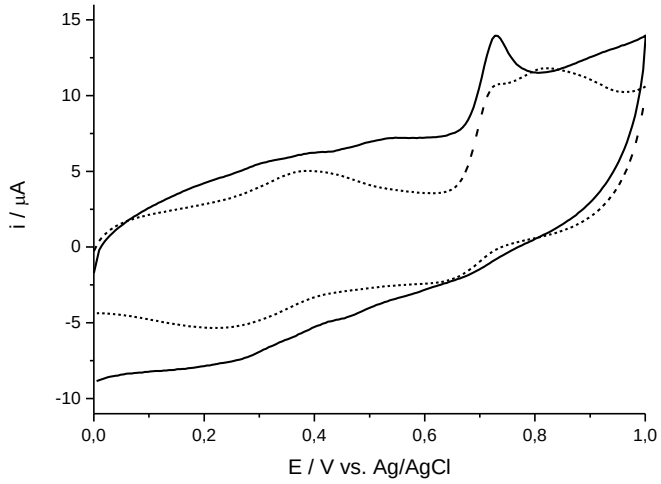
Supplementary Figure 1. Cyclic voltammograms of 5-ASA (5 x 10^{-5} M) at a bare gold electrode (dashed line) and a bare GCE (solid line). Scan rate: 100 mVs^{-1}



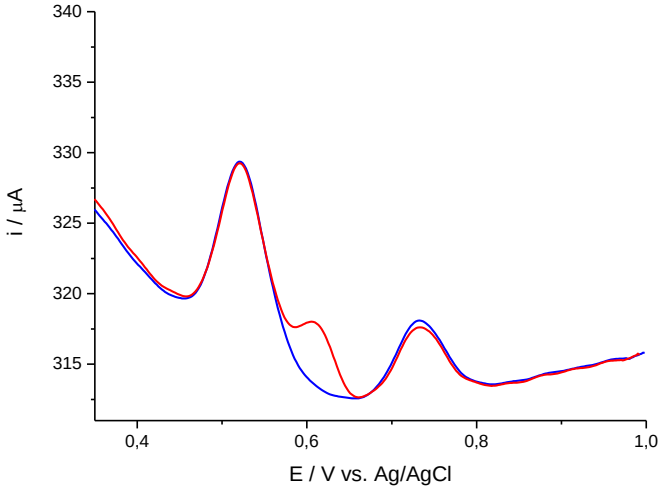
Supplementary Figure 2. Cyclic voltammograms of 5-ASA (5 x 10^{-5} M) at a gold electrode (dashed line) and GCE (solid line) modified with MWCNTs/Nafion composite film. Scan rate: 100 mVs^{-1}



Supplementary Figure 3. Cyclic voltammograms of Ac-5-ASA (5 x 10^{-5} M) at a bare gold electrode (dashed line) and a bare GCE (solid line). Scan rate: 100 mVs^{-1}



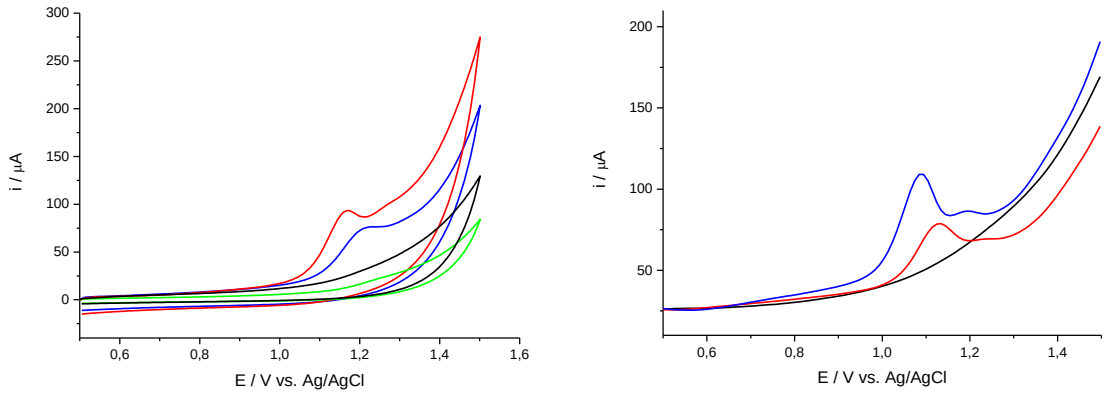
Supplementary Figure 4. Cyclic voltammograms of Ac-5-ASA (5 x 10^{-5} M) at a gold electrode (dashed line) and GCE (solid line) modified with MWCNTs/Nafion composite film. Scan rate: 100 mVs^{-1}



