GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

HABILITATION THESIS

ANALYTICAL INVESTIGATION OF SOME DRUGS AND HEAVY METALS' IONS

PROFESSOR NELA BIBIRE, PhD

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CONTENTS

ABSTRACT

The Habilitation Thesis is structured into three parts:

The first part presents the major scientific research activities I performed during my postdoctoral period (1999-2019), focusing mainly on the following research domains:

1. Development of some High Performance Liquid Chromatography (HPLC) analytical methods

The newly developed HPLC (High Performance Liquid Chromatography) methods used both UV detection (simultaneous analysis of some anti-tuberculosis drugs - isoniazid, pyrazinamide, rifampicin and analysis of some antihypertensive drugs - lisinopril, bisoprolol or analysis of some antibiotics - ciprofloxacin) and mass spectrometry detection (analysis of clopidogrel, alprazolam and bisoprolol); they were successfully applied for the quantitative analysis of pharmaceutical substances from different therapeutic classes in pharmaceutical products, as well as in biological fluids (clopidogrel, alprazolam and bisoprolol).

The HPLC methods were validated, complying with current validation criteria, proving to be suitable for use in any laboratory of analysis. These methods can be successfully applied in bioequivalence studies, for therapeutic monitoring of drugs and for pharmacokinetic parameters'determination.

2. Development of some UV-Vis Spectrophotometric analytical methods

These studies focused on two therapeutic classes - angiotensin converting enzyme (ACE) inhibitors (lisinopril and ramipril) and antihypertensive beta-blockers (bisoprolol); these substances have long been a research topic of interest for the members of my department.

The designed methods were based on the formation of complexes between ACE inhibitors and heteropoly acids (phosphotungstic and phosphomolybdic acids), in acidic medium, and between bisoprolol and tropeolin 00. The latter method was often cited in several scientific papers, because, at that time, it was the only reported Vis Spectrophotometric method for quantitative determination of bisoprolol.

All three UV-Vis Spectrophotometric methods are easy to apply and can be used in routine analysis in Drugs Analysis and Control Laboratories.

3. Development of some Atomic Absorption Spectroscopy (AAS) analytical methods

AAS is a spectro-analytical procedure specifically used for the determination of metals and, considering the biological and medical implications of heavy metals' ions from environmental elements and from various plant products, our research group meant to expand its research area and focused on AAS.

Therefore, we analyzed the heavy metals content from tobacco, because cigarette smoke is one of the main sources of heavy metals for the human body and for the environment, and we also evaluated the lead content in various food products of plant origin, that represent the basis of our diet (wheat, corn, beans, onion, carrot, potatoes, cabbage).

4. Development of some Ion-Selective Membrane Electrodes (ISE)

ISE represents my first scientific domain of interest and this quest began during my PhD studies, when my research objectives were directed towards the development and application of Ion-Selective Membrane Electrodes in tropane alkaloids analysis (atropine, homatropine, scopolamine and N-butylscopolammonium bromide).

Later on, I worked in collaboration with other colleagues on developing ion-selective membrane electrodes for other classes of therapeutic agents (H2-antihistamines - ranitidine, famotidine, nizatidine, purine alkaloids - caffeine, theophylline, ACE inhibitors - lisinopril, ramipril) and for some heavy metals' ions (copper, mercury, cadmium, lead and nickel ions).

The second part of the Habilitation Thesis presents my professional, scientific and academic contributions. This section includes a review of my career, a synthesis of my PhD Thesis, a brief presentation of my teaching activity and a concise overview of my research results.

My research results have materialized into scientific papers published in ISI and BDI journals or in ISI and BDI indexed supplements. 38 of these publications are in ISI indexed journals (cumulative impact factor = 19,278; 17 papers as main author; 21 papers as co-author; Hirsch index = 7). The visibility of my research is supported by more than 100 citations in the ISI Web of Science, Core Collection, Thomas Reuters.

The third part describes my future research directions, that will develop in parallel with the subjects of interest already described:

- ✓ introduction of new analytical reagents for the analysis of ions and pharmaceutical substances;
- ✓ correlation of physico-chemical parameters of newly synthesized chemical entities with their structure, in order to identify their utility in therapeutics;
- ✓ development and validation of drug analysis methods for prolonged-release dosage forms, micro-particulate and nano-particulate systems;
- ✓ characterization of some extracts from marine macroalgae along the Romanian Black Sea coast.

REZUMAT

Teza de abilitare este structurată în trei părți:

Prima parte este rezervată cercetărilor științifice majore din perioada postdoctorală (1999 - 2019), perioadă în care m-am concentrat asupra următoarelor domenii de cercetare:

1. Dezvoltarea unor metode de analiză cromatografică de lichide la înaltă performanță (HPLC)

Metodele de analiză prin cromatografie de lichide la înaltă presiune (HPLC) au fost atât cu detecție în UV (analiza simultană a unor antituberculoase - izoniazida, pirazinamida și rifampicina, analiza unor antihipertensive - lisinopril și bisoprolol și analiza unor antibiotice - ciprofloxacina), cât și cu detecție prin spectrometrie de masă (analiza clopidogrelului, alprazolamului și bisoprololului) și au fost aplicate la determinarea cantitativă a unor substanțe medicamentoase aparținând la clase terapeutice diferite atât din produse farmaceutice, dar și din lichide biologice (analiza clopidogrelului, alprazolamului și bisoprololului).

Metodele HPLC dezvoltate au fost validate conform criteriilor de validare în vigoare pentru a putea fi utilizate în siguranță de orice laborator de analize. Metodele respective pot fi aplicate cu succes în studiile de bioechivalență, pentru monitorizarea terapeutică a substanțelor medicamentoase vizate și stabilirea parametrilor farmacocinetici.

2. Dezvoltarea unor metode de analiză spectrofotometrică UV-Vis

Aceste studii au vizat două clase de substanțe medicamentoase, și anume lisinoprilul și ramiprilul dintre inhibitoarele enzimei de conversie a angiotensinei (IECA) și bisoprololul dintre antihipertensivele beta-blocante, substanțe medicamentoase ce constituie de mai mult timp subiectele unora dintre preocupările științifice ale colectivului disciplinei din care fac parte.

Metodele se bazează pe formarea de complecși cu heteropoliacizii în mediu acid și anume acidul fosfotungstic și acidul molibdofosforic în cazul celor două IECA, iar în cazul bisoprololului cu tropeolina 00. Această din urma metodă a fost deseori citată în literatura de specialitate pentru că la acea dată era singura medodă de determinare cantitativă a bisoprololului prin spectrofotometrie în vizibil. Toate cele trei metode spectrofotometrice UV-Vis sunt ușor de aplicat și pot fi utilizate în analiza de rutină din cadrul laboratoarelor de analiză și control al medicamentelor.

3. Dezvoltarea unor metode de analiză spectroscopică de absorbție atomică (AAS)

AAS este o metodă spectroscopică specifică analizei metalelor și de aceea colectivul nostru a dorit sa-și diversifice aria cercetării având în vedere implicațiile biologice și medicale ale ionilor metalelor grele din elemente de mediu și din diverse produse vegetale.

De aceea, pe de o parte am analizat ionii metalelor grele din tutun pentru că fumul de țigară este o sursă importantă de metale grele pentru organismul uman și pentru mediu, iar pe de altă parte ne-am îndreptat atenția asupra conținutului în plumb ale diverselor produse alimentare de origine vegetală care care constituie baza alimentației noastre (grâu, porumb, fasole, ceapă, morcovi, cartofi, varză).

4. Dezvoltarea unor electrozi membrană ion-selectivi (ISE)

ISE reprezintă cea mai veche dintre preocupările mele științifice, iar punctul de plecare l-a constituit doctoratul meu care a avut ca obiectiv al cercetării dezvoltarea și aplicarea

electrozilor membrană ion-selectivi în analiza alcaloizilor cu nucleu tropanic (atropină, homatopină, scopolamină și bromură de N-butil scopolamoniu).

În continuare am colaborat cu alți colegi care au ales la rândul lor, mai târziu, să dezvolte ISE pentru alte clase de substanțe medicamentoase (antihistaminice H2 - ranitidină, famotidină și nizatidină, alcaloizi purinici - cafeina, teofilina, IECA - lisinopril, ramipril) sau pentru unii ioni ai metalelor grele (ionii cuprici și mercurici, ionii de cadmiu, plumb și nichel).

A doua parte a tezei prezintă contribuțiile profesionale, științifice și academice. Această secțiune include o trecere în revistă a carierei, o sinteză a tezei mele de doctorat, o informare succintă asupra activității mele didactice și o scurtă prezentare a rezultatelor activității de cercetare.

Rezultatele cercetărilor mele s-au concretizat în lucrări ştiinţifice publicate în reviste cotate ISI, reviste indexate BDI sau suplimente ale revistelor indexate ISI sau BDI. Dintre acestea, 38 de lucrări publicate sunt indexate și cotate ISI (factor de impact cumulat 19, 278 / 17 lucrări ca autor principal, 21 lucrări coautor, indice Hirsch 7). Vizibilitatea cercetărilor mele este susținută de peste 100 de citări în ISI Web of Science, Core Collection, Thomas Reuters.

A treia parte descrie direcțiile viitoare de cercetare care se vor derula în paralel cu preocupările deja descrise:

- ✓ introducerea de noi reactivi analitici pentru determinarea unor ioni și a unor substanțe medicamentoase;
- ✓ corelarea parametrilor fizico-chimici ai unor entități chimice nou sintetizate cu structura lor, în scopul identificării utilității acestora în terapeutică;
- ✓ elaborarea și validarea unor metode de analiză pentru substanțe medicamentoase din forme farmaceutice cu cedare prelungită, sisteme micro- și nanoparticulate;
- ✓ caracterizarea unor extracte din macroalge marine prezente de-a lungul coastei românești a Mării Negre.

I. MAIN SCIENTIFIC ACHIEVEMENTS IN POSTDOCTORAL PERIOD (1999- 2019)

I.1. Development of some High Performance Liquid Chromatography (HPLC) analytical methods

The chromatographic methods are commonly used for the qualitative and quantitative analysis of raw materials, drug substances, drug products, and compounds in biological samples in pharmaceutical industry. The components monitored include chiral or achiral drugs, process impurities, residual solvents, excipients such as preservatives, degradation products, extractable and leachable from container and closure or manufacturing process, pesticide in drug product from plant origin, and metabolites.

HPLC play an important and critical role in the field of pharmaceutical industries and analysis, since it is used to test the products and to detect the raw ingredient used to make them i.e., qualitative and quantitative analysis. Moreover, the importance of HPLC uses in these fields falls under the stringent regulations established by the U.S. Food and Drug Administration (FDA) (1). This obligates all pharmaceutical companies to detect the quality of their products by using the HPLC before allowing them to sell it in the global market.

The most important benefits gain from the uses of HPLC technique in the industrial and analytical field that it is help in structure elucidation and quantitative determination of impurities and degradation products in bulk drug materials and pharmaceutical formulations (2). These benefits which gain from the using of HPLC not only limited for the synthetic drugs and formulas but also include herbal medicine too.

I.1.1. Development of some HPLC analysis methods with UV-Vis detection

In liquid chromatography the choice of detection approach is critical to guarantee that all the components are detected. One of the widely used detectors in HPLC is UV detector which is capable of monitoring several wavelengths concurrently; this is possible only by applying a multiple wavelength scanning program.

If present in adequate quantity, UV detector assures all the UV-absorbing components are detected. A UV visible HPLC detector uses light to analyze samples. By measuring the sample's absorption of light at different wavelengths, the analyte can be identified. HPLC UV detectors can be used by any lab using HPLC, including genomic, biology, and biochemistry laboratories, to analyze nucleic acids, proteins, and to do toxic and therapeutic drug testing.

Two types of HPLC-UV detectors are single and variable wavelength detectors. Single wavelength detectors measure the samples absorption of a single wavelength, while variable wavelength detectors measure absorption of multiple wavelengths and are therefore more sensitive (3). When deciding on a HPLC-UV detector, consider whether a single wavelength will be sufficient or if a variable wavelength detector is needed. The wavelength range that can be used and the sensitivity of the detector should also be factored into the decision.

A standard UV detector allows user to choose wavelength between 195 to 370 nm. Most commonly used is 254 nm. Compared to a UV detector, a VIS detector uses longer wavelength (400-700 nm). There are detectors that provide wider wavelength selection, covering both UV and Vis ranges (195-700 nm) called UV/Vis detector (4). UV/Vis detection is more rugged than many other detection systems.

Published papers in the field

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♦ **Nela Bibire**, Mădălina Vieriu, Alina Diana Panainte, Luminița Agoroaei, Livia Uncu, Cristina Victorina Vlase, Aurel Vlase. A New High Performance Liquid Chromatographic Method for Ciprofloxacine. *Rev Chim (Bucharest)* 2015; 66(9): 1463-1466.

♦ Alina Diana Panainte, Luminița Agoroaei, **Nela Bibire**, Gladiola Țântaru, Mihai Apostu, Mădălina Vieriu, Florin Adrian Șpac. A HPLC Method for the Determination of Bisoprolol in Tablets and its Application to a Bioequivalence Study. *Rev Chim (Bucharest)* 2015; 66(11): 1791-1795.

♦ Vieriu M, **Bibire N**, Peşte G, Dorneanu V, Potorac L. A High Performance Liquid Chromatographic Method Using UV Detection for the Determination of Lisinopril. *Rev Chim (Bucharest)* 2013; 64(3): 298-300.

♦ Gabriela Peşte, **Nela Bibire**, Rodica Diaconescu, Corneliu Oniscu. A New Liquid Chromatographic Method for Determination of 4-Hidroxibenzyl Alcohol in 2-Isopropoxi Ethanol Solution. *Buletinul Institutului Politehnic Iași* 2011; LVII(LXI), Fasc. 1: 171-178.

I.1.1.1. Simultaneous HPLC analysis of isoniazid, pyrazinamide and rifampicin

Introduction

Pulmonary tuberculosis, the chronic infectious disease caused by *Mycobacterium tuberculosis*, represents a global health emergency causing an increasing number of deaths in developing countries (5, 6). A resurgence of tuberculosis has been noticed in central and Eastern Europe in recent years and the increase in poverty, poor living conditions, malnutrition and lack of medication are among the main causes (7). Currently, more than one third of the world's population is infected with *Mycobacterium tuberculosis*, with approximately 8 million new cases and 2 million deaths reported each year (8). Presently, control of tuberculosis is still a challenge and it is estimated that between 2002 and 2020 nearly 1 billion people will have been affected by the disease if proper control measure will not be instated (9).

Isoniazid (I) (figure 1), pyrazinamide (P) (figure 2), rifampicin (R) (figure 3) are active pharmaceutical substances (APS) used as first-line anti-tuberculosis drugs. The use of fixed-dose combination tablets of the four active APS for tuberculosis treatment is preferred (10).

Various techniques have already been reported for the analysis of anti-tuberculosis APS in various pharmaceutical formulations and biological samples (11, 12). Among them, reversed phase-high performance liquid chromatography (RP-HPLC) is a sensitive enough and suitable method to quantify those pharmaceutical substances included in the study, as well as many others (13-21).

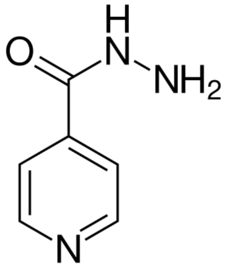


Fig. 1. Chemical structure of isoniazid

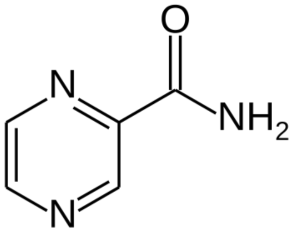


Fig. 2. Chemical structure of pyrazinamide

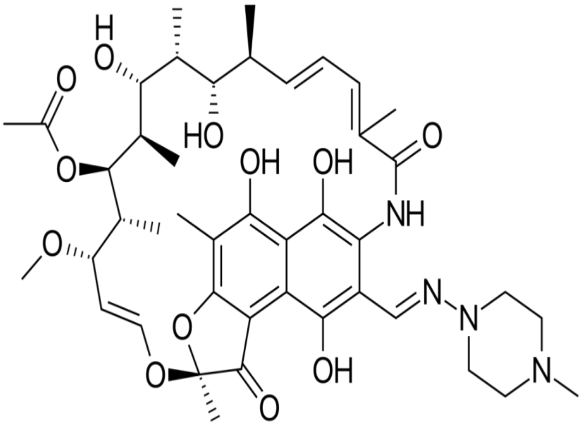


Fig. 3. Chemical structure of rifampicin

Materials and methods

Instruments

Chromatographic analysis has been performed with an Agilent 1100 Series HPLC system with UV-VIS detection by performing manual injections with a 20 µL sample loop. Separation of the interest compounds was achieved under gradient elution mode on a Zorbax SB-C18 (150×4 mm, 5 µm) stainless steel column with a guard column containing the same stationary phase.

Reagents

Pure active isoniazid, pyrazinamide and rifampicin were purchased from the Control Department of Pharmaceutical and Biological Products, Antibiotics Company, Iasi, Romania. APS tablets were purchased from the same company. Acetonitrile (CH_3CN) of HPLC reagent grade was obtained from Sigma Aldrich, and ultrapure water (18.2 MΩ·cm) was obtained with UltraPure Option Q Lab equipment.

Mobile phase

The gradient elution was carried out with a mobile phase of 10% aqueous solution CH_3CN for channel A and 50% CH_3CN in pH = 6.8 phosphate buffer (20 mM), (plus 1.5 mL triethylamine) for channel B.

The analytes can be analyzed in a chromatographic run of 10 minutes at a flow rate of 1 mL/min.

The gradient profile (A:B) was 100:0 (v/v) for 4 minutes, then 0:100 for 0.5 minutes and then it was kept constant at 0:100 (v/v) for 5.5 minutes.

Preparation of solutions

Stock standard solutions were prepared by dissolving accurately weighed reference isoniazid (99.65%), pyrazinamide (99.48%) and rifampicin (99.86%) in mobile phase B in order to achieve 1000 mg/L concentration levels.

Mixed standards were prepared by appropriate dilution and by mixing mobile phase B with each stock solution in a 10 mL volumetric flask to achieve selected final concentrations in the calibration range.

Results

In order to determine the optimum wavelength for detection, absorption spectra of all analytes were registered in the 200-600 nm range using a SPECORD 210 UV-VIS spectrophotometer (figure 4).

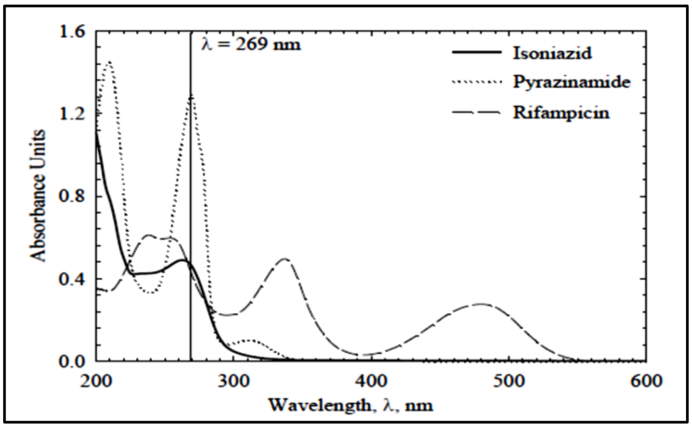


Fig. 4. UV-VIS absorption spectra of isoniazid, pyrazinamide and rifampicin

For the determination of the specific retention time (tR) single component standards were injected into the HPLC system under the chromatographic conditions described previously (figure 5).

The calibration curves for mixtures containing standards of isoniazid, pyrazinamide and rifampicin were investigated in the 0.5-50 mg/L concentration range. The calibration curves of standard isoniazid, pyrazinamide and rifampicin were generated by plotting each analyte peak area against concentration.

Linear regression analysis has been applied to plots of the interest APS peak area versus concentration. Calibration curves for isoniazid, pyrazinamide, and rifampicin are presented in figure 6. Very good correlation coefficients were obtained and the linearity data are reported in table 1.

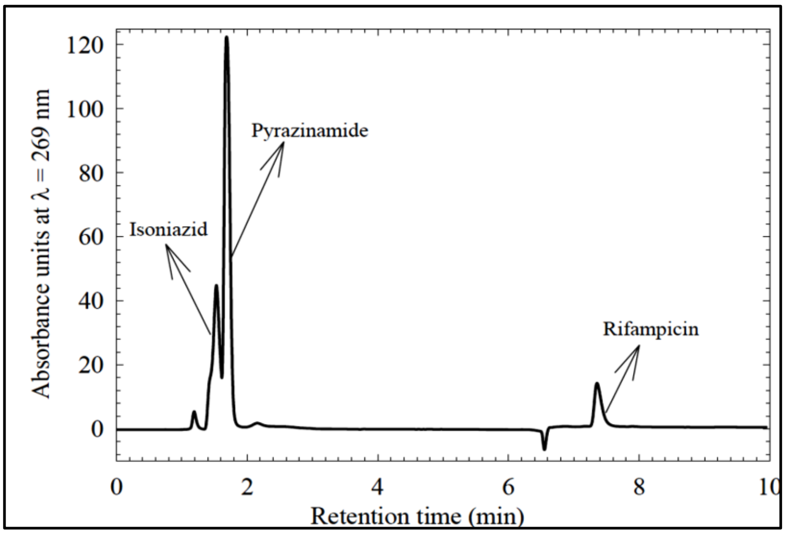


Fig. 5. Chromatogram of isoniazid, pyrazinamide and rifampicin

The limit of detection (LOD) has been estimated based on the ratio between three times the standard deviation, estimated from 10 successive injections of the lowest concentration standard, and the sensitivity of the method for each one of the APS.

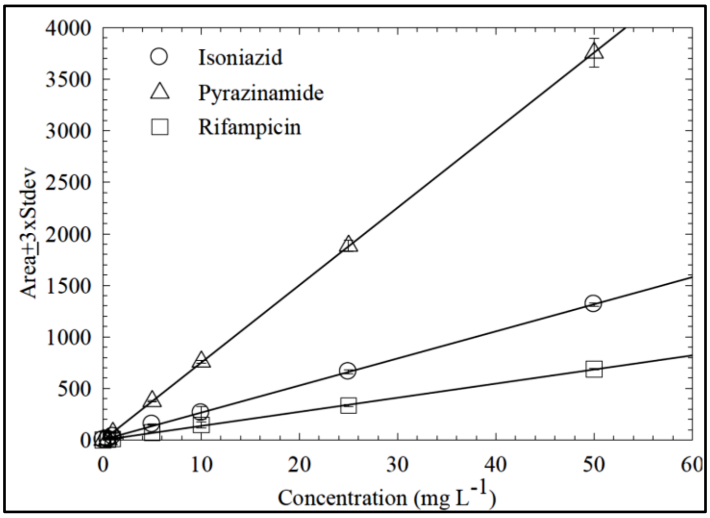


Fig. 6. Calibration curves for isoniazid, pyrazinamide and rifampicin

Table 1. Calibration results and LOD values for isoniazid, pyrazinamide and rifampicin

APS	Formula	M (g/mol)	Points	R^2	Slope	Offset	LOD (mg/L)
isoniazid	$C_6H_7N_3O$	137	6	0.9997	26.239	4.098	0.472
pyrazinamide	$C_5H_5N_3O$	123	6	0.9997	75.424	-8.395	0.542
rifampicin	$C_{43}H_{58}N_4O_{12}$	823	6	0.9995	13.687	-0.377	0.456

The amounts of each APS have been compared to the labeled value (table 2).

Table 2. Quantified amount of APS per tablet/capsule

Batch N^o	APS	Labelled amount (mg)	Quantified amount (mg)	STDEV×2	% APS
1	isoniazid	300	303.2	6.66	101.07
	pyrazinamide	500	498.18	9.64	99.64
	rifampicin	150	149.43	1.15	99.62
2	isoniazid	300	299.96	5.78	99.99
	pyrazinamide	500	501.10	4.90	100.22
	rifampicin	150	150.64	5.49	100.43
3	isoniazid	100	100.03	6.80	100.03
	pyrazinamide	500	501.34	9.46	100.27
	rifampicin	150	150.55	9.76	100.37

Discussions

As figure 4 shows, detection of isoniazid, pyrazinamide and rifampicin was best done at 269 nm.

Figure 5 shows a separation chromatogram of all three APS within 10 minutes, including the detection at 269 nm.

The elution order was $tR_{isoniazid} < tR_{pyrazinamide} < tR_{rifampicin}$ in agreement with the polarity of the interest APS expressed as log P: -0.64 for isoniazid (18), -1.884 for pyrazinamide (19) and 3.719 for rifampicin (20).

The quantification of the interest APS in pharmaceutical samples was performed by means of external standard procedure. Three batches of isoniazid, pyrazinamide and rifampicin tablets were analyzed using the described method. Five tablets for each of the APS were weighed to determine the average tablet weight. After homogeneous grounding to a fine powder, a certain amount of each APS was accurately weighted and quantitatively transferred into a 100 mL volumetric flask by adding mobile phase B.

The samples were ultrasonicated to achieve better dissolution and then filtered through a 0.45 µm Millipore filter to obtain clear solutions before injecting. For each sample, three replicate injections were made to evaluate the reproducibility of the preparation of the sample, of injecting and that of tablet content. Neither of the investigated tablets contained compound that interfered with the determination of either of the APS. The averaged concentration of APS in each sample and the associated RSDs showed good reproducibility of the method and of the injection (table 2).

The admissibility criteria from Pharmacopoeia specifies that there should not be less than 99.0% and no more than 101% of isoniazid, 99.0% and 100.5% of pyrazinamide, and 97.0% and 102.0% of rifampicin in the drug product. The data in table 2 showed that the analyzed tablets met the criteria (22).

Conclusions

The performed work shows that a new and accurate HPLC method can be used to assay isoniazid, pyrazinamide and rifampicin used as active pharmaceutical ingredients in drug tablets used for the treatment of tuberculosis. The method saves time (10 minutes chromatographic run for the analysis of all three APS) as well as organic solvents and the proposed method showed low detection limits (isoniazid 0.472 mg/L, pyrazinamide 0.542 mg/L, rifampicin 0.456 mg/L) and very good linearity.

The present method is simple and fast. Moreover, the analytical equipment used is an integral part of the infrastructure presently existent in almost any analytical laboratory. The proposed method can be used for the simultaneous analysis of isoniazid, pyrazinamide and rifampicin in pharmaceutical formulations used for the treatment of tuberculosis.

I.1.1.2. HPLC analysis of lisinopril

Introduction

Lisinopril - (S)-1-[N^2-(1-carboxy-3-phenylpropyl)-L-lysyl]-L-proline dihydrate - (figure 7) is a drug of the angiotensin converting enzyme inhibitor (IECA) class that is primarily used in treatment of hypertension, congestive heart failure, heart attacks and also in preventing renal and retinal complications of diabetes. Lisinopril has a number of properties that distinguish it from other ACE inhibitors: it is hydrophilic, it has a long half-life and good tissue penetration and it is not metabolized by the liver.

Several assay methods are available for the assay of lisinopril, including HPLC-UV (23-29). The HPLC assays are sensitive enough to measure lisinopril levels in biological fluids after oral administration of therapeutic doses to humans.

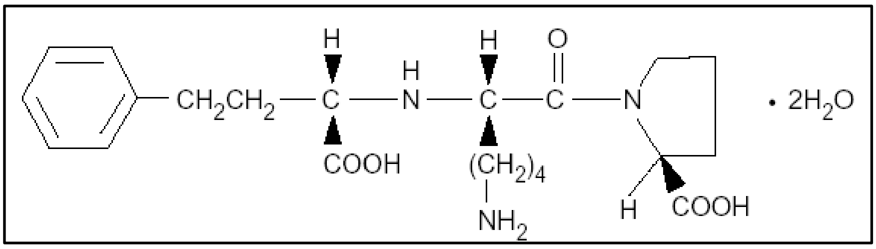


Fig. 7. Chemical structure of lisinopril

Materials and methods

Instruments

All analyses were performed using the HPLC Agilent 1200 system. The system components included the Agilent 1200 Degasser, Agilent 1200 Binary Pump, Agilent 1200 Autosampler and Agilent 1200 UV Detector.

The Agilent Chem 32 software was used for system control and data acquisition. An analytical scale Mettler-Toledo XP56, a Sigma 2-16 K centrifuge and a Vibramax 110 shaker were used. The separation was performed using a Lichrospher® 100 RP 8 (125 X 4.6), 5µM.

Reagents

The standard lisinopril dihydrate used in this study was supplied by European Pharmacopoeia, with a purity of 100.00%.

All solvents and other chemicals were HPLC grade provided by Merck, Germany.

Mobile phase

The mobile phase consist in a mixture of pH 2 phosphate buffer containing 0.125% sodium hexanesulphonate and acetonitrile (72:28, v/v) with a flow rate of 1 mL/min.

Preparation of solutions

A stock solution of lisinopril dihydrate with a concentration of 0.3 mg/mL was prepared by dissolving an appropriate quantity of lisinopril dihydrate reference substance in methanol:water (1:1). This solution was kept at 5°C. In these conditions, it was stable for at least 7 days.

Seven lisinopril solutions of various concentrations (0.6, 1.2, 9, 18, 24, 48, 60 µg/mL) were prepared for the study of the linearity response in pH 1.2 buffer, covering the concentrations range of 0.6-60 µg/mL.

The quality control samples with theoretical concentrations of 3 µg/mL (the low-QC1), 30 µg/mL (the medium-QC2) and 45 µg/mL (the high-QC3) were used to validate the analytical method.

Results

Method validation

<u>Selectivity/Specificity</u>

The study of the Selectivity/Specificity: a placebo mixture containing non-active ingredients usually found in lisinopril tablets was carried through the extraction procedure and chromatographed to determine the extent to which non active ingredients may interfere with the chromatographic analysis of lisinopril (figure 8).

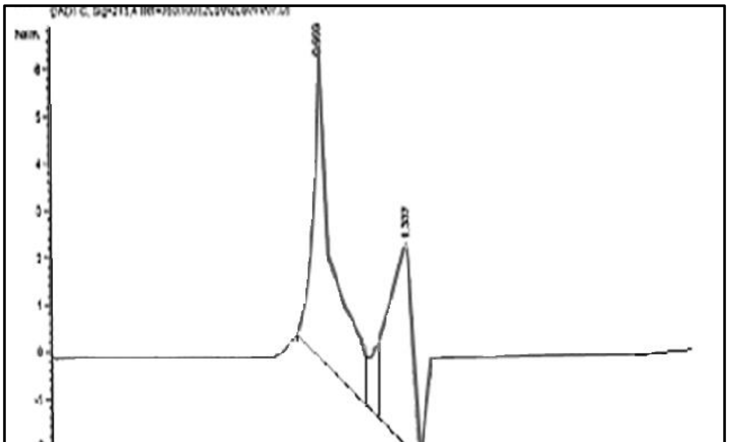
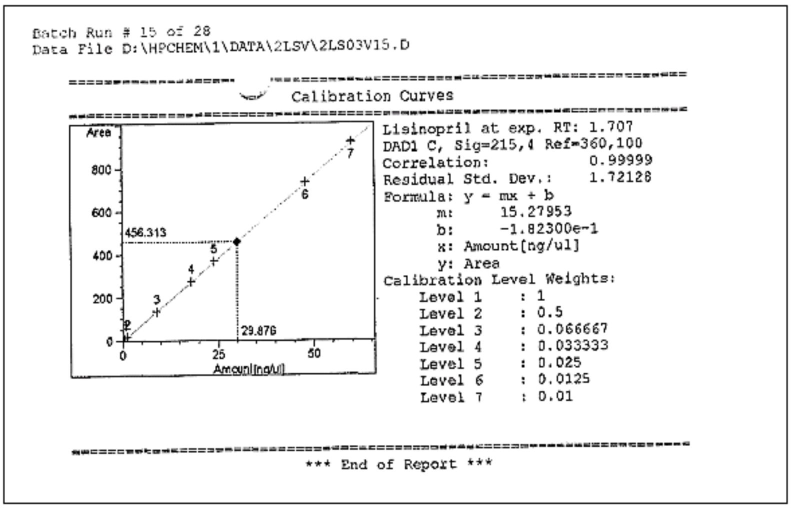