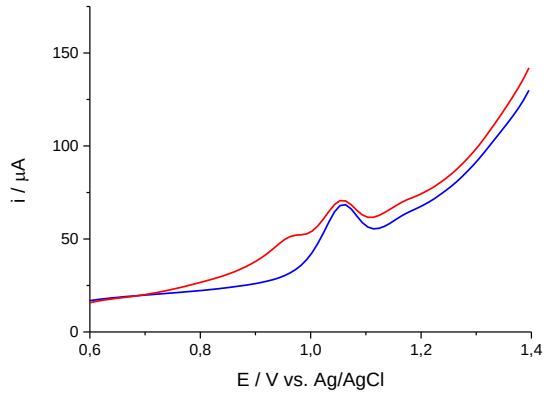
Supplementary Figure 5. Square-wave voltammograms of 5-ASA and Ac-5ASA (2.5 x 10^{-6} M) at the MWCNTs/Nafion-GCE with 5-fold uric acid (red) and without uric acid (blue). SWV settings: f = 70 Hz, E_{sw} = 50 mV and ΔE = 2 mV (E_{acc} = 0.1 V, t_{acc} = 30 s)

9.4. Supplemental data 4: Development of electrochemical platform based on carbon nanotubes decorated with zirconium oxide nanoparticles for determination of nebivolol

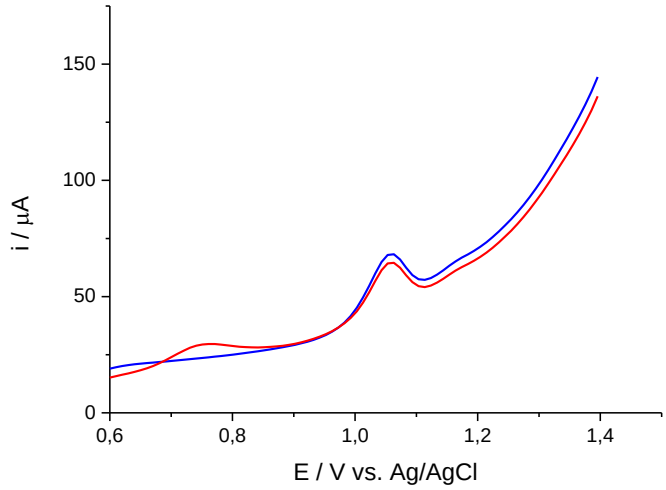
a) b)



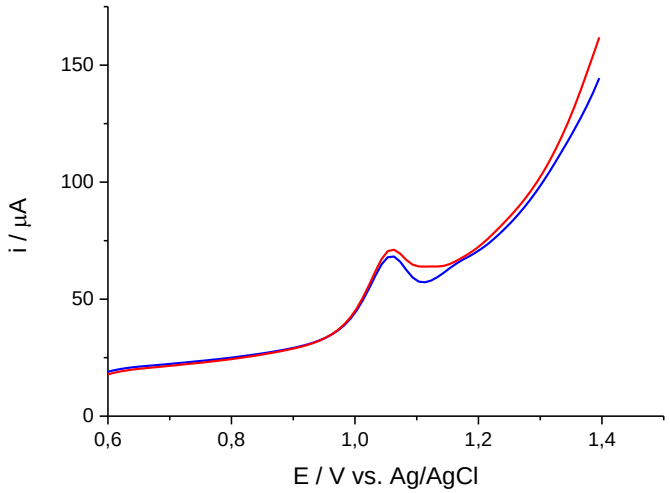
Supplementary Figure 1. Cyclic voltammograms (**a**) of NBV (1 x 10^{-4} M) in 0.1 M H_2SO_4 at a bare GCE (black), the BiNP/GCE (green), the MWCNTs/Nafion-GCE (red) and BiNP-MWCNTs/GCE (blue). Differential pulse voltammograms (**b**) of NBV (1 x 10^{-4} M) in 0.1 M H_2SO_4 at the MWCNTs/Nafion-GCE (blue) and BiNP-MWCNTs/GCE (red) together with corresponding background current (black)