Fig. 8. Chromatogram recorded for blank placebo sample

The study of linearity and lower limit of quantification: calibration curves are found to be consistently accurate over 0.600 to 60.000 µg/mL calibration range (figure 9).



```
Batch Run # 15 of 28
Data File D:\HPCHEM\1\DATA\2LSV\2LS03V15.D

        ====================================================================
                          Calibration Curves
        ====================================================================
                                   Lisinopril at exp. RT: 1.707
                                   DAD1 C, Sig=215,4 Ref=360,100
                                   Correlation:            0.99999
                                   Residual Std. Dev.:     1.72128
                                   Formula: y = mx + b
                                          m:      15.27953
                                          b:      -1.82300e-1
                                          x: Amount[ng/ul]
                                          y: Area
                                   Calibration Level Weights:
                                       Level 1    : 1
                                       Level 2    : 0.5
                                       Level 3    : 0.066667
                                       Level 4    : 0.033333
                                       Level 5    : 0.025
                                       Level 6    : 0.0125
                                       Level 7    : 0.01

        ====================================================================
                          *** End of Report ***
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Fig. 9. Representative calibration curve for lisinopril dissolved in pH 1.2 buffer

The lower limit of quantification or the lowest standard level with a coefficient of variation less than 20%, is 0.6 µg lisinopril/mL (figure 10). Results are presented in table 3.

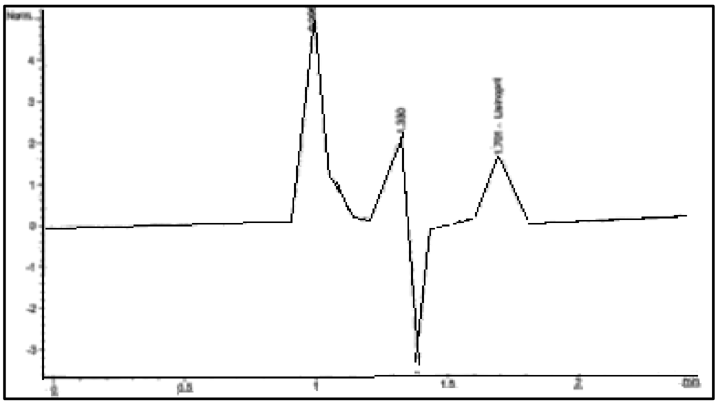


Fig. 10. The chromatogram recorded for sample containing 0.6 µg/mL lisinopril

Table 3. Lower limit of quantification

	Calculated concentration (µg/mL)	Accuracy %
	0.610	101.667
	0.610	101.667
	0.625	104.167
	0.625	104.167
	0.600	100.000
	0.615	102.500
N	6	6
Mean	0.614	102.361
SD (±)	0.010	
CV(%)	1.580	
Acceptance criteria: 4 out of 6 LLQC must be 100±20% nominal value, nominal mean % 100±20%, CV % ≤ 20%		

Within-run accuracy and precision evaluations were performed by analyzing replicate concentrations of lisinopril. The run consisted of a calibration curve and a total of 18 quality control samples, 6 replicates of each of low (QC1), medium (QC2) and high (QC3) quality control samples. Results are presented in table 4.

The between-run accuracy and precision were assessed by repeated analysis of quality control samples containing different concentrations of lisinopril on separate occasions. A single run consisted of a calibration curve and 6 replicates of the low (QC1), medium (QC2) and high (QC3) quality samples for lisinopril. Results are presented in table 5.

Table 4. Intra-day precision and accuracy for lisinopril quality control samples

	Theoretical concentration QC1(3 µg/mL)		Theoretical concentration QC2 (30 µg/mL)		Theoretical concentration QC3 (45 µg/mL)	
No.	Calculated (µg/mL)	Accuracy %	Calculated (µg/mL)	Accuracy %	Calculated (µg/mL)	Accuracy %
1.	2.980	99.333	29.876	99.587	45.241	100.536
2.	2.961	98.700	30.095	100.317	44.855	99.678
3.	2.966	98.867	30.159	100.530	44.636	99.191
4.	3.100	103.333	30.200	100.667	44.200	98.222
5.	3.110	103.667	31.100	103.667	45.950	102.111
6.	2.990	99.667	29.500	98.333	44.775	99.500
n	6	6	6	6	6	6
Mean	3.018	100.594	30.155	100.517	44.943	99.873
SD	0.068		0.530		0.598	
CV %	2.265		1.758		1.329	
Acceptance criteria: 67% Total QCs must be 100±15% nominal values; 50% QCs per level must be 100±15% nominal values ; Mean % nominal 100±15%; CV(%) ≤15%						

Table 5. Inter-day precision and accuracy for lisinopril spiked quality control samples

	Theoretical concentration QC1 (3 µg/mL)		Theoretical concentration QC2 (30 µg/mL)		Theoretical concentration QC3 (45 µg/mL)	
No.	Calculated (µg/mL)	Accuracy %	Calculated (µg/mL)	Accuracy %	Calculated (µg/mL)	Accuracy %
1.	2.950	98.333	30.454	101.513	45.414	100.920
2.	2.921	97.367	30.141	100.470	45.299	100.664
3.	2.970	99.000	30.311	101.037	45.360	100.800
4.	2.910	97.000	30.617	102.057	45.729	101.620
5.	2.926	97.533	30.260	100.867	45.482	101.071
6.	2.933	97.767	30.219	100.730	45.413	100.918
n	6	6	6	6	6	6
Mean	2.935	97.833	30.334	101.112	45.450	100.999
SD	0.022		0.174		0.150	
CV %	0.740		0.573		0.330	
Acceptance criteria: 67% Total QCs must be 100±15% nominal values, 50% QCs per level must be 100±15% nominal values, Mean % nominal 100±15%, CV(%) ≤ 15%						

Discussions

The method was validated according to The Guidance for Industry: Bioanalytical Method Validation (30). The parameters usually examined in the validation process are selectivity/specificity, linearity, limit of quantification, accuracy and precision.

The study of the selectivity/specificity proved that no significant interferences were observed in 3 different extracted samples (figure 8). Thus the analytical method proved to be selective.

The coefficient of correlation (r^2) is greater than or equal to 0.99998, as it can be observed in figure 9.

The analytical method proved to be sensitive, allowing a precise quantification of concentrations as low as 0.6 µg/mL as seen in figure 10.

Accuracy of the analitycal method represents the degree of closeness of the determined values of an analyte to the nominal /or known true value declared from an individual sample. The accuracy of a bioanalytical method is expressed as a percentage of the nominal value (% Nominal).

Precision of the analitycal method represents the degree of dispersal of the values determined of an analyte, from a series of samples processed and analyzed individually from a homogeneous volume of biological matrix. Precision of a bioanalytical method is expressed as the coefficient of variation of the concerned series of determinations, CV (%).

The within-run coefficient of variation ranged between 1.329 and 2.265%. The within-run percentages of nominal concentration ranged between 98.873 and 100.594% (table 4.

The between-run coefficient of variation ranged between 0.33 and 0.74 %. The between-run percentages of nominal concentrations ranged between 97.833 and 101.112% (table 5).

Conclusions

A reversed phase HPLC method has been developed and validated for the determination of lisinopril dissolved in pH 1.2 buffer solution.

This chromatographic assay fulfilled all the requirements of a reliable method, including accuracy and precision, linearity, selectivity/specificity.

The assay has proven to be sensitive, specific and reproducible and it can be used for the therapeutic drug monitoring of lisinopril.

I.1.1.3. HPLC analysis of bisoprolol

Introduction

Bisoprolol fumarate (BF) is a synthetic beta 1 - selective blocking agent. It is used in cardiovascular diseases such as high blood pressure (hypertension), reduced blood flow to the heart (cardiac ischemia); congestive heart failure, preventive treatment before and primary treatment after heart attacks decreasing the chances of recurrence (31). Bisoprolol has a higher degree of β1-selectivity compared to other β1-selective β-blockers such as atenolol, metoprolol and betaxolol (32-34). Beta 1 - selectivity of bisoprolol fumarate has been demonstrated in both animal and human studies.

The chemical name for bisoprolol fumarate is (±)-1-[4-[[2-(1-methylethoxy) ethoxy] methyl]phenoxyl-3-[(1-methylethyl)amino]-2-propanol(E)-2-butenedioate (35). Its empirical formula is $(C_{18}H_{31}NO_4)_2$•$C_4H_4O_4$ and its structure is presented in figure 11.

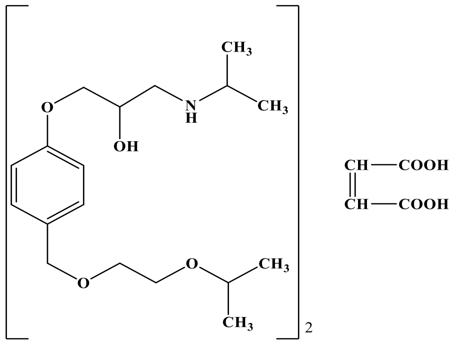


Fig. 11. Chemical structure of bisoprolol fumarate

Various methods for bisoprolol determination are reported in literature. There are UV-Vis (36, 37), HPLC (38-40), HPTLC (41, 42) methods for the bisoprolol determination from tablets and biological fluids.

The aim of this study was to develop a fast, efficient, accurate, precise and robust RP-HPLC method for separating and determining bisoprolol in a tablet sample (43).

Materials and methods

Instruments

High Performace Liquid Chromatograph, Agilent Technologies 1100 with Diode Array Detector. For the sample preparation an analytical balance Kern 770 and a Cole Parmer ultrasonic bath were used.

Chromatographic separation of bisoprolol was achieved using an isocratic elution and a reverse phase column (Zorbax SB-C18 100×3 mm, 3.5 µm). The chromatographic assay was performed at a temperature of 25°C and using a 20 µL sample loop.

Reagents

The solvents and other materials were HPLC grade provided by Merck's Chemical Co and Fluka. The reference substance of bisoprolol was supplied from Unichem Laboratories LTD, India. Pharmaceutical formulations of 5 and 10 mg were obtain from the pharmacy.

Mobile phase

The mobile phase consisting from a mixture of methanol:acetonitrile:45mM potassium dihydrogen phosphate buffer at pH = 3.0 (30:25:45) was pumped into the system with a flow of 0.30 mL/min.

Preparation of solutions

Stock standard solution of bisoprolol was prepared in mobile phase at a concentration of 100 µg/mL and stored at room temperature. This stock standard solution was diluted by using mobile phase to give appropriate working solution (10 µg/mL).

A phosphate buffer (45 mM) was prepared by dissolving 6.12 g of phosphate monopotasic in 900 mL water. The solution was completed at 1000 mL with water and the pH value was adjusted at 3.0 with ortophosphoric acid.

Sample preparation

The developed method for HPLC determination of bisoprolol with UV detection was applied for the investigation of some pharmaceutical tablets from different manufacturers. A number of 20 tablets containing bisoprolol fumarate were crushed in a mortar, and a quantity of powder equivalent at 5mg bisoprolol fumarate was dissolved in

25 mL methanol under agitation using an ultrasonic bath. The obtained solution was filtered in order to separate the excipients and completed into a volumetric flask of 100 mL using mobile phase. Three successive weightings were performed.

Application to bioequivalence study

The monography for tablets provides a quantitative determination of the active substance released after the tablets dissolution. Were used tablets of Concor® (original) and Bisotens® (generic) containing 10 mg of BF for both pharmaceutical products. The dissolution test was carried out on 6 tablets using phosphate buffer as the dissolution medium, as provided in USP 29 (35), at three different pH values (1.2, 4.5 and 6.8) using a dissolution apparatus Agilent Technologies 708. The dissolution medium with different pH values were prepared according to European Pharmacopoeia 6.0 (44). Determination of the amount of active substance dissolved from tablets was performed in the conditions of the method.

Method validation

Validation was carried out following the International Conference on Harmonization (ICH) (45) guidelines for determination of selectivity, linearity, detection limit, quantification limit, system suitability, precision, accuracy, recovery, stability and robustness.

The linearity was investigated in the 0.10-15 µg/mL concentration range and the calibration curve was obtained by plotting the peak area values against the BF concentration using linear regression.

The detection limit (LOD) and the quantification limit (LOQ) of the drug were calculated using the calibration curve statistical parameters with following formulas (1):

$$LOD = \frac{3.3 \times SD}{S} \qquad\qquad LOQ = \frac{10 \times SD}{S} \quad (1),$$

Where: S is the slope of the calibration curve and SD is the standard deviation of the intercept of from regression equation (45).

The precision and accuracy were tested by determining the active compound concentration at three concentration levels in the same day and on three different days. The precision of the method was expressed by standard deviation (SD) and covariance (CV) (%), respectively. The accuracy was expressed by recovery.

The extraction recovery for tablet at three different concentrations of BF was determined. Known amounts of BF were added to tablets solutions. After the chromatographic analysis, the peak areas were compared with those obtained for standard solutions of BF at the same concentration. Than the recovery was estimated.

To evaluate the robustness of the developed RP-HPLC method, some small deliberate variations in the optimized method parameters were done. The effect of change in flow rate and column temperature on the peak area were studied.

A series of standards samples was prepared from freshly made stock solutions in the mobile phase to evaluate the stability of samples at room temperature.

Results

Optimization of chromatographic conditions

The first step was to establish the composition of the mobile phase and the retention time (figure 12).

Then was studied the influence of the flow rate of the mobile phase on the peak normalization (figure 13).

The detection was performed at a wavelength of 225 nm as it was determined from the absorbtion spectra of BF (figure 14).

The effect of pH on the peak area for a sample containing 5 μg/mL BF was examined (figure 15).

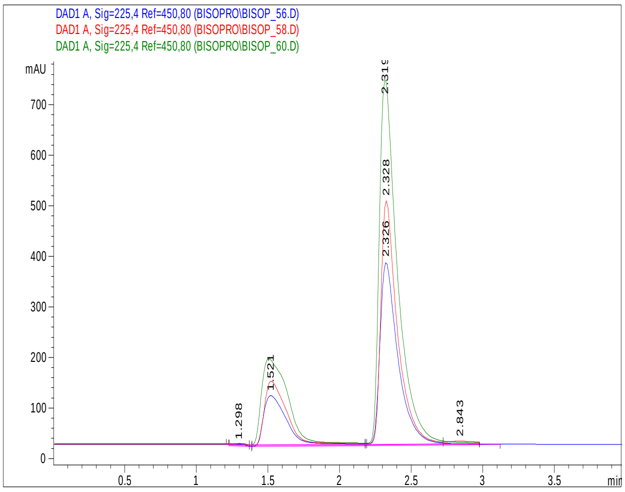


Fig. 12. Retention time

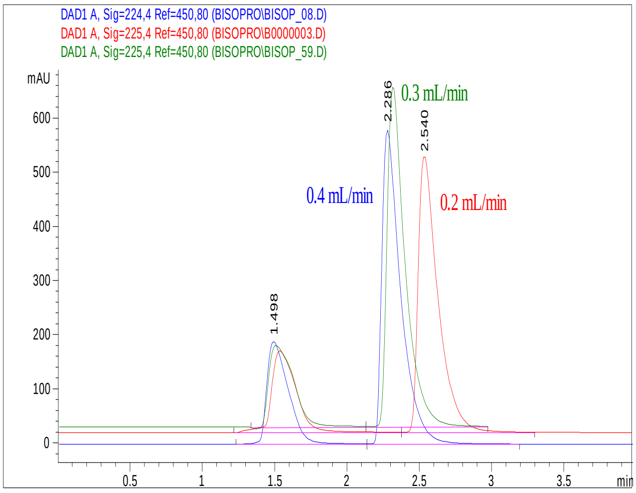


Fig. 13. The flow rate study

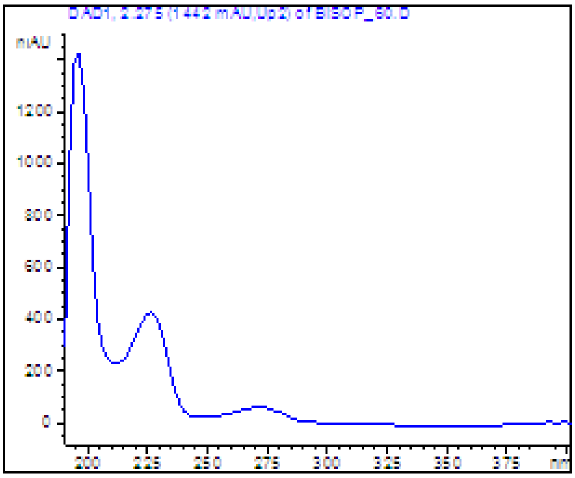


Fig. 14. The absorbtion spectra of BF

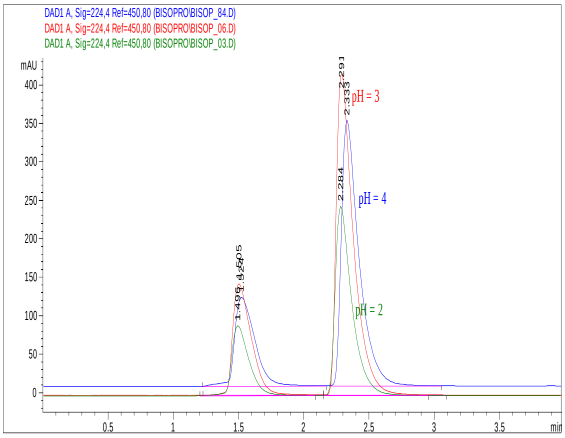


Fig. 15. The effect of pH

Method validation

<u>Selectivity</u>

The selectivity was asesed by comparing the chromatograms for the standard, sample and blank solutions (figure 16).

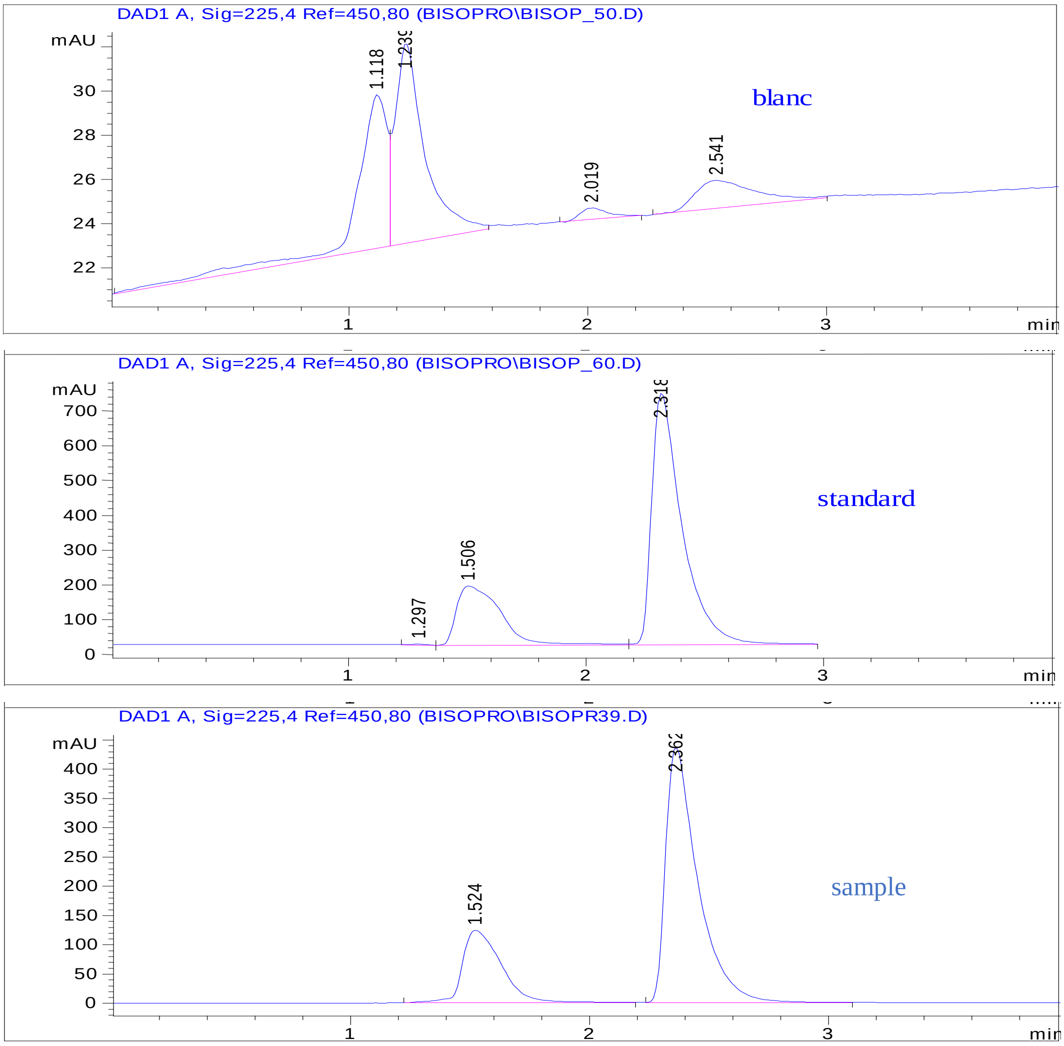


Fig. 16. Chromatograms for blank (0.10%), standard (9µg/mL), and sample (0.60µg/mL)

Linearity

Calibration plots (figure 17) were constructed by plotting the concentration against peak area of BF.

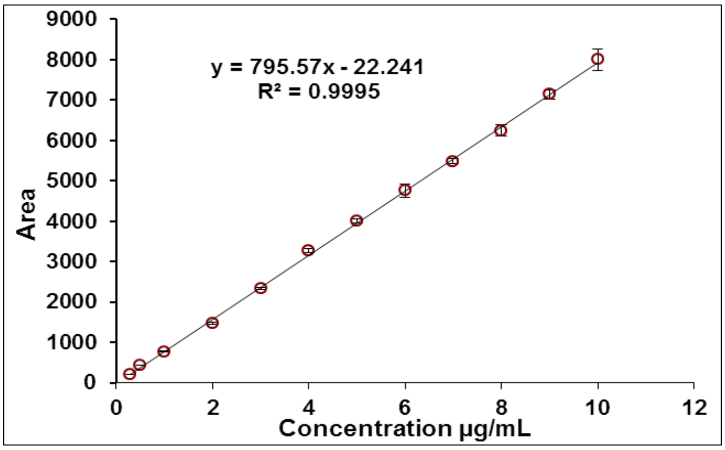


Fig. 17. Calibration curve

System suitability

Using the standard solution of BF the system suitability was analysed after the determination of standard error (SD), theoretical plate number (N), capacity factor (k') and tailing factor (T). The results are all within acceptable limits (table 6).

Table 6. System suitability

Capacity factor (k')	Tailing factor (T)	Theoretical plates (N)	SD
9.02	1.02	1.563	6.63

Precision and Accuracy

Precision and accuracy were tested at three different concentrations (3, 5 and 7 µg/mL). Table 7 summarizes the results obtained for the intra-day parameters.

The interday precision and accuracy was evaluated for six aliquots for three sample concentration in three different days. The results are in table 8.

Table 7. Intra-day precision and accuracy

No. det.	c_{th} = 3µg/mL		c_{th} = 5µg/mL		c_{th} = 7µg/mL	
	c_{exp} (µg/mL)	%nominal	c_{exp} (µg/mL)	%nominal	c_{exp} (µg/mL)	%nominal
1	2.96	98.67	5.05	100.98	6.87	98.13
2	3.01	100.33	4.97	99.40	6.93	98.98
3	2.96	98.67	5.07	101.32	6.94	99.12
4	2.99	99.67	5.05	100.99	6.97	99.57
5	3.04	101.33	4.94	98.74	6.99	99.92
6	3.11	103.67	5.10	102.00	7.02	100.29
Mean	3.01	100.39	5.03	100.57	6.95	99.34
SD	0.06	1.90	0.06	1.24	0.05	0.77
CV%	1.89	1.89	1.23	1.23	0.75	0.77

C_{th} = theoretical concentration. C_{exp} = experimental concentration. SD = standard deviation. CV % = coefficient of variation

Table 8. Inter-day precission and accuracy

No. det.	c_{th} = 3µg/mL		c_{th} = 5µg/mL		c_{th} = 7µg/mL	
	c_{exp} (µg/mL)	%nominal	c_{exp} (µg/mL)	%nominal	c_{exp} (µg/mL)	%nominal
1	2.89	96.33	4.98	99.60	7.23	103.29
2	2.97	99.00	4.87	97.40	6.97	99.55
3	3.03	101.00	5.03	100.52	6.89	98.48
4	2.93	97.67	5.05	100.99	7.12	101.71
5	3.10	103.33	4.91	98.20	6.99	99.92
6	2.91	97.00	5.11	102.20	7.02	100.29
Mean	2.97	99.06	4.99	99.82	7.04	100.54
SD	0.08	2.67	0.09	1.79	0.12	1.71
CV%	2.70	2.69	1.80	1.80	1.71	1.70

C_{th} = theoretical concentration. C_{exp} = experimental concentration. SD = standard deviation. CV % = coefficient of variation

Recovery

Table 9. Recovery results of BF from tablet

Concentration added (µg/mL)	Concentration found mean±SD (µg/mL)	Recovery (%)	RSD (%)
Tablet			
3	2.96±0.05	98.66	1.68
6	5.98±0.09	99.79	1.50
9	9.06±0.13	99.83	1.50

Robustness

Table 10. Robustness study

No. det.	BF concentration (µg/mL)	The flow rate (mL/min)	Temperature (°C)	Average area (n = 3)	Recovery BF (%)
1	4	0.28	20	3198.87	99.75
2		0.30	25	3201.68	99.85
3		0.32	30	3203.62	100.36
4	5	0.28	20	3913.21	101.08
5		0.30	25	3914.23	99.65
6		0.32	30	3920.02	98.38
7	6	0.28	20	4681.66	100.82
8		0.30	25	4690.28	99.85
9		0.32	30	4687.15	99.69

Stability tests

To test the stability of bisoprolol in solutions, a number of 6 replicates with 5 µg/mL concentration of the quality control were used and compared with 6 replicates of stability samples with the same concentration. The solutions were kept at room temperature for 8 hours. The obtained results are summarised in table 11.

Table 11. Stability of analyte in solutions at room temperature

Analyte		bisoprolol
Solvent		mobile phase
Storage conditions		8 hours at room temperature
Comparison samples		Stability samples
5µg/mL		5µg/mL
Response of the analyte		
4021.31		3999.95
3987.45		3978.87
3913.21		3913.21
3967.68		3914.23
3896.41		3920.02
3954.23		3893.41
3977.68		3934.23
4054.52		4013.65
Statistics		
Mean	3971.56	3945.94
SD	52.11	42.17
CV(%)	1.31	1.06

<u>Dissolution profiles</u>

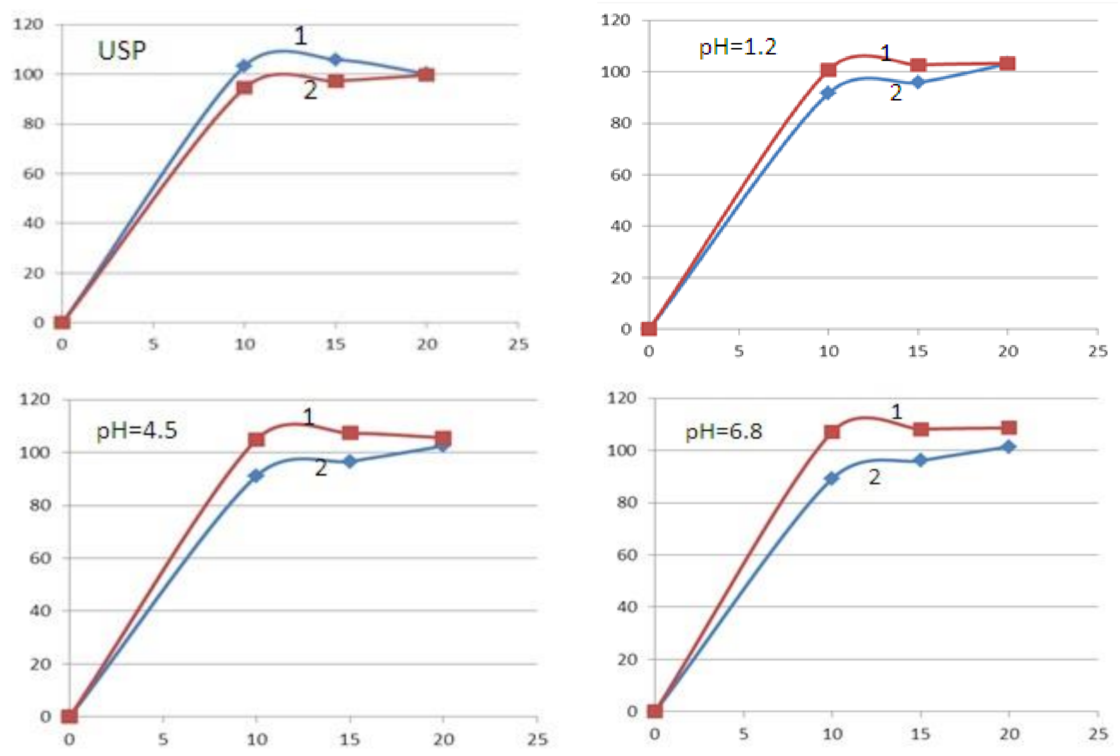


Fig 18. Dissolution profiles from Concor ® (1) and Bisotens® (2)

Discussions

Several parameters were examined for the optimization of HPLC analysis of the BF.