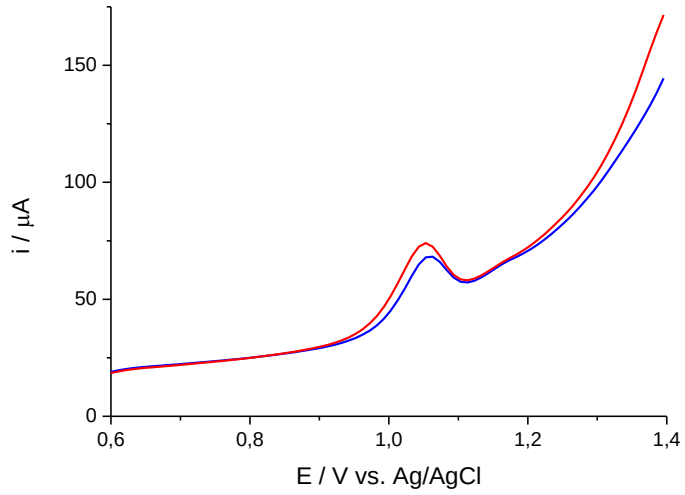
Supplementary Figure 2. Differential pulse voltammograms of NBV (2.5 x 10^{-6} M, blue) and mixture of NBV with carvedilol (1:1, red) in 0.1 M H_2SO_4. DPV settings: pulse amplitude of 50 mV, pulse width of 75 ms and scan rate of 20 mV s^{-1} (E_{acc} = 0 V, t_{acc}= 240 s)



Supplementary Figure 3. Differential pulse voltammograms of NBV (2.5 x 10^{-6} M, blue) and mixture of NBV with propranolol (1:1, red) in 0.1 M H_2SO_4. DPV settings: pulse amplitude of 50 mV, pulse width of 75 ms and scan rate of 20 mV s^{-1} (E_{acc} = 0 V, t_{acc}= 240 s)



Supplementary Figure 4. Differential pulse voltammograms of NBV (2.5 x 10^{-6} M, blue) and mixture of NBV with atenolol (1:1, red) in 0.1 M H_2SO_4. DPV settings: pulse amplitude of 50 mV, pulse width of 75 ms and scan rate of 20 mV s^{-1} (E_{acc} = 0 V, t_{acc}= 240 s)



Supplementary Figure 5. Differential pulse voltammograms of NBV (2.5 x 10^{-6} M, blue) and mixture of NBV with bisoprolol (1:1, red) in 0.1 M H_2SO_4. DPV settings: pulse amplitude of 50 mV, pulse width of 75 ms and scan rate of 20 mV s^{-1} (E_{acc} = 0 V, t_{acc}= 240 s)

10. BIOGRAPHY

Mirela Sadiković was born on 3rd of May, 1988 in Zagreb, Croatia. She finished elementary school and the Fourth Gymnasium in Zagreb. In 2007, she started studying Pharmacy at the Faculty of Pharmacy and Biochemistry, University of Zagreb. During her studies, she has been awarded several different scholarships and graduated in 2012 among the top 10% of students in her generation. With her Master thesis, titled "Electrochemical Properties of Lovastatin and Corresponding β-hydroxycarboxylic Acid", she has began her scientific research in the field of pharmaceutical analysis. In 2012 and 2013, she did an internship in a pharmacy, passed the state exam and obtained the Licence for independent work. Up to now, she has worked at the pharmacy and as a teaching assistant at the Department of Pharmaceutical Analysis at the Faculty of Pharmacy and Biochemistry, where she started her PhD in Pharmacy. Her main field of interest is the development of new electroanalytical methods for determination of selected pharmaceuticals in dosage forms and biological fluids using novel sensors based on carbon nanotubes. In 2016, she began her postgraduate specialization in Analysis and control of medicinal products. She is the co-author of four research papers and has participated in several international scientific congresses.