According to the results obtained (figure 12), it was thought that the mobile phase should consist of a mixture of acetonitrile, methanol and phosphate buffer with pH adjustment at 3.0 for which the retention time for BF was 2.87 min.

Different mixtures of acetonitrile - methanol - phosphate buffer were tried as mobile phase, including with a v/v/v ratios of 20:30:50, 25:30:45, 30:25:45 or 30:20:50. The most appropriate peaks appear for a mobile phase with a ratio between the mentionated components of 25:30:45 (v/v/v).

The optimum flow rate was 0.3 mL/min (figure 13) and figure 15 shows the variation of pH.

Figure 16 showed that the method is selective because it has the ability to separate the signal corresponding of bisoprolol. In addition, the excipients used in pharmaceutical formulation have not retention times which interfere with the retention time of bisoprolol.

The graph 17 showed a good linearity in 0.30-10 µg/mL concentration range. Correlation coefficient was 0.9995.

The LOD and LOQ of BF were 0.10 and 0.28 µg/mL, respectively. These values are lower than those obtained by many other reported methods.

The intraday and interday precision of measurements were lower than the permissible values (CV ≤ 15%) and for accuracy the variation of recovery was between 99.33-102.00% (table 7 and table 8).

Recovery and RSD% values (table 9) obtained at the study of BF from tablet showed that the method can be applied with good results in drug control laboratories.

Robustness study conducted by deliberate changes in flow rate (±0.2 mL) and temperature of the column (±5ºC), revealed that there was no significant variation in % assay (table 10).

The results of the stabilitity tests showed that bisoprolol is found to be stable in solution for 8 hours at room temperature (CV% ≤ 15%) (table 11).

Analyzing the dissolution profiles from figure 18 it was observed that the dissolution percentage of both types of tablets in specified conditions of USP 29 and at the three values of pH have values was higher than 85% (as a percentage of the amount claimed). The average amount of BF dissolved after 15 minutes was higher than 95% for all the tablets (in accordance with USP criteria's).

Conclusions

A sensitive HPLC method with UV detection was developed and validated for BF determination from tablets.

In this study, the purpose of the method was to raise the sensitivity and thus the possibility of working at low concentrations.

The sensibility and recovery percentage of BF are high according to the other methods and the retention time is quite short.

The method has demonstrated validity during in vitro bioequivalence study.

I.1.1.4. HPLC analysis of ciprofloxacin

Introduction

Ciprofloxacin,1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3quinolone carboxylic acid, is a synthetic chemotherapeutic drug from the second-generation fluoroquinolone class (figure 19). It kills bacteria by interfering with the enzymes that cause DNA to rewind after being copied, which stops synthesis of both DNA and proteins (46).

Several assay methods are available for ciprofloxacin, including HPLC-UV and LC-MS/MS methods (47-52).

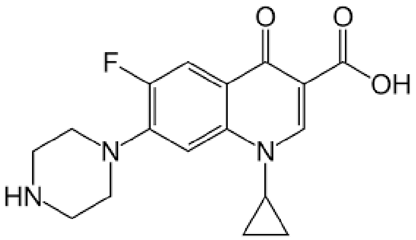


Fig. 19. Chemical structure of ciprofloxacin

Materials and methods

Instruments

All analyses were performed on an Agilent 1200 system. The system components included an Agilent 1200 Degasser, an Agilent 1200 Binary Pump, an Agilent 1200 Autosampler and an Agilent 1200 UV Detector.

The Agilent Chem 32 software was used for system control and data acquisition. An analytical balance Mettler-Toledo XP56, a Sigma 2-16 K centrifuge and a Vibramax 110 shaker were used for the sample preparation.

The separation was performed using a reverse phase column Zorbax XDB-CN, (100 mm x 4.6 mm, 3.5 µm) supplied by Agilent, USA.

Reagents

The standard ciprofloxacin hydrochloride used in this study was supplied by Hiran Orgochem Ltd. (batch number FPCPF070979) with a purity of 99.50%.

All solvents and other chemicals were HPLC grade provided by Merck, Germany.

Mobile phase

The mobile phase consisted in a mixture of phosphoric acid solution (pH 3.0) and acetonitrile 90:10 (v/v), at a flow rate of 0.8 mL/min.

Preparation of solutions

A stock solution of ciprofloxacin hydrochloride with a concentration of 1.4 mg/mL was prepared by dissolving an appropriate amount of ciprofloxacin hydrochloride reference substance in the mobile phase. This solution was kept at 5±3°C. In those conditions, it was found to be stable for at least 7 days.

For the study of linearity response, six ciprofloxacin solutions were prepared in pH 6.8 buffer solution, covering the concentration range between 0.28-0.70 µg/mL.

The theoretical concentrations of ciprofloxacin calibration standard solutions were 0.28, 0.42, 0.49, 0.56, 0.63 and 0.70 µg/mL, respectively.

The quality control samples of ciprofloxacin theoretical concentrations of 0.350 µg/mL (low - QC1), 0.525 µg/mL (medium - QC2) and 0.595 µg/mL (the high - QC3) were considered to be appropriate to be used in order to validate the analytical method.

Results

The analytical method for the assay of ciprofloxacin dissolved in pH 6.8 buffer solution was validated. The parameters usually examined in the validation process were selectivity/specificity, linearity, limit of quantification, accuracy and precision, recovery and stability (53, 54).

Method Validation

Selectivity/Specificity study

A placebo mixture containing all the non-active ingredients of tablets containing 20 mg ciprofloxacin was carried through the extraction procedure and chromatographed to determine the extent to which non active ingredients could interfere with the assay of ciprofloxacin.

Linearity

Calibration curves were found to be consistently accurate over the calibration range between 0.28 and 0.70 µg/mL (figure 20).

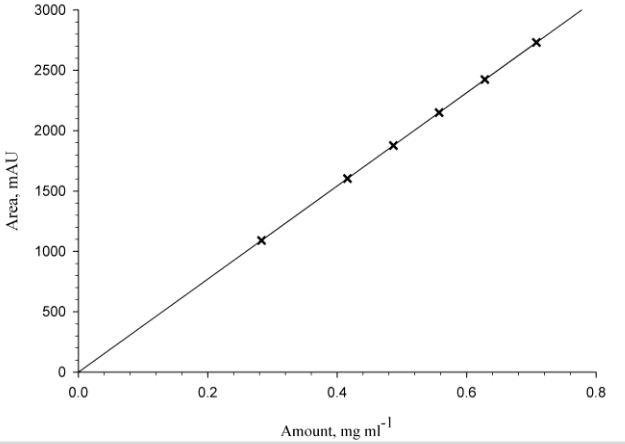


Fig. 20. Calibration curve for ciprofloxacin dissolved in pH 1.2 buffer

The lower limit of quantification, i.e. the lowest standard level with a coefficient of variation less than 2% and a signal to noise ratio higher than or equal to 5 was 0.28 µg/mL (figure 21). Results are presented in table 12.

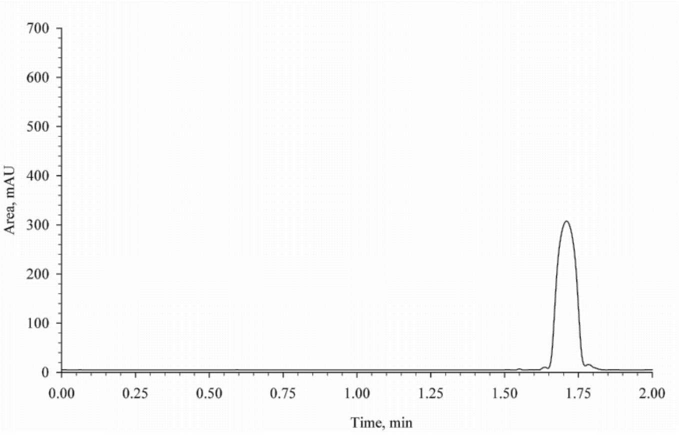


Fig. 21. The chromatogram recorded for a 0.28 µg/mL ciprofloxacin sample

Table 12. Lower limit of quantification

	Ciprofloxacin 0.280 mg/mL		
	Concentration (mg/mL)	Nominal (%)	Signal/Noise Ratio
n = 3	0.28421	101.503	213.200
	0.28771	102.753	232.500
	0.28536	101.915	260.000
Mean	0.28576	102.057	235.233
SD (±)	0.002		
CV (%)	0.624		

Accuracy and Precision

Intra-day accuracy and precision evaluations were performed by analyzing replicate concentrations of ciprofloxacin. The run consisted of a calibration curve and a total of 18 quality control samples, 6 replicates of each of low (QC1), medium (QC2) and high (QC3) quality control samples. Results are presented in table 13.

Table 13. Intra-day precision and accuracy

	QC1 0.350 mg/mL		QC2 0.525 mg/mL		QC3 0.595 mg/mL	
n = 3	Found (μg/mL)	Nominal (%)	Found (μg/mL)	Nominal (%)	Found (μg/mL)	Nominal (%)
Mean	0.347	99.036	0.526	100.142	0.616	103.521
SD (±)	0.003		0.002		0.012	
CV (%)	0.756		0.401		1.868	
Acceptance criteria: 67% Total QCs must be 100 ± 15% nominal values; 50% QCs per level must be 100 ± 15% nominal values;						

The inter-day accuracy and precision were assessed by the repeated analysis of quality control samples containing different concentrations of ciprofloxacin on separate occasions.

A single run consisted of a calibration curve and 6 replicates of the low (QC1), medium (QC2) and high (QC3) quality samples for ciprofloxacin. Results are presented in table 14.

Table 14. Inter-day precision and accuracy

	QC1 0.350mg/mL		QC2 0.525mg/mL		QC3 0.595mg/mL	
n = 15	Found (μg/mL)	Nominal (%)	Found (μg/mL)	Nominal (%)	Found (μg/mL)	Nominal (%)
Mean	0.347	99.165	0.517	98.537	0.598	100.497
SD (±)	0.006		0.009		0.014	
CV (%)	1.697		1.796		2.269	
Acceptance criteria: 67% Total QCs must be 100 ± 15% nominal values; 50% QCs per level must be 100 ± 15% nominal values; Mean % nominal 100 ± 15%; CV(%) ≤ 15%.						

The recovery of ciprofloxacin was evaluated by comparing analyte response of three extracted samples of low, medium and high quality control samples to those of three appropriately diluted standard solutions. Results are presented in table 15.

Table 15. Recovery of ciprofloxacin

Samples	R_QC1		R_QC2		R_QC3	
	unextracted	extracted	unextracted	extracted	unextracted	extracted
Mean	1281.51	1300.37	1924.56	1957.53	2200.15	2192.17
SD (±)	2.723	2.497	5.291	41.295	13.470	26.540
CV (%)	0.212	0.192	0.275	2.110	0.612	1.211
C (mg/mL)	0.0350	0.0350	0.0525	0.0525	0.0595	0.0595
Mean Recovery (%)	101.471		101.713		99.637	
Acceptance criteria: 95 ≤ Mean recovery (%) ≤ 105; CV(%) ≤ 5.						

<u>Stability of analyte after sample processing at room temperature</u>: samples prepared at low (QC1) and high (QC3) quality control levels were submitted to the extraction procedure and kept at room temperature. A calibration curve and 3 replicates of low and high quality control samples (comparison samples) were freshly processed and analyzed with 3 replicates of the stability samples in a single run. Concentrations were calculated to determine % change over time. Ciprofloxacin was found to be stable for 73 hours at room temperature after sample processing with changes of -0.371 and -1.685%. Results are presented in table 16.

Table 16. Stability at room temperature

n = 3	QC1 0.350 µg/mL			QC3 0.595 µg/mL		
	comparison samples		stability samples	comparison samples		stability samples
	Measured	Nominal (%)	Measured	Measured	Nominal (%)	Measured
Mean	0.352	100.560	0.351	0.592	99.468	0.582
SD (±)	0.003		0.003	0.013		0.006
CV (%)	0.730		0.812	2.129		1.031
Change (%)		-0.371			-1.685	
Acceptance criteria: CV(%) ≤ 5%; % Change ± 5%.						

<u>Short Term Stability of Analyte in Dissolution Medium at Room Temperature</u>: samples were prepared at low (QC1) and high (QC3) quality control levels. Three replicates of low and high quality control samples were left at room temperature for approximately 1 hour (stability samples). A calibration curve and 3 replicates of low and high quality control samples (comparison samples) were freshly processed with the stability samples and analyzed in a single run. Concentrations were calculated to determine % change over time. Ciprofloxacin is found to be stable in dissolution medium for 1 hour at room temperature with % changes of 0.346 and 1.241%. Results are presented in table 17.

Table 17. Short term stability in dissolution medium

n = 3	QC1 0.350 mg/mL			QC3 0.595 mg/mL		
	comparison samples		stability samples	comparison samples		stability samples
	Measured	Nominal (%)	Measured	Measured	Nominal (%)	Measured
Mean	0.353	100.801	0.354	0.594	99.913	0.602
SD (±)	0.001		0.001	0.009		0.002
CV (%)	0.393		0.316	1.541		0.329
Change (%)		0.346			1.241	
Acceptance criteria: CV(%) ≤ 5%; % Change ± 5%.						

Short term stability of ciprofloxacin in solution at 5 ± 3°C:

The short term stability of ciprofloxacin in the mobile phase consisting a mixture of phosphoric acid solution (pH 3) and acetonitrile (90:10, v/v) was evaluated.

A stock solution of ciprofloxacin was prepared in mobile phase, aliquoted and stored at 5±3°C (stability samples). Replicates of stability sample and comparison sample (freshly prepared stock solution) were diluted at approximately the same analyte concentration and analyzed in a single run. Analyte responses were used to determine % change over time.

Ciprofloxacin was found to be stable in mobile phase for 4 hours at 5 ± 3°C with % changes of 1.063. Results are presented in table 18.

Table 18. Short term stability at 5 ± 3°C

1.4000 mg/mL	Comparison samples	Stability samples
Analyte response	2609.11621	2637.44922
	2618.85327	2647.31128
	2619.25488	2645.86621
3	3	3
Mean	2615.741	2643.542
SD (±)	5.741	5.326
Corrected mean	-	2643.542
CV (%)	0.219	0.201
Change (%)	1.063	
Acceptance criteria: CV(%) ≤ 5%; % Change ± 5%.		

Long term stability of ciprofloxacin in solution at 5±3°C

The short term stability of ciprofloxacin in the mobile phase consisting a mixture of phosphoric acid solution (pH 3.0) and acetonitrile (90:10, v/v) was evaluated.

A stock solution of ciprofloxacin was prepared in mobile phase, aliquot and stored at 5±3°C (stability samples). Replicates of stability sample and comparison sample (freshly prepared stock solution) were diluted at approximately the same analyte concentration and analyzed in a single run. Analyte responses were used to determine % change over time.

Ciprofloxacin was found to be stable in mobile phase for 7 days at 5±3°C with % changes of 2.379%. Results are presented in table 19.

Table 19. Long term stability at 5±3ºC

Concentration, 1.4000 mg/mL	Comparison samples	Stability samples
Analyte response	2584.48706	2646.09888
	2595.25903	2656.83862
	2589.53320	2651.19336
Mean	2589.76010	2651.3771
SD (±)	5.390	5.372
CV (%)	0.208	0.203
Change (%)	2.379	
Acceptance criteria: CV(%) ≤ 5%; % Change ± 5%.		

Discussions

The analytical method for the assay of ciprofloxacin dissolved in pH 6.8 buffer solution was validated. The parameters usually examined in the validation process were selectivity/specificity, linearity, limit of quantification, accuracy and precision, recovery and stability (53, 54).

Selectivity - no significant interferences were observed in 3 different extracted samples.

The coefficients of correlation were greater than or equal to 0.99993.

The lower limit of quantification was 0.28 µg/mL and the analytical method proved to be sensitive, allowing a precise quantification of concentrations as low as 0.28 µg/mL (figure 21 and table 12).

The intra-day coefficient of variation ranged between 0.401 and 1.868%. The intra-day percentages of nominal concentration ranged between 99.036 and 100.142% (table 13).

The inter-day coefficient of variation ranged between 1.697 and 2.269%. The inter-day percentages of nominal concentrations ranged between 98.537 and 100.497% (table 14).

Mean recovery values for ciprofloxacin were 101.471, 101.713 and 99.637% at low, medium and high quality control levels, respectively (table 15).

Conclusions

A reversed phase HPLC method using UV detection has been developed and validated for the determination of ciprofloxacin dissolved at pH 6.8.

This chromatographic assay fulfilled all the requirements for being a reliable method, including accuracy and precision, linearity, selectivity/specificity and stability.

The assay has proven to be sensitive, specific and reproducible and it can be used for the assay of ciprofloxacin in various pharmaceuticals products.

I.1.2. Development of some HPLC analysis methods with MS detection

Liquid chromatography coupled with mass spectrometry detection is one of the most powerful analytical tools for organic compound analysis. The advantages of using LC/MS methods over HPLC methods include: selectivity, chromatographic integrity, peak assignment, structural information, rapid method development.

In the pharmaceutical industry, accurately identifying the components of a mixture can be the difference between life and death. Mass spectrometry delivers that capability and more. Mass spectrometry plays many key roles in the pharmaceutical industry, from the very early stages of drug discovery through to late-stage development and clinical trials (55). When new therapeutic agents are first "synthesized", be they small molecules or large proteins, their structural composition must be determined/confirmed. Mass spectrometry plays a key role in these determinations. MS can reveal a compound's efficacy and toxicology. It can be used to study a compound's pharmacokinetics - its distribution in the body over time (56).

The unique degree of sensitivity and selectivity in complex matrices that is afforded by mass spectrometry makes it the technique of choice for these extensive experiments that take place from drug discovery, where animal studies are conducted, through clinical trials, where these parameters are determined in humans. The technique can also disclose if a drug causes health problems or is broken down into compounds that do (57). After a drug is tested and approved, companies use MS to make sure that a manufacturing process makes the right compound and that dangerous chemicals from packaging do not end up in the drug.

Published papers in the field

♦ Panainte Alina Diana, Morariu Ionela Daniela, **Bibire Nela**, Vieriu Mădălina, Ţântaru Gladiola, Luca Alina Costina, Apostu Mihai. Studies on Chromatographic Fractioning by Cations Exchangers of a Bovine Hemoglobin Hydrolysate. *Rev Chim (Bucharest)* 2018; 69(10): 2794-2798.

♦ **Nela Bibire**, Mădălina Vieriu, Gladiola Țântaru, Mihai Apostu, Luminița Agoroaei, Alina Diana Panainte, Alexandru Znagovan, Aurel Vlase. A New and Sensitive LC-MS/MS Method for the Determination of Clopidogrel in Human Plasma. *Rev Chim (Bucharest)* 2014; 65(7): 807-810.

♦ **Nela Bibire**, Gladiola Ţântaru, Mihai Apostu, Luminița Agoroaei, Mădălina Vieriu, Alina Diana Panainte, Aurel Vlase. A New Bioanalytical Method for the Determination of Alprazolam in Human Plasma. *Rev Chim (Bucharest)* 2013; 64(6): 587-592.

♦ Gabriela Peşte, **Nela Bibire**, Mihai Apostu, Aurel Vlase, Corneliu Oniscu**.** A New Liquid Chromatography - Tandem Mass Spectrometry Method for Determination of Bisoprolol in Human Plasma Samples. *Journal of Biomedicine and Biotechnology*, Volume 2009; Article ID 736327: 8 pag.

I.1.2.1. HPLC Analysis of clopidogrel

Introduction

Clopidogrel hydrogen sulfate: [methyl(+)-(S)-α-(2-chloro-phenyl)-6,7-dihydrothienol[3,2-*c*]pyridin-5(4H)-acetate hydrogen sulfate] (figure 22), is a potent antiplatelet drug, prescribed for the prevention of vascular thrombotic events. Following oral administration, clopidogrel is rapidly absorbed and undergoes extensive hepatic metabolism.