List of publications:

B. Nigović, M. Sadiković, M. Sertić, Multi-walled carbon nanotubes/ Nafion composite film modified electrode as a sensor for simultaneous determination of ondansetron and morphine, Talanta 122 (2014) 187-194.

M. Sadiković, B. Nigović, S. Jurić, A. Mornar, Voltammetric determination of ropinirole in the presence of levodopa at the surface of a carbon nanotubes based electrochemical sensor in pharmaceuticals and human serum, J. Electroanal. Chem. 733 (2014) 60-68.

B. Nigović, M. Sadiković, S. Jurić, Electrochemical sensing of mesalazine and its N-acetylated metabolite in biological samples using functionalized carbon nanotubes, Talanta 147 (2016) 50-58.

M. Sadiković, B. Nigović, Development of electrochemical platform based on carbon nanotubes decorated with zirconium oxide nanoparticles for determination of nebivolol, Int. J. Electrochem. Sci. 12 (2017) 9675-9688.

Basic documentation card

PhD thesis

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NOVEL ELECTROCHEMICAL METHODS IN ANALYSIS OF SELECTED DRUGS USING CARBON NANOTUBES-BASED SENSORS

Mirela Sadiković

SUMMARY

Due to strict requirements regarding quality control of pharmaceuticals and with the aim of obtaining maximal efficacy and safety of drug therapy for patients, there is a constant demand for developing new reliable analytical methods to determine drug concentrations in complex samples. Electroanalytical techniques, especially voltammetric, represent a powerful analytical tool. Electrochemical nanosensors have recently found extensive applications in pharmaceutical analysis. Therefore, the aim of this doctoral thesis was to develop new electroanalytical methods for the determination of selected pharmaceuticals in dosage forms and biological fluids using novel sensors based on multi-walled carbon nanotubes dispersed in Nafion matrix, as well as their combination with zirconium oxide nanoparticles. Electrochemical behaviour of selected drugs was examined by cyclic voltammetry to get an insight into their redox mechanism at modified electrodes. Experimental conditions and instrumental parameters that could affect the electroanalytical performance of proposed sensors were carefully optimised. New methods were validated according to ICH guidelines. Direct pulse voltammetric techniques (square-wave or differential pulse) were used to determine the content of ropinirole, L-dopa, mesalazine and nebivolol in tablets, except for ondansetron in which case adsorptive stripping procedure was employed. The good recoveries indicated that excipients did not interfere with the assay of active ingredient in any case. In comparison to the high performance liquid chromatographic methods, there were no significant differences regarding the accuracy and precision; however voltammetric techniques offer high sensitivity, rapid response and simplicity. Finally, nanosensors were applied for the simultaneous quantification of selected pharmaceuticals with other co-administered drugs in therapy (such as ondansetron with morphine and ropinirole with L-dopa) or their metabolites (mesalazine and N-acetylated metabolite) in human serum samples using adsorptive stripping voltammetry. Integration of the nanoparticles in the sensor improved notably the determination of therapeutic concentrations of nebivolol in serum. Excellent recoveries in the range of 98.7 – 102.6 % were obtained in all cases, without the need for sample pretreatment. A considerable enhancement effect on voltammetric responses of selected drugs, is due to synergy of remarkable properties of nanomaterials and cation-exchange polymer, that enhanced the preconcentration of positively charged drug molecules.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 121 pages, 4 schemes, 58 figures, 13 tables and 160 references. Original is in English language.