Several assay methods are available for determination of clopidogrel, including liquid chromatography using UV detection (58-63). The liquid chromatographic assays are not sensitive enough to measure clopidogrel levels in biological fluids after oral administration to humans in therapeutic doses. Recently, LC-MS/MS technology is beginning to meet the challenges of quickly detecting, identifying and quantifying drugs (64).

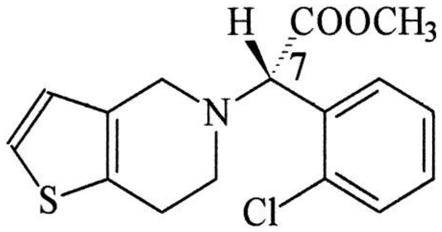


Fig. 22. Chemical structure of clopidogrel

Materials and methods

Instruments

All analyses were performed using the Agilent 1200 Triple Quad 6410 System. The system components included the Agilent 1200 Degasser, Agilent 1200 Binary Pump, Agilent 1200 Autosampler, Agilent 1200 Mass Selective Detector. The Agilent MassHunter software was used for system control and data acquisition. The separation was performed using a reverse phase column Zorbax SB C8, supplied by Agilent, USA.

Reagents

The standard clopidogrel hydrogen sulfate used in this study was supplied by U.S. Pharmacopoeia, with a purity of 98.8% as clopidogrel. The standard clopidogrel-d3 hydrogen sulfate was supplied by SynFine Research. All solvents and other chemicals were HPLC grade provided by Merck, Germany. The human plasma (anticoagulant K_3-EDTA) was obtained from SCIPAC, U.K.

Sample preparation

A stock solution of clopidogrel with a concentration of 0.2 mg/mL was prepared by dissolving the appropriate quantity of clopidogrel bisulphate reference substance in 10 mL acetonitrile. A stock solution of clopidogrel-d3 with a concentration of 0.2 mg/mL was prepared by dissolving an appropriate quantity of clopidogrel-d3 hydrogen sulphate reference substance in 10 mL acetonitrile. These solutions were kept at -25°C. In these conditions, they were stable for 95 days.

Nine solutions of different concentration were prepared for the study of the linearity response in human plasma, covering the range of concentrations 10-10000 pg/mL. The theoretical concentrations of clopidogrel calibration standards were 10; 20; 500; 1000; 2000; 4000; 6000; 8000; 10000 pg/mL.

Mobile phase

The mobile phase consists of a mixture of 0.1% formic acid aqueous solution and acetonitrile (50-50, v/v), at flow rate of 1 mL/min.

Mass Spectrometry

The mass transition of the ion-pair was followed as m/z 322.1→ 212.1, for clopidogrel and m/z 327 → 217, for clopidogrel-d3, in multiple-reaction monitoring mode.

Method validation

The quality control samples having clopidogrel theoretical concentrations of 30, 2500 and 7500 pg/mL were considered to be appropriate to be used to validate the analytical method.

As plasma sample, the internal standard solution was added to each plasma sample in borosilicate glass tube. Firstly, the plasma samples were buffered (pH 2) and then extracted by using a mixture of hexan and ethyl acetate on a vortex mixer for 5 minutes.

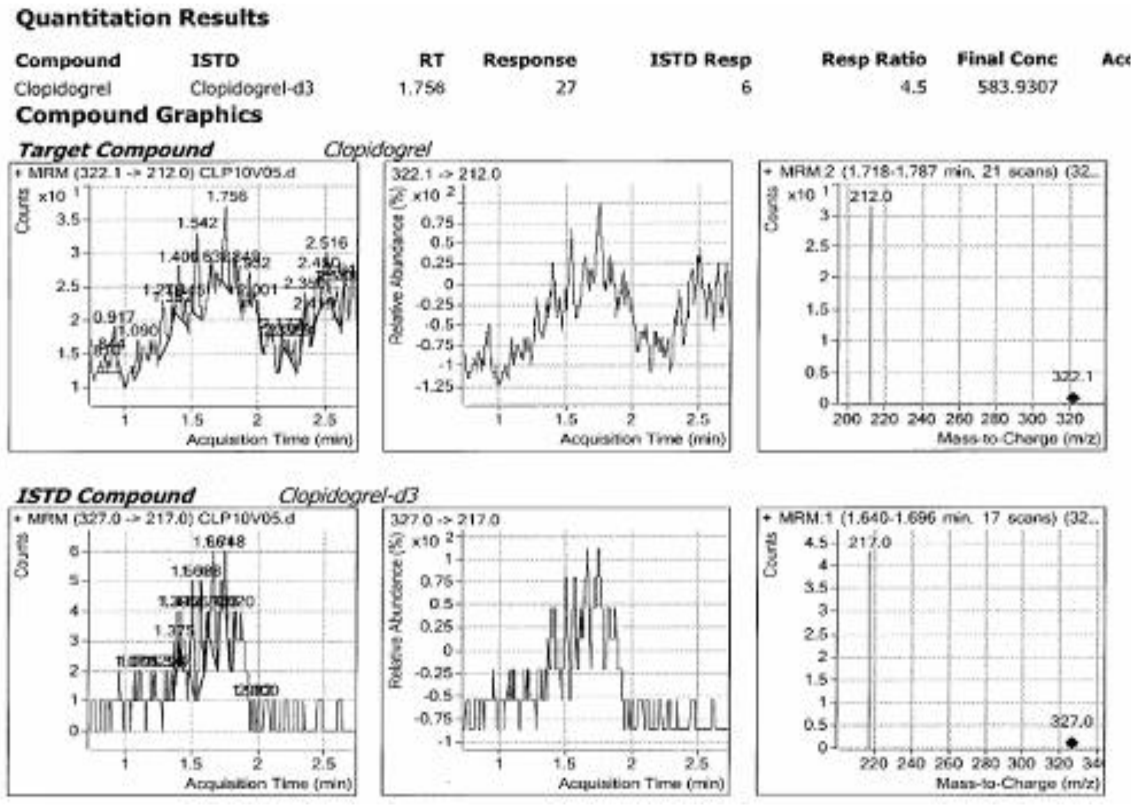
The upper organic layer was transferred to a clean test tube and evaporated at 40˚C under nitrogen. The residue was dissolved in a mixture water-acetonitrile 50:50 (v/v). 10 µL of this solution was injected into the chromatographic system.

Results

Method validation

Selectivity/Specificity

The method described in this paper has been tested for possible interferences from other plasma factors (figure 23).



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	Resp Ratio	Final Conc	Acc
Clopidogrel	Clopidogrel-d3	1.758	27	6	4.5	583.9307	

Compound Graphics

Target Compound Clopidogrel

ISTD Compound Clopidogrel-d3

Fig. 23. Chromatogram recorded for blank sample

Linearity

The linearity was investigated for theoretic concentration between 10-10000 pg/mL clopidogrel and the calibration curve was derived by plotting the peak-height ratio of the analyte and the internal standard against the concentration of clopidogrel, using linear regression analysis (figure 24).

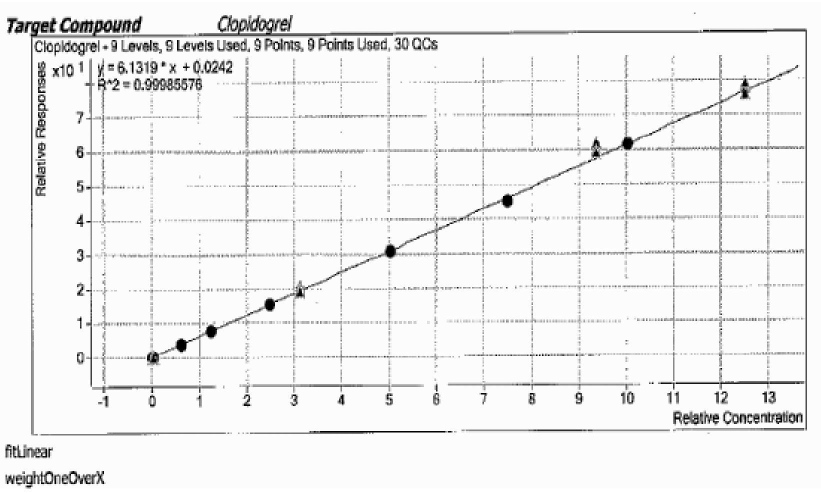
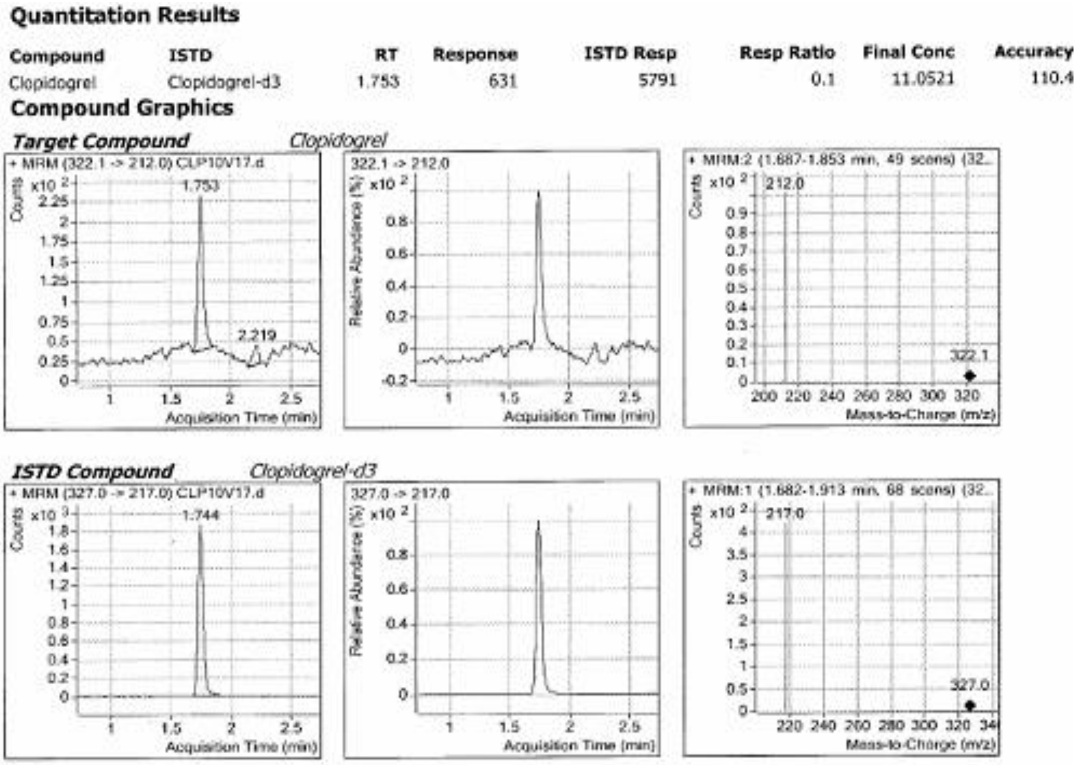


Fig. 24. The clopidogrel calibration curve obtained for plasma samples

Limit of quantification

The lower limit of quantification, i.e. the lowest standard level with a coefficient of variation less than 20%, is 10 pg/mL for clopidogrel (figure 25). Results are presented in table 20.


Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	Resp Ratio	Final Conc	Accuracy
Clopidogrel	Clopidogrel-d3	1.753	631	5791	0.1	11.0521	110.4

Fig. 25. The chromatogram recorded for sample containing 10 pg/mL clopidogrel

Table 20. Lower limit of quantification

Analyte concentration: 10 pg/mL		
	Calculated concentration (pg/mL)	Accuracy %
	10.674	106.74
	11.052	110.52
	10.249	102.49
	9.508	95.08
	7.853	78.53
	7.527	75.27
n	6	6
Mean	*9.477*	*94.772*
SD (±)	*1.480*	
CV(%)	*15.615*	
Acceptance criteria: 4 out of 6 LLQC must be within 100±20% nominal value; Mean % nominal 100±20%. CV (%)≤20%		

Accuracy and Precision

The accuracy of the analytical method represents the degree of closeness of the determined values of the analyte to its nominal or known true value from an individual sample. The accuracy of the analytical method is expressed as a percentage of the nominal value (% Nominal). Precision of the analytical method represents the degree of dispersal of the determined values of an analyte, from a series of samples analyzed individually. Precision of an analytical method is expressed as the coefficient of variation of the concerned series of determinations, CV (%). The accuracy and precision of the method were calculated for three concentration levels of clopidogrel in human plasma. Six clopidogrel samples with theoretical concentration levels of 30 pg/mL (QC1), 2500 pg/mL (QC2) and 7500 pg/mL (QC3) were repeatedly injected into the system. Table 21 summarizes the results obtained for the intra-day parameters. The inter-day precision and accuracy was evaluated also using six aliquots for each quality control sample concentration, prepared and analyzed on six different days. The results are presented in table 22.

Table 21. Intra-day precision and accuracy for clopidogrel quality control sample

	Theoretical concentration - QC1 (30 pg/mL)		Theoretical concentration - QC2 (2500 pg/mL)		Theoretical concentration - QC3 (7500 pg/mL)	
No.	Calculated conc. (pg/mL)	Accuracy %	Calculated conc. (pg/mL)	Accuracy (%)	Calculated conc. (pg/mL)	Accuracy (%)
1.	29.428	98.027	2540.724	101.578	7759.080	103.403
2.	28.132	93.712	2564.092	102.512	7828.556	104.329
3.	30.819	102.662	2488.762	99.501	7710.387	102.754
4.	27.122	90.345	2644.044	105.709	7848.035	104.588
5.	28.557	95.128	2560.978	102.388	8040.836	107.158
6.	25.855	86.127	2662.261	106.437	8087.979	107.786
n	6	6	6	6	6	6
Mean	*28.319*	*94.396*	*2576.810*	*103.072*	*7879.146*	*105.055*
SD (±)	*1.736*		*65.252*		*152.432*	
CV(%	*6.132*		*2.532*		*1.935*	
Acceptance criteria: 67% Total QCs must be 100±15% nominal values.50% QCs per level must be 100±15% nominal values. Mean % nominal 100±15%; CV(%) ≤15%						

Table 22. Inter-day precision and accuracy for clopidogrel spiked quality control sample

No.	Theoretical concentration - QC1 (30 pg/mL)		Theoretical concentration - QC2 (2500 pg/mL)		Theoretical concentration - QC3 (7500 pg/mL)	
	Calculated conc. (pg/mL)	Accuracy %	Calculated conc.(pg/mL)	Accuracy %	Calculated conc. (pg/mL)	Accuracy %
1.	32.853	109.510	2431.698	97.268	7727.130	103.028
2.	31.685	105.617	2455.572	98.223	7412.419	98.832
3.	29.323	97.743	2450.892	98.036	7557.611	100.768
4.	29.097	96.990	2386.714	95.469	7876.531	105.020
5.	28.763	95.877	2438.067	97.523	7321.038	97.614
6.	29.858	99.527	2496.953	99.878	7424.827	98.998
n	6	6	6	6	6	6
Mean	30.263	100.877	2443.316	97.733	7553.259	100.710
SD (±)	1.636		35.919		212.138	
CV(%)	5.407		1.470		2.809	
Acceptance criteria: 67% Total QCs must be 100±15% nominal values. 50% QCs per level must be 100±15% nominal values. Mean % nominal 100±15%. CV(%) ≤ 15%.						

Discussions

The parameters usually studied in the validation process of the LC-MS/MS method are: selectivity/specificity, linearity, limit of quantification, accuracy and precision, recovery (30, 65).

As it can be seen in figure 23, no overlapping peaks were detected at clopidogrel and internal standard retention time, 1.7 min. The bioanalytical method proved to be selective.

The least-square linear regression revealed that the relationship was linear in the investigated domain, with a regression coefficient of 0.99985, meeting the acceptance criteria ($r^2 \geq 0.990$), as it can be seen in figure 24.

The bioanalytical method proved to be sensitive, allowing a precise quantification of clopidogrel as low as 10 pg/mL (figure 25).

Conclusions

A liquid chromatography - tandem mass spectrometry method has been developed and validated for the determination of clopidogrel in human plasma samples.

This chromatographic assay fulfilled all the requirements of accuracy and precision, linearity, selectivity/specificity.

The assay has proven to be sensitive, specific and reproducible and it can be used for therapeutic drug monitoring of clopidogrel.

I.1.2.2. HPLC analysis of alprazolam

Introduction

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazole [4,3,-α]-[1,4] benzodiazepine) (figure 26) is a new generation 1.4-benzodiazepine belonging to the nitrogen heterocycle compounds class. It is a benzodiazepine mainly used to treat anxiety disorders. On a short time basis it is used to palliate symptoms of anxiety or anxiety associated to symptoms of depression. Besides this, alprazolam is also used to treat panic disturbances with or without agoraphobia (66, 67).

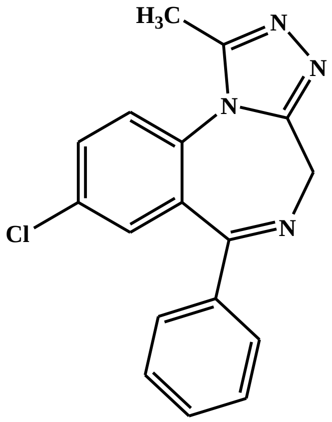


Fig. 26. Chemical structure of alprazolam

Several methods for the quantitative determination of alprazolam in human plasma have been reported. Mainly, liquid chromatography methods with UV detection were described (68-70).

LC-MS methods have been widely accepted as the most used method for the quantitative determination of drugs due to their sensitivity and specificity (71-73).

Materials and methods

Reagents

The standard Alprazolam (batch number 2) used in this study was supplied by Official European Pharmacopeia, with a purity of 100.00 %.

The internal standard Lorazepam (batch number 1b) was supplied by Official European Pharmacopeia with a purity of 100.00 %.

All solvents and other chemicals were HPLC grade provided by Merck, Germany. The human plasma was obtained from Center for Blood Drawing and Preservation Iaşi, Romania.

Preparation of stock solutions, calibration standards and quality control samples

The reference substance, alprazolam, accurately weighed, was dissolved in methanol, obtaining 1 mg/mL stock solution. Alprazolam intermediate solutions (50 μg/mL, 5 μg/mL and 0.1 μg/mL) were prepared by diluting the alprazolam stock solution with methanol. The alprazolam intermediate solutions were diluted with methanol to give the alprazolam working solutions for calibration standards and quality control samples, at the following concentrations: 10, 20, 250, 500, 1000, 2000, 3000, 4000, 5000 ng/mL (the working solutions for calibration standards) and 30,1250,3500,10, 5000 ng/mL (the working solutions for quality control samples).

Lorazepam stock solution (0.2 mg/mL) was prepared by accurately weighing the lorazepam reference substance and dissolution in methanol to give a final concentration of 0.2 mg/mL. A lorazepam intermediate solution (1000 ng/mL) was prepared by diluting the lorazepam stock solution with methanol. A lorazepam working solution (100 ng/mL) was prepared by diluting 1 mL lorazepam intermediate solution (1000 ng/mL) up to 10 mL with distilled water. The stock solutions, the intermediate solutions and the working solutions for calibration standards and quality control samples were stored in the refrigerator at -25±10°C. The internal standard working solution was stored at 5±3°C when not in use.

The calibration and QC samples were prepared by spiking the blank plasma with an appropriate amount of alprazolam, as shown in table 23 and table 24. Replicate 200 μL solutions of each calibration standards and quality control samples were stored at 25±10°C.

Table 23. Preparation of calibration standards solutions

Working solution for calibration standard			Plasma blank	Calibration standard
Theoretical concentration, ng/mL	Volume, mL	Volume, mL	Remove, mL	Theoretical concentration, ng/mL
10	0.1	10	0.1	0.1
20	0.1	10	0.1	0.2
250	0.1	10	0.1	2.5
500	0.1	10	0.1	5
1000	0.1	10	0.1	10
2000	0.1	10	0.1	20
3000	0.1	10	0.1	30
4000	0.1	10	0.1	40
5000	0.1	10	0.1	50

Table 24. Preparation of quality control solutions

Working solution for quality control			Plasma blank	Quality control
Theoretical concentration, ng/mL	Volume, mL	Volume, mL	Remove, mL	Theoretical concentration, ng/mL
10	0.1	10	0.1	0.1
30	0.1	10	0.1	0.3
1250	0.1	10	0.1	12.5
3500	0.1	10	0.1	35
5000	0.1	10	0.1	50

Human plasma extraction

Samples were extracted in the following manner: lorazepam internal standard working solution (50 μL) was added to 200 μL human plasma mixed with EDTA-K_3 anticoagulant and the solution was vortex stirred at 1200 rpm for approx. 30 seconds. 2 mL isobutyl-methyl ether was added and the tubes were horizontally stirred (for approx. 5 min.), then samples were centrifuged (approx. 3900 rpm for 5 min.), the organic layer was transferred to a clean glass tube and evaporated to dryness at normal pressure and 40°C. 100 μL of the mobile phase were added to the dry residue and mixed at 1.200 rpm for around 2 minutes. Extracts were transferred to vials prior to injection into the LC-MS/MS system.

LC-MS/MS method

All analyses were performed using the Agilent 1200 Triple Quad 6410A System. The system components included the Agilent 1200 Degasser, Agilent 1200 Binary Pump, Agilent 1200 Autosampler, Agilent 1200 Mass Selective Detector. The Agilent MassHunter software was used for system control and data acquisition.

The LC-MS/MS method for determination of alprazolam in human plasma samples described in this paper was performed using a reverse phase column Zorbax SB-CN (4.6 x 100 mm, 3.5 μm). The mobile phase consisted of a mixture of 0.5% formic acid solution (pH = 3) and acetonitrile (30:70, v/v).

The LC system was operated at 0.8 mL/min, using the binary pump. The column temperature was 40°C. The injection volume was 10 μL and represented no more 5% of the total sample available for injection. The tandem mass spectrometer settings are detailed in table 25.

Table 25. Mass Spectrometer settings

Mode	MRM
Ionization mode	Positive
Source	API-ESI
Drying temperature	350°C
Nebulizer pressure	60 psi
Flow rate of drying gas	11 L/min

Alprazolam and lorazepam were monitored at m/z transitions of 309→281 and 321→275 for lorazepam, respectively, with dwell times of 100 ms and collision energy of 14, for each.

LC-MS/MS peaks were observed at approximately 2 min for both the alprazolam and lorazepam, respectively. Integration was performed using the Agilent MassHunter software associated with the mass spectrometer.

The calibration curve was constructed over the range 0.1-50 ng/mL by plotting the nominal concentration of each calibrant against the peak area ratio with reference to the internal standard.

The regression type used was linear with a weighting factor of 1/X applied. The concentration of alprazolam in unknown, validation and QC samples was back-calculated from the calibration curve.

Results

Method validation

The parameters usually examined in the validation process are selectivity, specificity, linearity, limit of quantification, accuracy and precision, recovery, stability (65).

Selectivity/Specificity

The method described in this paper has been tested for possible interferences from other plasma factors. Plasma aliquots from six different sources were assessed for analysis in order to investigate the plasma components behavior (figure 27).

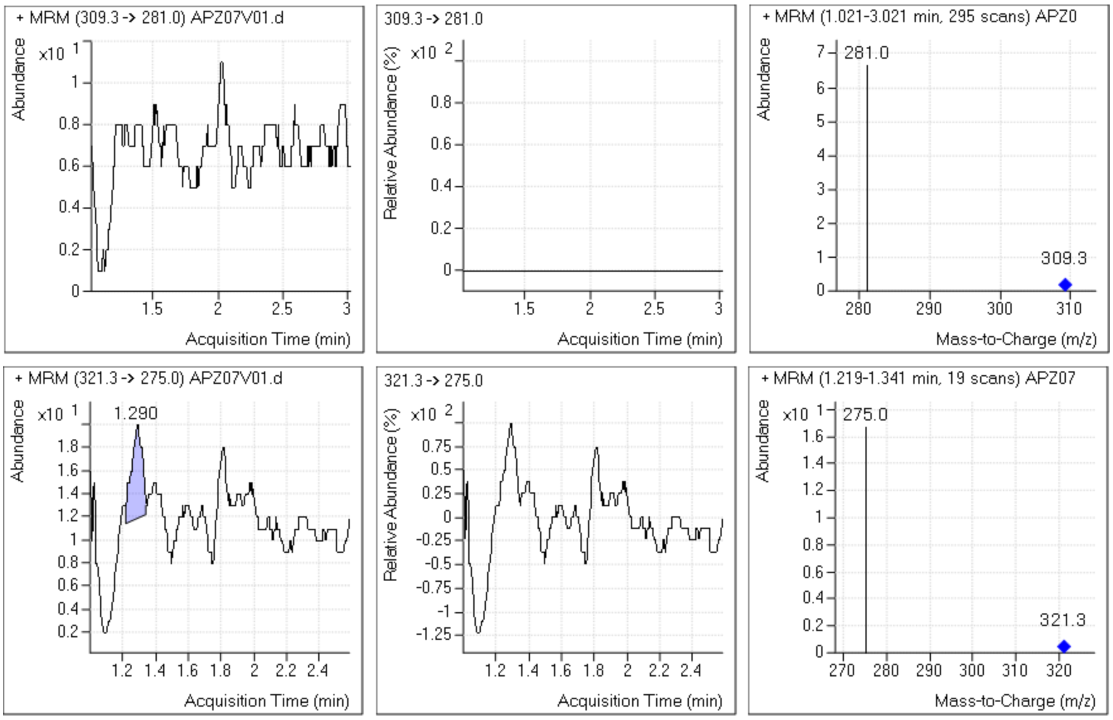


Fig. 27. Chromatogram of blank sample

Linearity

The linearity was investigated for alprazolam theoretical concentration levels in the range between 0.1 ng/mL and 50 ng/mL and the calibration curve was derived by plotting the peak-height ratios of the analyte and the internal standard, using linear regression analysis (figure 28).

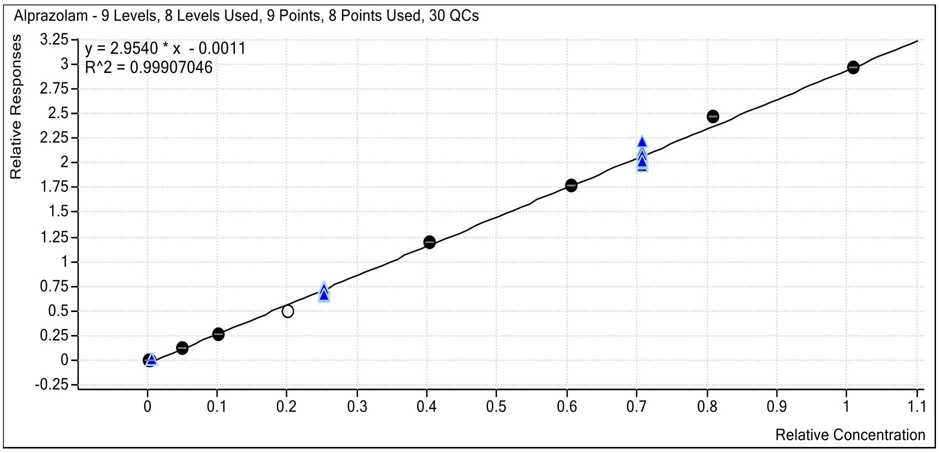


Fig. 28. The alprazolam calibration curve obtained for plasma samples

Quantification limit

The lower limit of quantitation, i.e. the lowest standard level with a coefficient of variation less than 20% and a signal to noise ratio higher than or equal to 5, was 0.1 ng/mL alprazolam (figure 29).

The results are shown in table 26.

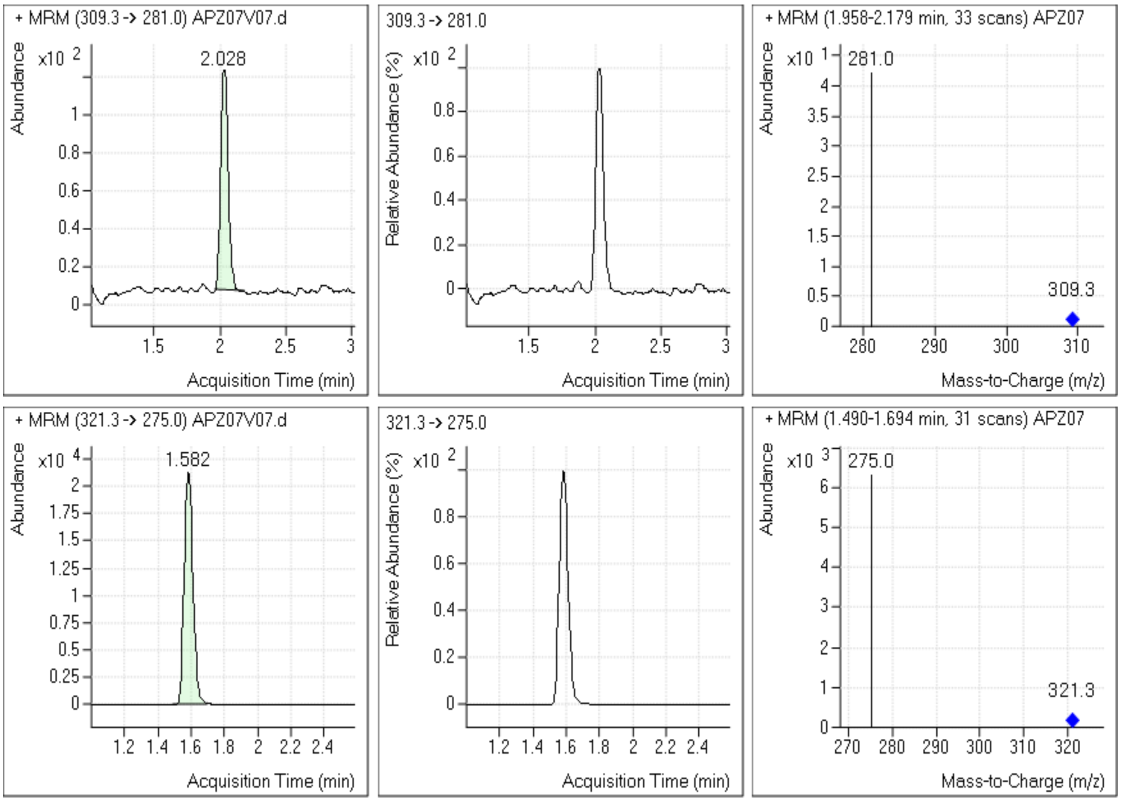


Fig. 29. The chromatogram recorded for 0.1 ng/mL alprazolam sample

Table 26. Lower limit of quantification

	Calculated concentration (ng/mL)	Accuracy %	Signal/Noise ratio
Alprazolam 0.1 ng/mL	0.112	110.748	11.204
	0.147	145.452	6.567
	0.105	104.165	8.839
	0.114	112.392	8.206
	0.109	107.861	7.590
	0.116	115.225	11.204
Average	0.117	115.974	8.668
% Deviation	0.015		
% RSD	12.876		

Accuracy and Precision

The accuracy and precision of this method were calculated for three concentration levels of alprazolam in human plasma. Six replicate samples having alprazolam theoretical concentration levels of 0.303 ng/mL (QC1), 12.625 ng/mL (QC2) and 35.350 ng/mL (QC3) were injected into the system. Table 27 summarizes the results obtained for the intra-day parameters.

The inter-day precision and accuracy were evaluated also using six aliquots for each quality control sample concentration, prepared and analyzed in six different days. The results are summarized in table 28**.** Recovery

Recovery of alprazolam was evaluated by comparing analyte response of six extracted samples of low, medium and high quality control samples to those of six appropriately diluted standard solutions. Results are presented in table 29.

Table 27. Evaluation of precision and accuracy for alprazolam quality control samples

No.	QC1 0.303 ng/mL		QC2 12.625 ng/mL		QC3 35.350 ng/mL	
	Found (ng/mL)	Recovery %	Found (ng/mL)	Recovery %	Found (ng/mL)	Recovery %
1	0.272	89.627	11.984	94.921	40.010	113.182
2	0.294	97.073	13.157	104.215	34.164	96.644
3	0.309	102.081	11.964	94.763	38.870	109.958
4	0.311	102.488	13.969	110.646	37.816	106.977
5	0.301	99.307	12.521	99.175	46.504	131.553
6	0.310	102.167	13.492	106.864	36.510	103.282
Average	0.299	98.791	12.848	101.764	38.979	110.266
% Deviation	0.015		0.825		4.203	
% RSD	5.022		6.421		10.783	
Overall % deviation	1.7					
% RSD	5.0-10.7					

Table 28. Evaluation of inter-day precision and accuracy for alprazolam spiked quality control samples

No.	QC1 0.303 ng/mL		QC2 12.625 ng/mL		QC3 35.350 ng/mL	
	Found (ng/mL)	Recovery %	Found (ng/mL)	Recovery %	Found (ng/mL)	Recovery %
1	0.278	91.812	11.643	92.224	30.560	86.451
2	0.294	96.981	11.333	89.767	30.296	85.703
3	0.348	114.720	10.946	86.701	32.214	91.127
4	0.271	89.538	11.652	92.296	28.954	81.906
5	0.295	97.260	10.282	81.445	32.719	92.558
6	0.295	97.489	11.169	88.471	32.484	91.894
Mean	0.297	97.965	11.171	88.482	31.205	88.273
% Deviation	0.027		0.514		1.500	
% RSD	9.108		4.602		4.808	
Overall % deviation	0.7					
% RSD	4.6-9.1					

Table 29. Recovery of alprazolam from biological matrix

	Extracted peak area	Unextracted peak area	Mean recovery (%)
QC1 (0.303 ng/mL)			
Mean	1306.878	1705.395	76.632
S.D.	52.629	32.303	
% CV	4.027	1.894	
n	6	6	
QC3 (35.350 ng/mL)			
Mean	161342.967	198222.845	81.395
S.D.	14489.582	3844.347	
% CV	8.981	1.939	
N	6	6	

For internal standard, average internal standard response of eighteen extracted samples was compared to the average internal standard responses of eighteen appropriately diluted internal standard solutions. Results are presented in table 30.

Table 30. Recovery of internal standard from biological matrix

	Internal standard response for extracted sample	Internal standard response for unextracted sample
Average	75404.589	84942.473
SD (±)	2427.919	1992.693
CV (%)	3.220	2.346
Concentration (ng/mL)	50.500	50.50
Mean recovery (%)	88.771	

Discussions

As it can be seen in the figure 27, no overlapping peaks were detected at the retention time of alprazolam and internal standard, 2 min and 1.6 min, respectively. No endogenous components interfering with the analyte and the internal standard were found in the chromatograms obtained from blank plasma samples.

The least-square linear regression revealed that the relationship in between concentration and peak-height was linear in the investigated domain, with a correlation coefficient of 0.9988, meeting the acceptance criteria ($r^2 \geq 0,990$), as it can be seen in figure 28.

The bioanalytical method proved to be sensitive, allowing a precise quantification of concentration as low as 0.1 ng/mL (figure 29 and table 26).

Average recovery values for alprazolam were 76.632, 71.783 and 81.395% at low, medium and high quality control levels, respectively (table 29).

Mean recovery value for the internal standard was 88.771% (table 30).

There were no interferences from endogenous plasma components or from other sources.

Alprazolam and the internal standard (lorazepam) were well separated and their peaks were narrow and symmetrical.

The simple sample-preparation procedure and short retention time enable determination of many samples per day.

Conclusions

A new liquid chromatography-tandem mass spectrometry method has been developed and validated for the assay of alprazolam in human plasma in the concentration range 0.1-50 ng/mL.

The precision and accuracy of the assay were both good.

The developed assay was successfully applied to a pharmacokinetic study after oral administration of alprazolam to healthy volunteers.

I.1.2.3. HPLC analysis of bisoprolol

Introduction

Bisoprolol fumarate is a synthetic cardioselective β_1-adrenergic blocker. It possesses an asymmetric carbon atom in its structure and is provided as a racemic mixture. The S(-) enantiomer is responsible for most of the beta-blocking activity (74).

The objectives of this work was to develop and to validate a simple, accurate, rapid and economic LC-tandem mass spectrometry method for the determination of bisoprolol in human plasma samples, using liquid-liquid extraction, and to present some of this method applications (75).

Materials and methods

Instruments

All analyses were performed using the Agilent 1100 LC/MSD Trap XCT system. The system compenents included the Agilent 1100 Degasser, Agilent 1100 Binary Pump, Agilent 1100 Autosampler, Agilent 1100 Mass Selective Detector. The Bruker Daltonik software was used for system control and data acquisition. An analytical balance Mettler-Toledo XP56, a Sigma 2-16 K centrifuge and a Vibramax 110 shaker were used for the sample preparation.

The separation was performed using a reverse phase column (Zorbax SB-C18 Solvent Saver Plus, 3 x 100mm, 3.5μm, supplied by Agilent, USA).

Reagents

All solvents and other chemicals (acetonitrile, methanol, sodium hydroxide, tert-butyl methyl ether, water, formic acid) were HPLC grade provided by Merck's Chemical Co., Darmstadt, Germany. The reference substances of bisoprolol and metoprolol (internal standard) were supplied from the USP Pharmacopoeia. The human plasma was obtained from Center for Blood Drawing and Preservation, Iasi, Romania.

Bisoprolol stock solution

Bisoprolol was dissolved in methanol, obtaining a bisoprolol stock solution of 500 μg/mL.

Metoprolol stock solution

The internal standard, metoprolol, was dissolved in methanol, obtaining a metoprolol stock solution of 500 μg/mL.

Solutions for linearity response

Eight bisoprolol concentrations were prepared in human plasma, covering the expected range of observed concentrations (1-100 ng/mL). The theoretical concentrations of bisoprolol calibration standards were 1.0; 2.0; 10.0; 20.0; 40.0; 60.0; 80.0 and 100.0 ng/mL.

Quality control samples

Plasma samples having bisoprolol theoreticals concentrations of 3 ng/mL, 25 ng/mL and 75 ng/mL were considered to be appropriate to be used to validate the bioanalytical method.

Samples for recovery

In order to determine the analyte and the internal standard recovery from the plasma, water samples containing the same bisoprolol concentrations as the quality control samples were prepared.

Samples preparation

After alkalization with sodium hydroxide and addition internal standard solution, the 0.250 mL plasma sample was extracted with 2 mL tert-butyl methyl ether. The solvent was evaporated using a flow air at 40ºC. The solid residue was dissolved in a 0.250 mL mixture 0.1% formic acid solution - acetonitrile (50-50, v/v).

Results

The development of the LC- Tandem Mass Spectrometry method

The LC-MS/MS method for determination of bisoprolol in human plasma samples described in this paper was performed using a mobile phase consist in mixture 0.1% formic acid solution (pH 3,0) - acetonitrile (50-50, v/v). The LC system was operated at 0.3 mL/min, using the binary pump. The column temperature was 40°C. The injection volume was 5 μL and represented no more 5% of the total sample available for injection. Short run times of about 3 minutes were achieved for both bisoprolol and internal standard, metoprolol. Bisoprolol was eluted at 1.7 minutes and metoprolol at 1.9 minutes. The peaks of interest were free from interfering peaks at their respective retention time.

To minimize undesirable fragmentation voltages were tested from 80 to 200 V. At 100V, the MS response of bisoprolol and metoprolol showed both minimal undesirable fragmentation and highest response.

The mass transition ion-pair was followed as m/z 326.2 →116.1 for bisoprolol and m/z 268.2→191.0, for metoprolol, as sustained Bhatt et al. (78).

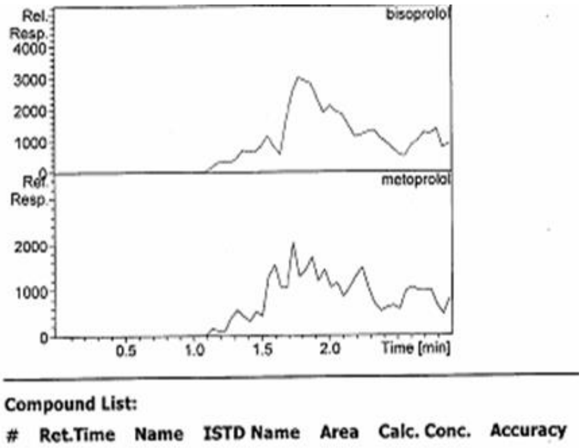
The protonated molecular ion of bisoprolol $[M+H]^+$ (m/z 326.3) was tested to give the highest sensitivity. Based on the optimization results, m/z 116.2 was selected as the quantifier ion. Also, the protonated molecular ion of metoprolol $[M+H]^+$ (m/z 268) was tested and m/z 116.2 was selected as the quantifier ion. We have chosen products ions with the same m/z value for bisoprolol and metoprolol, respectively, because a possible suppression effect would influence the quantification of both the analyte and internal standard in the same way. Other mass spectrometric parameters (gas temperature, gas pressure and gas flow) were adjusted to get a maximum signal for bisoprolol . The nebulizing gas flow rate was set at 10 L/min, drying gaz temperature at 350°C and the capillary voltage at 4000 V. The response of bisoprolol and metoprolol were measured by MRM in the positive ionization mode with a collision energy of 20 V.

Method validation

The parameters usually examined in the validation process are selectivity /specificity, linearity, limit of quantification, accuracy and precision, stability.

Selectivity

Plasma aliquots from six different sources were assessed for analysis in order to investigate the plasma components behavior. As it can be seen in figure 30, no overlapping peaks were detected at bisoprolol and internal standard retention time, 1.7 min and 1.9 min, respectively.



Compound List:

Ret.Time Name ISTD Name Area Calc. Conc. Accuracy

Fig. 30. Chromatogram recorded for blank plasma sample

<u>Linearity and Lower limit of quantification</u>

The linearity was investigated for a bisoprolol theoreticals concentrations range between 1 ng/mL and 100 ng/mL and the calibration curve was derived by plotting the peak-height ratios of the analyte and the internal standard against the concentration of bisoprolol, using linear regression analysis (figure 31).

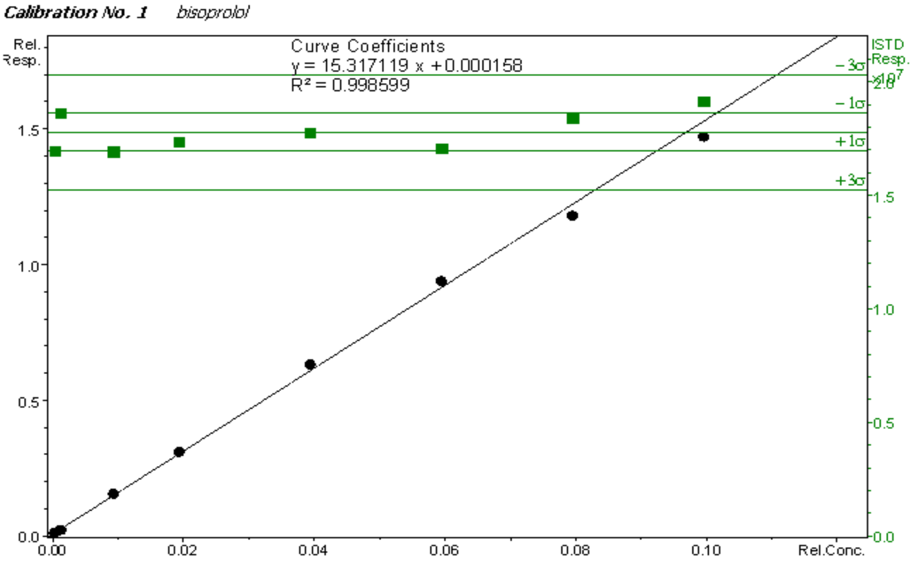
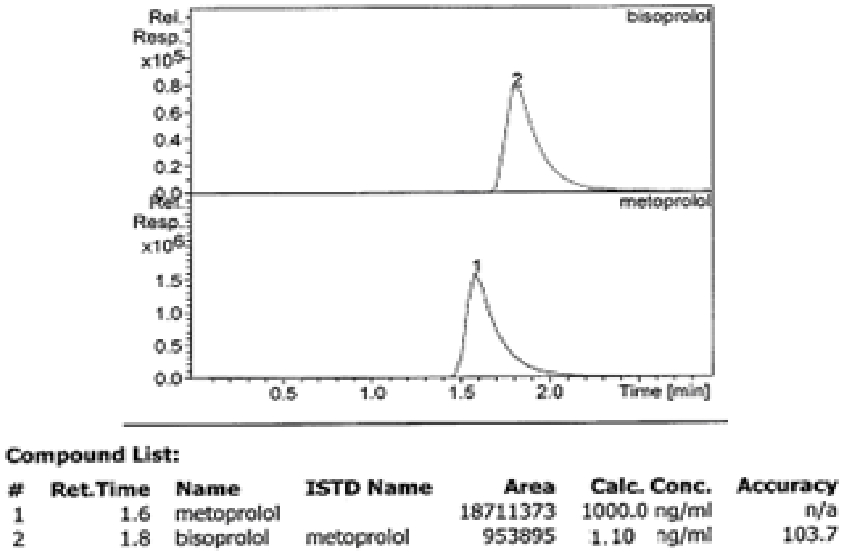


Fig 31. The bisoprolol calibration curve obtained for plasma samples

The lower limit of quantification, i.e. the lowest standard level with a coefficient of variation less than 20%, is for bisoprolol 0.990 ng/mL with 41.433 signal to noise ratio (figure 32). Results are presented in table 31.



Compound List:

#	Ret.Time	Name	ISTD Name	Area	Calc. Conc.	Accuracy
1	1.6	metoprolol		18711373	1000.0 ng/ml	n/a
2	1.8	bisoprolol	metoprolol	953895	1.10 ng/ml	103.7

Fig. 32. Chromatogram recorded for plasma containing bisoprolol (0.990 ng/mL, lower limit of quantification) and internal standard

Table 31. Lower limit of quantification

	Analyte concentration (ng/mL)		0.990
	Conc. (ng/ml)	% Nominal	Signal/Noise ratio
	0.989	99.945	44.500
	1.407	142.094	36.000
	0.958	96.802	46.100
	1.167	117.863	31.700
	1.175	118.723	41.500
	1.241	125.400	48.800
n	6	6	6
Mean	1.156	116.804	41.433
SD (±)	0.166		
CV(%)	14.339		
Acceptance criteria:			
4 out of 6 LLQC must be 100±20% nominal value. Mean % nominal 100±20% CV (%)≤20% Signal/Noise ratio ≥ 5			

Accuracy and Precision

The accuracy and precision of this method were calculated for three concentrations of bisoprolol in human plasma.

Six replicate samples having bisoprolol theoreticals concentrations of 3 ng/mL (QC1), 25 ng/mL (QC2) and 75 ng/mL (QC3) were injected into the system. Table 32 summarizes the results obtained for the intra-day parameters.

Table 32. Evaluation of intra-day precision and accuracy for bisoprolol spiked quality control samples

Cth = 3 ng/mL			Cth = 25 ng/mL		Cth = 75 ng/mL	
	Cexp (ng/mL)	% Nominal	Cexp (ng/mL)	% Nominal	Cexp (ng/mL)	% Nominal
1.	2.912	98.391	22.524	91.263	69.203	93.468
2.	3.003	101.439	24.862	100.737	68.525	92.552
3.	3.262	110.192	23.762	96.281	66.781	90.196
4.	2.829	95.568	25.189	102.062	76.691	103.580
5.	3.018	101.960	21.739	88.085	65.844	88.931
6.	2.854	96.431	21.371	86.593	74.009	99.958
Mean	2.980	100.664	23.241	94.170	70.176	94.781
SD	0.158		1.610		4.268	
CV,%	5.296		6.927		6.082	
Acceptance criteria:						
67% Total QCs must be 100±15% nominal values 50% QCs per level must be 100±15% nominal values Mean % nominal 100±15% CV(%) ≤15%						

C_{th} = theoretical concentration; C_{exp} = experimental concentration; SD = standard;

The inter-day precision and accuracy was evaluated also using six aliquots for each quality control sample concentration, prepared and analysed in six different days. The results are presented in table 33.

Table 33. Evaluation of inter-day precision and accuracy for bisoprolol spiked quality control samples

Cth = 3 ng/mL			Cth = 25 ng/mL		Cth = 75 ng/mL	
	Cexp (ng/mL)	Accuracy (%)	Cexp (ng/mL)	Accuracy (%)	Cexp (ng/mL)	Accuracy (%)
1.	3.015	101.860	24.331	98.586	70.921	95.788
2.	2.879	97.276	23.332	94.539	77.073	104.097
3.	3.257	110.048	23.109	93.636	77.932	105.256
4.	2.964	100.136	24.013	97.297	70.590	95.340
5.	2.943	99.439	28.411	115.117	75.582	102.083
6.	3.152	106.480	24.910	100.933	74.865	101.115
Mean	3.035	102.540	24.684	100.018	74.494	100.613
SD	0.142		1.940		3.092	
CV,%	4.686		7.860		4.151	
Acceptance criteria:						
67% Total QCs must be 100±15% nominal values 50% QCs per level must be 100±15% nominal values Mean % nominal 100±15% CV(%) ≤15%						

C_{th} = theoretical concentration; C_{exp} = experimental concentration; SD = standard;

Recovery

Recovery of bisoprolol was evaluated by comparing analyte response of six extracted samples of low, medium and high quality control samples to those of six appropriately diluted standard solutions. Mean recovery values for bisoprolol are 76.529, 78.479 and 79.863% at low, medium and high quality control levels, respectively.

For internal standard, mean internal standard response of eighteen extracted samples was compared to the mean internal standard responses of eighteen appropriately diluted internal standard solutions. Mean recovery value for the internal standard is 90.568%.

Stability tests

To test stability, a series of standards samples were prepared from freshly made stock solutions in the same solvent used for the assay. The lowest and highest concentration of the quality control (3 ng/mL and 75 ng/mL), including the analyte and internal standard (when appropiate) were used.

Human plasma samples of each concentration were prepared in enough volume to have multiple aliquots.The aliquots of each concentration were processed and quantified immediately in order to provide the reference (fresh) values and other six aliquots of each concentration were processed for the desired tests.

The following sub-sections present the procedure carried out and the corresponding results.

Stability of the analyte after sample processing at room temperature

Samples prepared at low (QC1) and high (QC3) quality control levels were submitted to the extraction procedure and kept at room temperature under ambient laboratory conditions (stability samples). A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed and analyzed with 6 replicates of stability samples in a single run. Concentrations were calculated to determine % change over time.

Bisoprolol is found to be stable for 31 hours at room temperature under ambient laboratory conditions after sample processing with %changes (ratio between mean concentration of stability samples and mean concentration of comparison samples) of - 11.483 and - 2.808%. Results are presented in table 34.

Stability of analyte in biological matrix at room temperature

Samples were prepared at low (QC1) and high (QC3) quality control levels. Six replicates of low and high quality control samples were left at room temperature for approximately 4 hours (stability samples).

A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed with 6 replicates of stability samples and analyzed in a single run. Concentrations were calculated to determine % change over time.

Bisoprolol is found to be stable in human plasma for 4 hours at room temperature with % changes of 3.522 and 3.566 %. Results are presented in table 35.

Table 34. Stability of analyte following sample processing at room temperature

	Analyte:		Bisoprolol			
	Biological matrix:		human plasma			
	Storage condition:		31 hours at room temperature			
	QC1 (3 ng/mL)			QC3 (75 ng/mL)		
	Comparison samples		Stability samples	Comparison samples		Stability samples
	Measured	% Nominal	Measured	Measured	% Nominal	Measured
	Conc.		Conc.	Conc.		Conc.
	2.682	90.611	2.838	80.224	108.352	82.798
	2.330	78.719	2.639	82.755	111.771	63.262
	3.082	104.134	2.434	78.821	106.457	75.379
	2.380	80.392	2.079	79.826	107.815	80.616
	2.117	71.504	2.047	91.323	123.343	58.511
	2.619	88.475	2.745	84.105	113.595	79.410
n	6	6	6	6	6	6
Mean	2.535	85.639	2.464	82.842	111.889	73.329
SD (±)	0.338		0.338	4.597		10.049
CV(%)	13.314		13.734	5.549		13.704
% Change		-2.808			-11.483	
Acceptance criteria						
67% Comparison samples must be 100±15% nominal values Mean % nominal of comparison samples 100±15% CV(%) ≤15% ; % Change ± 15%						

Table 35. Stability of analyte in biological matrix at room temperature

	Analyte:		bisoprolol			
	Biological matrix:		human plasma			
	Storage condition:		4 hours at room temperature			
	QC1 (3 ng/mL)			QC3 (75 ng/mL)		
	Comparison samples		Stability samples	Comparison samples		Stability samples
	Measured	% Nominal	Measured	Measured	% Nominal	Measured
	Conc.		Conc.	Conc.		Conc.
	3.509	118.561	3.540	61.764	83.419	72.020
	2.839	95.898	3.502	74.312	100.368	67.462
	3.453	116.639	3.281	73.697	99.537	90.117
	3.187	107.684	3.514	72.186	97.495	73.715
	4.310	145.604	3.616	68.557	92.595	69.225
	3.123	105.510	3.688	75.832	102.421	69.012
n	6	6	6	6	6	6
Mean	3.403	114.983	3.523	71.058	95.973	73.592
SD (±)	0.506		0.138	5.182		8.405
CV(%)	14.864		3.925	7.292		11.421
% Change		3.522			3.566	
Acceptance criteria						
67% Comparison samples must be 100±15% nominal values Mean % nominal of comparison samples 100±15% CV(%) ≤15% ; % Change ± 15%						

Stability of analyte in biological matrix after 3 freeze-thaw cycles