Keywords: multi-walled carbon nanotubes, zirconium oxide nanoparticles, Nafion, voltammetry, electrochemical nanosensors, ondansetron, morphine, ropinirole, L-dopa, mesalazine, metabolite of mesalazine, nebivolol

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The thesis was accepted: January 24th, 2018

Temeljna dokumentacijska kartica

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Farmaceutsko-biokemijski fakultet
Zavod za analitiku i kontrolu lijekova
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NOVE ELEKTROKEMIJSKE METODE U ANALITICI ODABRANIH LIJEKOVA PRIMJENOM SENZORA S UGLJIKOVIM NANOCJEVČICAMA

Mirela Sadiković

SAŽETAK

Uslijed strogih zahtjeva u pogledu kontrole kakvoće lijekova, a s ciljem postizanja maksimalne učinkovitosti i sigurnosti terapije za pacijente, postoji neprestani zahtjev za razvojem novih pouzdanih analitičkih metoda za određivanje koncentracija lijekova u složenim uzorcima. Elektroanalitičke tehnike, među kojima posebno voltametrijske, predstavljaju moćan analitički alat u tom cilju. S druge strane, elektrokemijski nanosenzori su u posljednje vrijeme našli značajnu primjenu u analitici lijekova. Stoga je cilj ovog doktorskog rada bio razvoj novih elektroanalitičkih metoda za određivanje odabranih lijekova u dozirnim oblicima i biološkim tekućinama primjenom inovativnih senzora s ugljikovim nanocjevčicama dispergiranima u matriksu Nafiona, kao i u kombinaciji s nanočesticama cirkonijeva dioksida. Elektrokemijsko ponašanje odabranih lijekova ispitano je cikličkom voltametrijom radi stjecanja uvida u mehanizam redoks procesa na modificiranim elektrodama. Eksperimentalni uvjeti i instrumentalni parametri koji bi mogli utjecati na elektroanalitičku izvedbu predloženih senzora pažljivo su optimirani. Nove metode validirane su u skladu s ICH smjernicama. Za određivanje sadržaja ropinirola, levodope, mesalazina i nebivolola u tabletama primijenjene su direktne pulsne voltametrijske tehnike (pravokutnovalna ili diferencijalno pulsna), osim u slučaju ondansetrona kada je primijenjena adsorptivna tehnika. Vrijednosti analitičkih prinosa ukazuju da pomoćne tvari ne interferiraju s određivanjem aktivnih tvari odabranih lijekova. Usporedbom s rezultatima tekućinske kromatografije visoke djelotvornosti, nisu nađene značajne razlike između tehnika što se tiče točnosti i preciznosti, ali su voltametrijske tehnike jednostavnije, brže i nude visoku osjetljivost. Nadalje, nanosenzori su korišteni i za simultanu kvantifikaciju lijekova koji se primijenjuju zajedno u terapiji (poput ondansetrona s morfinom i ropinirola s levodopom) te za simultanu kvantifikaciju lijekova s njihovim metabolitima (mesalazin s N-acetiliranim metabolitom mesalazina) u humanim uzorcima seruma primjenom adsorptivne voltametrije. Integracijom nanočestica u senzore omogućeno je osjetljivije određivanje terapijskih koncentracija nebivolola u serumu. Postignute su izvrsne vrijednosti analitičkih prinosa u rasponu od 98.7 % do 102.6 % u svim slučajevima, bez potrebe za predobradbom uzoraka. Znatno pojačanje voltametrijskih odgovora odabranih lijekova posljedica je sinergističkog učinka izvanrednih svojstava nanomaterijala i kationskog izmjenjivača, koji je omogućio ukoncentriravanje pozitivno nabijenih molekula lijekova.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

Rad sadrži: 121 stranicu, 4 sheme, 58 slika, 13 tablica i 160 literaturnih navoda. Izvornik je na engleskom jeziku.

Ključne riječi: ugljikove nanocjevčice, nanočestice cirkonijeva dioksida, Nafion, voltametrija, elektrokemijski nanosenzori, ondansetron, morfin, ropinirol, levodopa, mesalazin, *N*-acetilirani metabolit mesalazina, nebivolol

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Rad prihvaćen: 24. siječnja 2018.