Samples were prepared at low (QC1) and high (QC3) quality control levels, aliquoted and frozen at - 25±10°C. Some of the aliquots of quality control samples were subjected to three freeze-thaw cycles (stability samples).

The remaining aliquots were not thawed (comparison samples). A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed with 6 replicates of stability samples and analyzed in a single run.

Concentrations were calculated to determine % change over freeze-thaw cycles. Bisoprolol is found to be stable in human plasma after three freeze-thaw cycles with % changes of 1.920 and 6.212%. Results are presented in table 36.

Table 36. Stability of analyte in biological matrix after 3 freeze-thaw cycles at -25±10 ºC

	Analyte:		Bisoprolol			
	Biological matrix:		human plasma			
	Storage condition:		-25±10 ºC			
	QC1 (3 ng/mL)			QC3 (75 ng/mL)		
	Comparison samples		Stability samples	Comparison samples		Stability samples
	Measured	% Nominal	Measured	Measured	% Nominal	Measured
	Conc.		Conc.	Conc.		Conc.
	3.015	101.860	2.934	70.921	95.788	74.754
	2.879	97.276	3.239	77.073	104.097	73.574
	3.257	110.048	3.096	77.932	105.256	77.879
	2.964	100.136	3.180	70.590	95.340	83.159
	2.943	99.439	3.049	75.582	102.083	78.939
	3.152	106.480	3.063	74.865	101.115	86.426
N	6	6	6	6	6	6
Mean	3.035	102.540	3.093	74.494	100.613	79.121
SD (±)	0.142		0.107	3.092		4.921
CV(%)	4.686		3.454	4.151		6.220
% Change		1.920			6.212	
Acceptance criteria						
67% Comparison samples must be 100±15% nominal values Mean % nominal of comparison samples 100±15% CV(%) ≤15% ; % Change ± 15%						

The applications of LC- Tandem Mass Spectrometry method

The assay has proven to be suitable to determine the bisoprolol concentration in the bioequivalence study of Bisoprolol 10 mg coated tablets produced by Antibiotice S.A. (referred to as test drug) versus Concor® 10 mg coated tablets produced by Merck (referred to as reference drug). In figure 33, average bisoprolol concentrations recorded for 22 volunteers are plotted against time for both test and reference drugs.

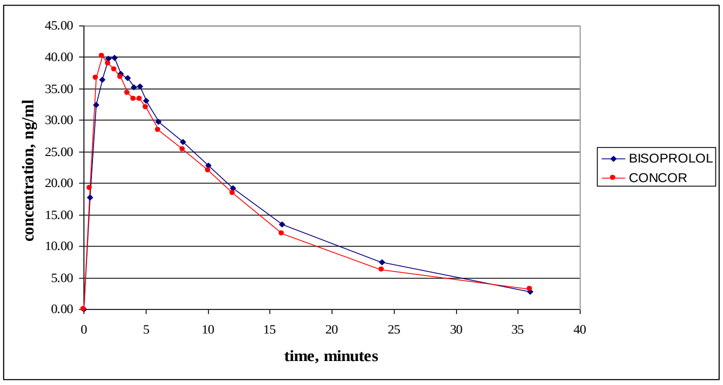


Fig. 33. Average bisoprolol concentrations recorded for the test and reference drugs in the bioequivalence study performed on 22 healthy volunteers

The concentration profiles are similarly, fitting the results obtained for the in vitro dissolution test (see figure 34).

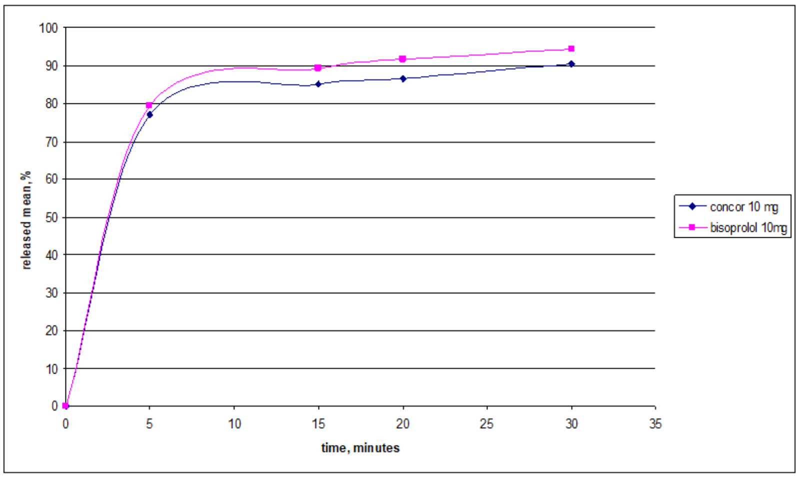


Fig. 34. Average dissolved bisoprolol recorded for test and reference drugs recorded for the *in vitro* dissolution test

Discussions

LC separation of bisoprolol and internal standard, metoprolol, has been carried out using the mobiles phases consisting of differents aqueous solutions and methanol or acetonitrile as organic phase. Hernardo et al. (76) used a acetonitrile as organic mobile phase to lead to shorter retention times and better resolution of the bisoprolol and internal standard. Formic acid solution as additive in water is have been so used by Shuijun et al. (77) to improve the sensitivity of MS detection.

The method was validated according Guidance for Industry: *Bioanalytical Method Validation* (30).

The reversed-phase HPLC method described in this paper has been tested for possible interferences from other plasma factors. The bioanalytical method proved to be selective (figure 30).

The reversed-phase HPLC method described in this paper has been tested for possible interferences from other plasma factors. Plasma aliquots from six different sources were assessed for analysis in order to investigate the plasma components behavior.

As it can be seen in figure 30, no overlapping peaks were detected at bisoprolol and internal standard retention time, 1.7 min and 1.9 min, respectively. The bioanalytical method proved to be selective.

The bioanalytical method proved to be sensitive, allowing a precise quantification of concentrations as low as 1 ng/mL (see figure 32).

Intra- and inter-day precision of analysis was < 8% and accuracy ranged was from 94.170% to 102.540%.

The values for the investigated parameters proved to be lower than the one reported by Oniscu et al. (79), employing a HPLC method with fluorescence detection. Also, Liu Mingyuan et al. (80) reported an accuracy ranged from 89.4%-113%, employing a precipitation with acetonitrile procedure for plasma sample preparations.

Based on the determined bisoprolol concentrations, the calculated pharmacokinetic parameters demonstrated that the drug produced by Antibiotice S.A. is bioequivalent with the one produced by Merck.

Conclusions

LC-tandem mass spectrometry method described and validated above is sensitive, accurate, precise, rapid and efficient.

The developed method can be applied for the determination of bisoprolol from human plasma samples (eg. for pharmacokinetic parameters).

I.2. Development of some UV-Vis Spectrophotometric analytical methods

Spectrophotometric analysis is ideal for measuring color consistency in pharmaceutical powders and plaques because it is non-destructive and can be utilized for on-line measurements. UV Spectrophotometry is the best method available for identification and comparison of organic compounds.

The pharmaceutical industry relies on spectrophotometric analysis for a variety of applications and choosing the right instrumentation is essential for consistent and quality results. Today's pharmaceuticals utilize organic compounds that have proven to block specific pain receptors. However, without qualitative analysis of these compounds using UV spectrophotometry, dangerous levels can become toxic to the human body, causing serious damage to internal organs or even death. Qualitative analysis of organic compounds can be achieved through the simple process of UV spectrophotometry (81, 82).

UV spectrophotometers measure the visible regions of ultraviolet light and can provide valuable information about the levels of active ingredients present in pharmaceutical compounds, as well as detect any impurities. By measuring the absorption of UV radiation of light, spectrophotometric analysis can quantify these levels at a highly accurate rate. The applications of quantitative analysis through spectroscopy allow for pharmaceutical researchers to clearly identify and compare organic compounds to ensure that the drug molecules are properly absorbed by the body and distributed to the right places. From the identification of active ingredients or protein analysis, each component of pharmaceutical research depends on spectrophotometers to provide qualitative analysis and exact drug formulations (83).

Published papers in the field

♦Țântaru Gladiola, Bibire Nela, Panainte Alina Diana, Vieriu Mădălina, Apostu Mihai. Aniline Derived Bis-Schiff Base - Analytical Reagent for the Assay of Fe (III). *Rev Chim (Bucharest)* 2018; 69(11): 3097-3099.

Țântaru G, Apostu M, **Bibire N**, Vieriu M, Panainte AD. Pyridine-Derived Schiff Base-Analytical Reagent for Iron (II) Ions. *Rev Med Chir Soc Med Nat Iași* 2018; 122(3): 640-646.

♦ Mădălina Vieriu, Gladiola Ţântaru, Mihai Apostu, Alina Diana Panainte, Luminița Agoroaei, Livia Uncu, **Nela Bibire**. A New Spectrometric Method for Quantitative Determination through Molecular Absorption of Lisinopril. *Rev Chim (Bucharest)* 2015; 66(10): 1563-1566.

♦ Mădălina Vieriu, Gladiola Țântaru, M. Apostu, Alina Diana Panainte, **Nela Bibire**. A New Spectrophotometric Method for the Assay of Lisinopril. *Rev Med Chir Soc Med Nat Iași* 2015; 119(4): 1199-1204.

♦ Mădălina Vieriu, **Nela Bibire**, Mihai Apostu, Alina Diana Panainte, Alexandru Znagovan, Ludmila Anghel, Gladiola Țântaru. Spectrophotometric Determination Method for Lisinopril Using Permanganate in Alkaline Medium. *Rev Chim (Bucharest)* 2014; 65(10): 1146-1148.

♦ Alina Diana Panainte, **Nela Bibire**, Gladiola Țântaru, Mihai Apostu, Mădălina Vieriu, Vasile Dorneanu. A New Method for the Assay of Bisoprolol Using Bromocresol Green. *Rev Chim (Bucharest)* 2014; 65(8): 916-920.

♦ Alina Diana Panainte (Gudruman), **Nela Bibire**, Gladiola Ţântaru, M. Apostu, Mădălina Vieriu, Vasile Dorneanu. Spectrophotometric Method for Estimation of Bisoprolol Fumarate in Tablets. *Rev Med Chir Soc Med Nat Iași* 2014; 118(2): 558-563.

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♦ Alina Diana Panainte (Gudruman), **Nela Bibire**, Gladiola Ţântaru, M. Apostu, Mădălina Vieriu. Validation of a Spectrophotometric Assay Method for Bisoprolol Using Picric Acid. *Rev Med Chir Soc Med Nat Iași* 2013; 117(2): 520-524.

♦ Mădălina Vieriu, **Nela Bibire**, Gladiola Ţântaru, M. Apostu, Alina Diana Panainte, V. Dorneanu. Spectrofotometric Method for the Assay of Ramipril Using Molybdophosphoric Acid. *Rev Med Chir Soc Med Nat Iași* 2012; 116(4): 1218-1222.

♦ Mădălina Vieriu, **Nela Bibire**, Gladiola Ţântaru, Mihai Apostu, Mariana Mândrescu, Alina Diana Gudruman, Vasile Dorneanu. A New Method for the Assay of Lisinopril Using Molybdophosphoric Acid. *Ovidius University Annals of Chemistry* 2011; 22(1): 32-36.

♦ **Nela Bibire**, Leonidas Christopoulos, Mihai Apostu, Vasile Dorneanu. Determinarea spectrofotometrică în vizibil a adrenalinei. *Rev Med Chir Soc Med Nat Iași* 2007; 111(3): 779-782.

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♦ M. Apostu, V. Dorneanu, **Nela Bibire**. Determinarea spectrofotometrică a ranitidinei în vizibil. *Rev Med Chir Soc Med Nat Iași* 2003; 107(1): 214-217.

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I.2.1. UV-Vis Spectrophotometric analysis of lisinopril

Introduction

Lisinopril is a derivative that belongs to nitrogen heterocycle compounds with the following chemical name [(S)-1-[N2-(1-carboxy-3-phenylpropyl)-L-lysyl]-L-proline dihydrate] (84).

This new spectrophotometric method for the quantitative determination of lisinopril through molecular absorption uses phosphotungstic acid in acidic medium. The developed method was validated using the following criteria: linearity, detection and quantification limits, precision, accuracy and robustness (85, 86).

Materials and methods

The reagents and apparatus used were: lisinopril as 100.03% pure reference substance (Lupin, India), phosphotungstic acid (Merck), hydrochloric acid (Merck), sodium lauryl sulphate (Merck), Kern 770 analytical balance, Hewlett Packard 8453UV-Vis Spectrophotometer.

Lisinopril forms with phosphotungstic acid in acidic medium an insoluble compound which can be spectrometrically measured at 335 nm.

In order to establish the optimum wavelength for the detection, a 24 µg/mL Lisinopril sample was analyzed. 2 mL of the 60 µg/mL lisinopril solution was mixed with 1 mL of 2% phosphotungstic acid solution and 1 mL of 0.5 M hydrochloric acid solution and 1 mL of 0.01% sodium lauryl sulphate solution. The UV-Vis absorption spectrum was recorded using 1cm quartz cell, after 15 minutes.

In order to establish the optimum acidity of the medium, it was first established that hydrochloric acid was the best option and then seven different concentration levels (0.2M, 0.3M, 0.4M, 0.5M, 0.6M, 0.7M, 0.8M) of reagent were used, while the parameters of the method were maintained unchanged.

In order to establish the optimum concentration of phosphotungstic acid, five solutions of reagent (0.50, 1.00, 1.50, 2.00, 2.50 and 3.00%) were used, while the parameters of the method were maintained unchanged.

In order to establish the optimum concentration of the sodium lauryl sulphate, six solutions of reagent (0.0050, 0.0075, 0.0100, 0.0200, 0.0300 and 0.0400%) were used, while the parameters of the method were maintained unchanged.

In order to assess the optimum time for measuring the absorbance values at 335 nm, 2 samples containing 9 µg/mL and 33 µg/mL of lisinopril were used. Their absorbances were measured every 5 minutes for 60 minutes at 335 nm, against a blank prepared in the same conditions in a 1 cm cell.

Method validation

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the following formulas:

$$\mathrm{LOD} = 3\cdot \mathrm{SD}\cdot \mathrm{Slope}^{-1}$$

$$\mathrm{LOQ} = 10\cdot \mathrm{SD}\cdot \mathrm{Slope}^{-1}$$

where:

SD = standard deviation of the intercept;

Slope = the slope of the calibration curve equation.

Method precision was evaluated through repeatability and reproducibility. Using the experimental data the sample concentration was calculated using the calibration curve equation.

Standard addition method was used to evaluate the accuracy of the method.

Results

From the analysis of the absorption spectra (figure 35), we observed a maximum of absorbance for the reaction product at 335 nm. That wave length was used for all the determinations.

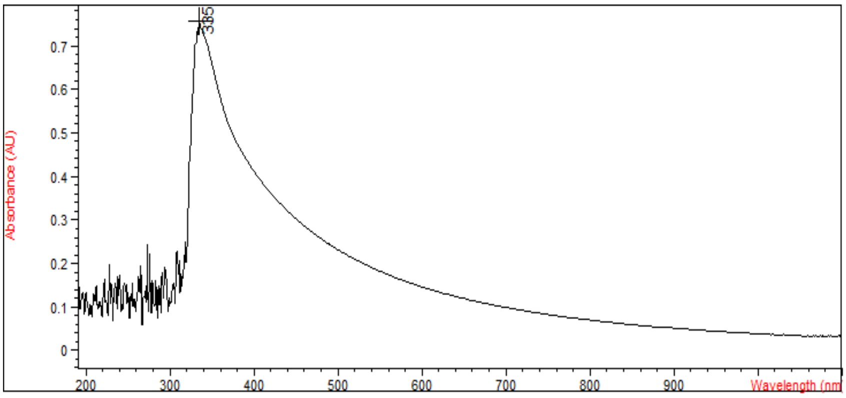

Fig. 35. The spectrum of the reaction product obtained for 10μg/mL lisinopril

The reaction in between lisinopril and phosphotungstic acid occurs in acidic medium (table 37). The optimum concentration of phosphotungstic acid solution was established based on the experimental data from table 38.

Table 37. Optimum concentration of hydrochloric acid

Hydrochloric acid (mol/L)	Lisinopril	
	9.0 μg/mL	33.0 μg/mL
	Absorbance (335 nm)	
0.2	0.05423	0.64528
0.3	0.09655	0.77582
0.4	0.15286	0.90155
0.5	0.17325	1.04980
0.6	0.15263	1.00990
0.7	0.14254	0.98122
0.8	0.13241	0.88311

Table 38. Optimum concentration of phosphotungstic acid

Phosphotungstic acid (%)	Lisinopril	
	9.0 μg/mL	33.0 μg/mL
	Absorbance (335nm)	
0.5	0.01965	0.28510
1.0	0.04356	0.58221
1.5	0.09252	0.79853
2.0	0.17256	1.05489
2.5	0.16642	0.99211
3.0	0.14265	0.91630

The optimum concentration of sodium lauryl sulfate was established based on the experimental data from table 39.

Table 39. Optimum concentration of sodium lauryl sulfate

Sodium lauryl sulfate (%)	Lisinopril	
	9.0 µg/mL	33.0 µg/mL
	Absorbance (335 nm)	
0.005	0.03781	0.65293
0.075	0.08945	0.89136
0.010	0.16956	1.05325
0.020	0.16715	1.00994
0.030	0.15262	0.99362
0.040	0.12565	0.89235

After studying the stability of the samples it was established that the chemical reaction between lisinopril and phosphotungstic acid was final after 15 minutes (table 40). Also, it was proved that the absorbance remains almost the same for at least another 15 minutes, time sufficient enough for the analysis to be performed.

Table 40. Stability study

Time (minutes)	Lisinopril	
	9.0 µg/mL	33.0 µg/mL
	Absorbance (335 nm)	
5	0.09026	0.76824
10	0.14236	0.89562
15	0.16331	1.0542
20	0.16242	1.0512
25	0.16542	1.0492
30	0.15821	1.0504
40	0.14742	1.0221
50	0.12655	0.99522
60	0.11502	0.89412

Optimum procedure: 2 mL of lisinopril working solutions were mixed with 1 mL of 0.5M hydrochloric acid solution, 1 mL of 0.2% phosphotungstic acid solution and 1 mL 0.01% sodium lauryl sulphate solution.

The final lisinopril concentration of the samples were in the 9 - 33 µg/mL range. The absorbance was measured after 15 minutes at 335 nm versus a blank solution prepared in similar conditions.

Method validation

Linearity was studied in the 3-36 µg/mL concentration range (figure 36). The obtained data were statistically evaluated (table 41) and the calibration curve was obtained (figure 37).

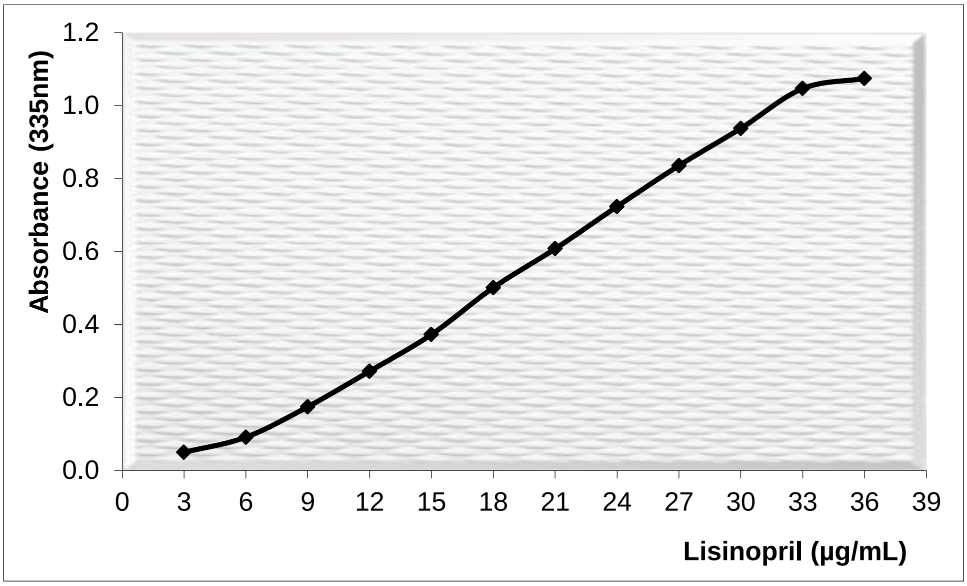

Fig. 36. Study of method linearity

Table 41. Study of method linearity

Lisinopril (µg/mL)	Absorbance (335 nm)				
	Ist Serie	IInd Serie	IIIrd Serie	IVth Serie	Average
3	0.06369	0.06025	0.06936	0.00594	0.04981
6	0.08506	0.08562	0.10535	0.08845	0.09112
9	0.15969	0.16969	0.19468	0.17456	0.17465
12	0.26235	0.27123	0.28459	0.27002	0.27204
15	0.36139	0.35139	0.40375	0.37452	0.37276
18	0.49511	0.48751	0.51833	0.50426	0.50130
21	0.59697	0.60697	0.62430	0.60254	0.60769
24	0.73912	0.72912	0.71146	0.71458	0.72357
27	0.81787	0.85787	0.83282	0.83450	0.83576
30	0.93735	0.93535	0.94347	0.93451	0.93767
33	1.04610	1.05020	1.05430	1.03650	1.04677
36	1.01260	1.05420	1.10620	1.12510	1.07452
Correlation and regression coefficients r = 0.9997, r^2 = 0.9994; Standard error = 0.0275; Intercept = -0.1658; Slope = 0.0368					

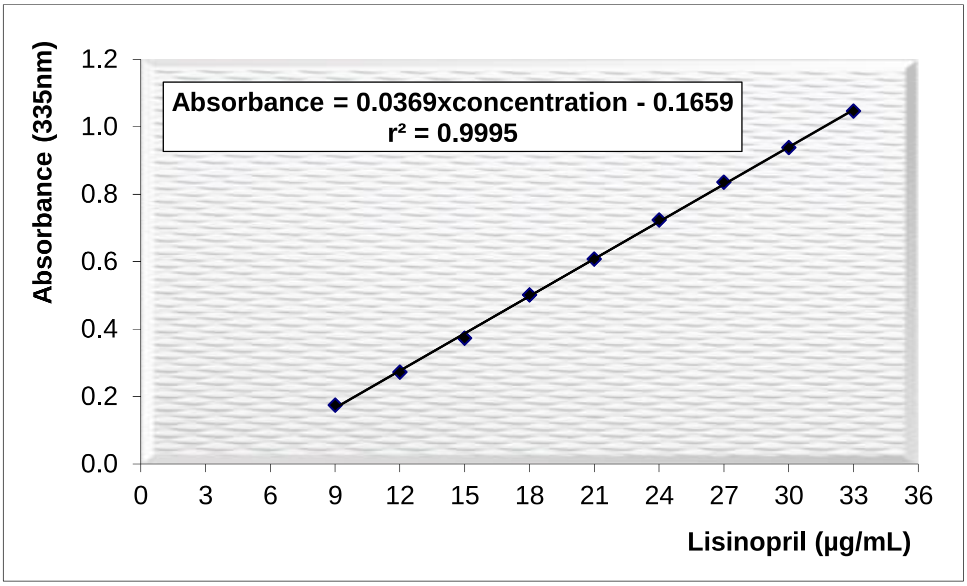

Fig. 37. Calibration curve

Detection and Quantification limits were calculated using the following formulas:

LOD = 3 x Standard error / Slope = 2.24 µg/mL

LOQ = 10 x Standard error / Slope = 7.47 µg/mL

Precision

Three samples of 18, 21 and 24 µg/mL lisinopril were used. Three assays were performed for each concentration. Two sets of assays were performed in different days in order to evaluate the *intermediary precision*.

The concentrations of the samples were calculated using the calibration curve equation (table 42).

Accuracy

In order to establish the accuracy of the method, lisinopril samples of 18, 21 and 24 µg/mL were analyzed. For each concentration, three determinations were performed (29).

The concentration of the samples was calculated from the experimental values of the absorbance, using the regression curve equation (table 42).

Table 42. Study of the precision and accuracy of the method

Lisinopril (µg/mL)	Precision		Intermediate precision		Accuracy	
	Found (µg/mL)	RSD (%)	Found (µg/mL)	RSD (%)	Absorbance	Recovery (%)
18	18.0999	0.94	0.49627	1.26	0.48217	100.07
	18.0223		0.51248		0.47956	99.74
	17.7754		0.49998		0.48917	100.96
21	21.0465	0.75	0.60982	0.86	0.60872	99.61
	20.9645		0.61025		0.61356	100.13
	21.2739		0.59854		0.59652	98.27
24	24.1629	0.91	0.72831	0.71	0.73215	98.96
	24.1865		0.71985		0.72971	98.73
	23.7944		0.71596		0.73654	99.38
					Mean	99.54

Robustness

This was done by varying the instrument, analyst, and time of study. The analysis was performed on a Shimadzu UV-Visible spectrophotometer, model-1700. Interday and intraday analysis was performed by changing the analyst.

Discussions

The best concentration of hydrochloric acid solution was established at 0.5M, because when using that solution the maximum absorbance measured at 335 nm had the greatest value (table 37).

To stabilize the suspension it was necessary to add a surfactant. We have undertaken a study for choosing the best surfactant of the following: glycerin, sodium lauryl sulfate, methyl cellulose, carboxymethyl cellulose. The best results were obtained when sodium lauryl sulfate was used (table 39).

According to the experimental data, the developed method for lisinopril determination was linear in 9-33 μg/mL concentration range (figure 36 and table 41).

When we compared this concentration range with that of other published methods (85) we found that it was very similar, but this new method has the following advantage: it does not involve rare or complex reagents. It is simple and easy to perform.

We observed that for each set of data and for both sets together the relative standard deviation was lower than 2% (at most RSD = 0.67%) (84). That proved that the proposed method was precise.

We observed that the recovery was 99.54% for the studied concentration range, the mean (minimum was 98.27% and maximum was 100.96%) and the relative standard deviation was under 2% (RSD = 0.81%). These values prove that the proposed method was accurate.

The evaluation of robustness was performed for system suitability to ensure the validity of analytical procedure, and reproducibility of the results confirmed the robustness of the method.

Another UV-Vis molecular absorbance spectrometric method was developed by our group for the assay of lisinopril using molybdophosphoric acid in hydrochloric acid medium. The reaction product showed a maximum absorbance at 369 nm. The optimum conditions of the reaction were established. The developed method was validated. The method showed a good linearity in the range of 8.0 - 32 μg/mL (correlation coefficient r = 0.9995). The LOD was 2.33 μg/mL and the LOQ was 7.79 μg/mL. The precision and the accuracy were determined, (RSD = 1.64%); mean recovery was 100.59% in the 98.51-102.39% concentration range (87).

A simple and accurate spectrophotometric method was developed for determination of lisinopril and the method was based on the oxidation reaction with potassium permanganate in alkaline medium when potassium manganate was obtained. Optimum working conditions were established and the new method was validated. The maximum absorbance was measured at 608 nm. The method presented a good linearity in the concentration range 2-14 μg/mL and regression coefficient r^2 = 0.9995. The RSD for the precision of the method was 1.50, the RSD for the intermediate precision was 1.12, and the recovery values were ranged between 99.18-100.74%. The proposed method for the determination of lisinopril was simple, rapid and cost effective compared with other techniques (88).

A spectrophotometric method for the assay of lisinopril using 2,4-dinitrophenol as reagent involved the addition of 2,4-dinitrophenol to lisinopril in methanol followed by absorbance measurement at 400 nm. Experimental conditions that provide wide linear range, maximum sensitivity, selectivity, accuracy and precisions were optimized. Beer's law was obeyed in the 2.0-14.0 µg/mL concentration range. Linear regression equation of calibration graph was A = 0.005 + 0.045 x concentration, with a regression coefficient of 0.9995 (n = 8). LOD and LOQ calculated according to the ICH guidelines were 0.42 and 1.42 µg/mL, respectively. Accuracy and precision of the assays were determined by computing the intra-day and inter-day variations at three different lisinopril concentrations; the intra-day and inter-day RSD was < 1.43% and accuracy was better than 1.72 % (89).

Conclusions

A spectrophotometric method was developed for the assay of lisinopril using phosphotungstic acid in acidic medium.

The reaction product showed a maximum molecular absorbance peak at 335 nm.

The analytical method was validated by establishing the linearity domain in the range of 9.0- 33.0 µg/mL, with a correlation coefficient of 0.9997, the detection limit is 2.24 µg/mL, the quantification limit is 7.47 µg/mL lower than the lowest concentration from the linearity domain.

The proposed method is simple, easy to perform, sensitive, linear, precise, accurate and robust.

I.2.2. UV-Vis Spectrophotometric analysis of ramipril

Introduction

Ramipril (figure 38) is a drug of the angiotensin converting enzyme (ACE) inhibitor class that is primarily used in the treatment of high blood pressure and congestive heart failure.

Ramipril, a prodrug, is converted to the active metabolite ramiprilat by liver esterase enzymes. Ramiprilat is mostly excreted by the kidneys. The half-life of ramiprilat is variable (3-16 hours), and is prolonged by heart and liver failure, as well as kidney failure (90).

This study presents a UV-Vis molecular absorbance spectrometric method for the assay of ramipril using molybdophosphoric acid in acidic medium. When comparing it to other assay methods in the literature, the proposed method is simple, easy to perform, sensitive, linear, precise, accurate and robust (91, 92).

The developed method was validated using the following criteria: linearity, detection and quantification limit, precision, accuracy and robustness (85, 93-96).

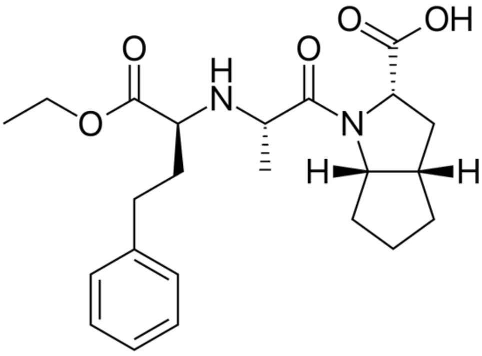

Fig. 38. Chemical structure of ramipril

Materials and methods

Instruments
- analytical balance (Kern 770);
- UV-Vis Spectrophotometer (Hewlett Packard 8453).

Reagents
- ramipril (99.21% reference substance, produced by Lupin, India);
- 0.1 M - 1.0 M hydrochloric acid aqueous solutions;
- 0.1% - 3.0% molybdophosphoric acid aqueous solutions;
- 0.01% sodium lauryl sulphate aqueous solution; Except ramipril, all other reagents were produced by Merck, Germany.

Principle of the method

Ramipril forms with molybdophosphoric acid in hydrochloric acid medium an insoluble compound which can be spectrometrically measured at 361 nm.

In order to establish the optimum wavelength for the detection, 2 mL of the 10 µg/mL working solution were mixed with 1 mL 0.2 M hydrochloric acid and 1% molybdophosphoric acid solution; then, 1 mL 0.01% sodium lauryl sulphate was added in order to prevent precipitation.

The UV-Vis absorption spectrum was recorded using 1 cm cell, after 20 minutes.

In order to establish the optimum working conditions, two solutions of 8 µg/mL and 32 µg/mL were used, while the parameters of the method were changed.

The optimum concentration of the hydrochloric acid solution and molybdophosphoric acid solution was established and the stability of the reaction product was evaluated.

Procedure: 1 mL 1M hydrochloric acid and 1 mL of 2% molybdophosphoric acid solution were added to 1 mL ramipril solution and 1 mL of 0.02% lauril sodium sulphate.

The absorbance was measured at 361 nm versus a blank solution prepared in identical conditions.

Results

The analysis of absorption spectra showed a maximum absorbance for the reaction product at 361 nm. This value was used for all the determinations (figure 39).

The best concentration of the hydrochloric acid was established at 1 M, because when using that solution the maximum absorbance measured at 361 nm had the greatest value (table 43).

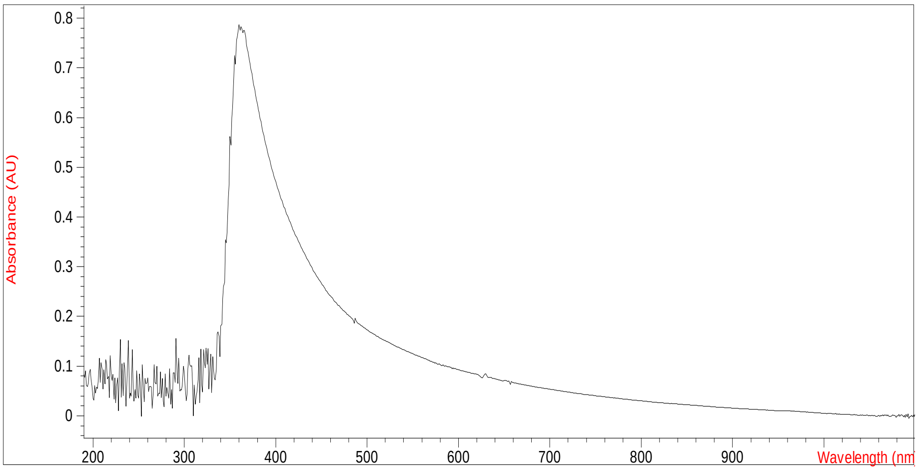

Fig. 39. Spectrum of the reaction product obtained for 32 µg/mL ramipril

The same criterion was used when optimum concentration of molybdophosphoric acid was set to 1% (table 44). By studying the stability of the samples it was established that the chemical reaction between ramipril and molybdophosphoric acid was complete 20 minutes after the last reagent was added. The results proved that absorbance remained almost the same for at least another 20 minutes, sufficient time for the measurements to be performed (table 45).

Table 43. Influence of medium acidity upon absorbance

HCl mol/L	Ramipril (361 nm)	
	8 µg/mL	36 µg/mL
0.50	0.03965	0.28511
0.75	0.08156	0.68221
1.00	0.12535	0.87965
1.25	0.11741	0.86963
1.50	0.09642	0.85211
1.75	0.08952	0.75152
2.00	0.06965	0.62353

Table 44. Influence of reagent concentration upon absorbance

Molybdo-phosphoric acid %	Ramipril (361 nm)	
	8 µg/mL	36 µg/mL
0.5	0.01965	0.29441
1.0	0.06743	0.49145
1.5	0.09021	0.77125
2.0	0.12742	0.88426
2.5	0.11096	0.85522
3.0	0.09832	0.78956
3.5	0.06153	0.68642
4.0	0.04123	0.57426

Table 45. Stability of the reaction product

Time (minutes)	Ramipril (361 nm)	
	8 µg/mL	36 µg/mL
5	0.05221	0.36854
10	0.12459	0.59562
15	0.12632	0.85422
20	0.12642	0.85429
25	0.12742	0.84522
30	0.11821	0.85421
40	0.12742	0.84985
50	0.12655	0.83456
60	0.12502	0.79412

Method validation

Linearity was studied in the 4-40 μg/mL concentration range (figure 40). The obtained data were statistically evaluated (table 46) and the calibration curve was obtained (figure 41).

Table 46. Linearity of the method

Ramipril (μg/mL)	Absorbance (361 nm)				
	Ist Series	IInd Series	IIIrd Series	IVth Series	Average
4	0.07375	0.07155	0.06893	0.07011	0.07108
8	0.12189	0.12524	0.11961	0.11926	0.12150
12	0.24722	0.24149	0.25028	0.24383	0.24571
16	0.35117	0.35107	0.36097	0.31686	0.34502
20	0.45176	0.45650	0.45190	0.45005	0.45255
24	0.56135	0.57117	0.56974	0.56546	0.56693
28	0.69654	0.68455	0.69581	0.67996	0.68922
32	0.78080	0.81412	0.78145	0.81617	0.79814
36	0.88693	0.87143	0.91045	0.89763	0.89161
40	0.89181	0.89302	0.92068	0.90763	0.90329
Correlation and regression coefficients r = 0.9996, r^2 = 0.9992; Standard error = 0.0079; Intercept = – 0.0951; Slope = 0.0277					

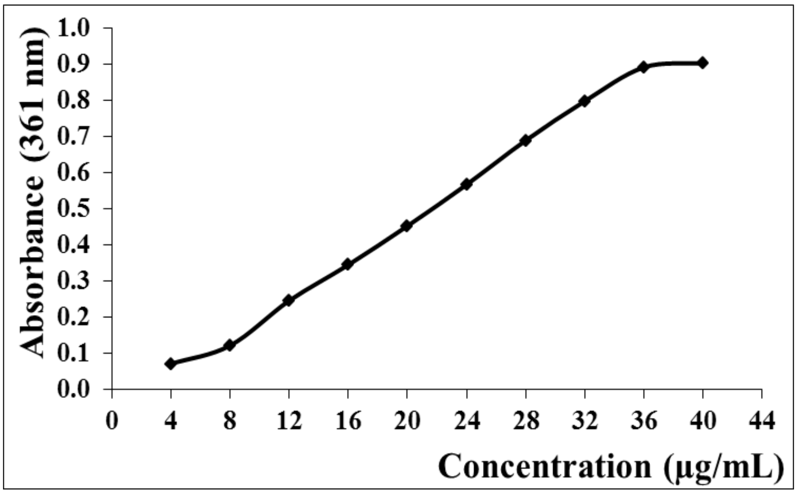

Fig. 40. Method linearity

The calibration curve equation was established (figure 41).

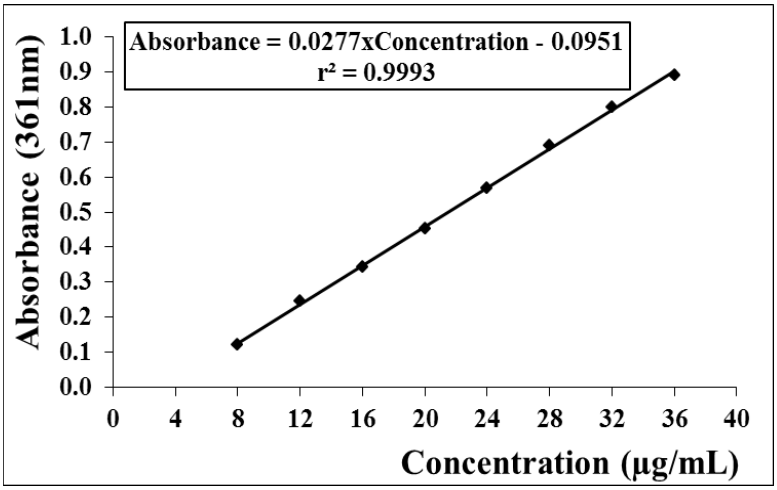

Fig. 41. Calibration curve

Detection and Quantification limits were calculated using the following formulas (85, 93-95):

LOD = 3 x Standard error / Slope = 0.86 µg/mL

LOQ = 10 x Standard error / Slope = 2.88 µg/mL

Precision

For precision determination, three solutions of 16, 20 and 24 µg/mL ramipril were used. Three assays were performed for each concentration. Two sets of assays were performed in different days in order to evaluate the intermediate precision.

Samples concentrations were calculated using the calibration curve equation (table 47).

Accuracy

In order to establish the accuracy of the method, ramipril solutions of 16, 20, and 24 µg/mL were analyzed. For each concentration, three determinations were performed (95).

The concentration of the samples was calculated based on the experimental absorbance values, using the regression curve equation (table 47).

Robustness

This was done by varying the instrument, analyst, and time of study. The analysis was performed on Shimadzu UV-Visible spectrophotometer, model-1700. Interday and intraday analysis was performed by changing the analyst.

Table 47. Precision and accuracy of the method

Concentration (µg/mL)	Precision		Intermediate precision		Accuracy	
	Real concentration (µg/mL)	RSD %	Real concentration (µg/mL)	RSD %	Real concentration (µg/mL)	Recovery (%)
16	16.2282	0.72	16.2877	0.97	15.8040	98.78
	15.9959		16.0248		15.8947	99.34
	16.0851		16.3095		16.1650	101.03
20	19.8079	0.77	19.6981	0.16	19.4260	97.13
	19.7111		19.7602		19.8000	99.00
	20.0120		19.7133		19.7427	98.71
24	23.9530	0.76	23.6412	0.40	23.6228	98.43
	23.6286		23.8349		23.9794	99.91
	23.6503		23.7532		23.4675	97.78
					Mean	98.90

Discussions

According to the experimental data, the developed method for ramipril determination was linear in the concentration range of 8-36 µg/mL (figure 40 and table 46), with a correlation coefficient of $r = 0.9996$, detection limit of 0.86 µg/mL, quantification limit of 2.88 µg/mL below the lowest concentration of the linearity range, method precision RSD% = 0.35%, and accuracy evaluated by recovery, R = 98.90%.

When we compared this concentration range with that of other published methods we found out that this new method has the following advantages: it does not involve rare or complex reagents, nor does it involve the use of toxic solvents. It is simple and easy to perform (91, 92).

We found that for each set of data and for both sets together the relative standard deviation was lower than 2%. This fact proved that the proposed method was precise.

A robustness study was performed to establish system suitability in order to ensure the validity of the analytical procedure. Reproducibility of the results confirmed the robustness of the method. These values prove that the proposed method was accurate (table 47).

Conclusions

A UV-VIS molecular absorbance spectrophotometric method was developed for the assay of ramipril using molybdophosphoric acid in hydrochloric acid medium. The reaction product showed a maximum absorbance at 361 nm.

The proposed method is simple, easy to perform, sensitive, linear, precise, accurate and robust.

We found that recovery was 100.59% for the studied concentration range, mean 98.90% (minimum 98.51% and maximum 102.39%), and the relative standard deviation was under 2% (RSD = 1.42 %).

I.2.3. UV-Vis Spectrophotometric analysis of bisoprolol

Bisoprolol fumarate can be quantitatively determined by various methods such as UV spectrophotometry, HPLC, HPTLC, RP-HPLC (97-100). Actually, no method has been reported for the quantitative determination of bisoprolol by visible region spectrophotometry.

This study presents a new spectrophotometric method for the assay of bisoprolol using tropaeolin 00 as reagent and the developed method was validated (93, 94, 100).

Materials and methods

Absorbance was measured in quartz cuvettes using a Hewlett Packard 8453 UV–VIS spectrophotometer while maintaining the temperature at 25^0C. Only analytical grade chemicals were used as reagents, such as: bisoprolol fumarate (100.07% pure substance provided by Unichem Laboratories LTD, India), hydrochloric acid (Tunic Prod, Romania), dichloromethane (Fluka, Germany); tropaeolin 00 (Tunic Prod, Romania).

A 100 μg/mL stock bisoprolol solution was then diluted to obtain standard solutions of various concentrations.

Assay procedure: 1 mL 0.05 M hydrochloric acid and 1 mL 0.01 % (w/v) tropaeolin 00 aqueous solution were added to each 1.0 mL of bisoprolol fumarate solution with a concentration in between 5-30 μg/mL.

The complex was then extracted using dichloromethane. Fifteen minutes later the absorbance was measured at 412 nm, using as reference a blank sample prepared in the same conditions.

Results

Analyzing the absorption spectra shown in figure 42, the wavelength of maximum absorption was established at 412 nm. It was used for all the measurements**.**

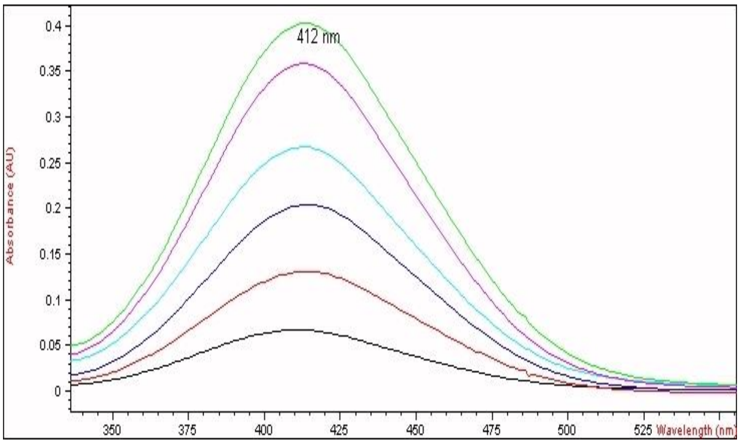

Fig. 42. Absorption spectra of reaction product against reagent blank

The optimal concentration of the tropaeolin 00 aqueous solution was found to be 0.01% (w/v) and 0.05 M hydrochloric acid was used with best results according to the data shown in table 48.

Table 48. Reagents concentration

Tropaeolin 00 (%)	Bisoprolol concentration		Hydrochloric acid (mol/L)	Bisoprolol concentration	
	5 µg/mL	30 µg/mL		5 µg/mL	30 µg/mL
	Absorbance	Absorbance		Absorbance	Absorbance
0.001	0.0568	0.4025	0.005	0.0698	0.4103
0.005	0.0572	0.4105	0.01	0.0702	0.4121
0.01	0.0627	0.4112	0.05	0.0712	0.4131
0.05	0.0712	0.4129	0.1	0.0710	0.4125
0.1	0.0702	0.4105	0.5	0.0698	0.0411
0.5	0.0697	0.4102	1	0.0692	0.4023

The complex formation reaction of bisoprolol and tropaeolin 00 was found to be finished after 5 minutes. The absorbance was measured after 15 minutes have passed since dichloromethane extraction according to the experimental data from table 49.

Table 49. The study of the stability of the complex

Bisoprolol concentration (µg/mL)		Time (minutes)						
		10	15	20	25	30	40	60
5	Absorbance	0.0705	0.0716	0.0712	0.0706	0.0685	0.0682	0.0682
30		0.4102	0.4125	0.4123	0.4112	0.4053	0.4026	0.3925

Method validation

Linearity was assessed by analyzing the obtained data shown in table 50 by linear regression and the calibration curve from figure 43 was obtained.

Table 50. Linearity determination

Bisoprolol concentration (µg/mL)	Absorbance						Mean
	I	II	III	IV	V	VI	
5	0.0703	0.0698	0.0705	0.0697	0.0680	0.0620	0.0684
10	0.1361	0.1354	0.1405	0.1357	0.1372	0.1380	0.1371
15	0.1998	0.2096	0.2013	0.2087	0.2008	0.1979	0.2014
20	0.2698	0.2711	0.2654	0.2685	0.2701	0.2685	0.2689
25	0.3430	0.3461	0.3397	0.3413	0.3453	0.3431	0.3431
30	0.4119	0.4099	0.4120	0.4099	0.4135	0.4198	0.4128

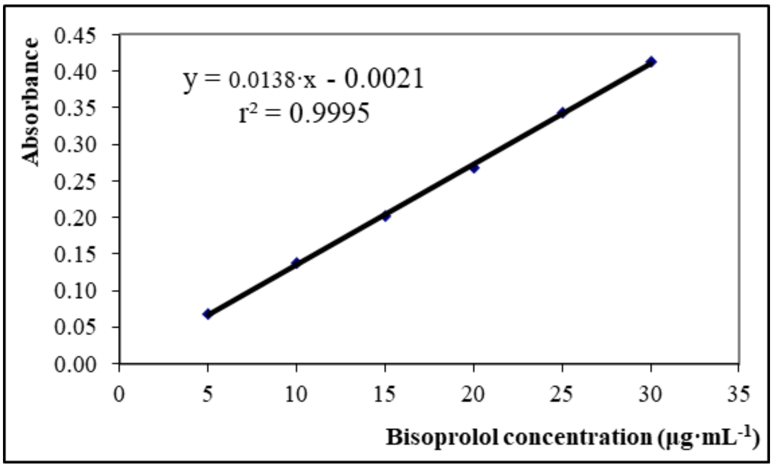

Fig. 43. Calibration curve

The parameters obtained for the validation of the method are summarized in table 51.

The parameters obtained for the precision of the method (table 52) and for the accuracy of the method (table 53) have demonstrated a precise and accurate analytical method.

Table 51. Method validation summary

Parameter	Values
Wavelength (nm)	402
Linearity domain (μg/mL)	5-30
Limit of detection (μg/mL)	0.67
Limit of quantification (μg/mL)	2.23
Regression equation	Y= a · X – b where: Y= absorbance X= concentration
Intercept (a)	0.0138
Slope (b)	0.0021
Correlation coefficient (r)	0.9997
Standard deviation	0.002866117

Table 52. Precision

Bisoprolol concentration (µg/mL)	Repeatability		Reproducibility	
	Absorbance	Recovery %	Absorbance	Recovery %
10	0.1381	100.39	0.1373	101.01
10	0.1375	101.47	0.1380	101.52
10	0.1376	101.54	0.1369	100.72
15	0.2078	101.71	0.2081	101.54
15	0.2068	101.23	0.2105	102.27
15	0.2081	101.86	0.2068	100,91
20	0.2749	100.67	0.2753	100.51
20	0.2721	99.66	0.2723	99.42
20	0.2758	101.00	0.2746	100.25
Statistical data	Mean = 101.05 SD = 0.71 RSD = 0.70		Mean = 100.95 SD = 0.87 RSD = 0.86	

Table 53. Accuracy

Theoretical concentration (µg/mL)	Absorbance	Calculated concentration (µg/mL)	Recovery %
10	0.1374	10.14	101.41
	0.1367	10.06	100.60
	0.1353	9.98	99.87
11.25	0.1538	11.30	100.44
	0.1525	11.21	99.64
	0.1519	11.16	99.20
15	0.2081	15.28	101.88
	0.2047	15.03	100.23
	0.2026	14.88	99.23
18.75	0.2588	18.91	100.85
	0.2548	18.62	99.31
	0.2574	18.81	100.32
Statistical Data		Mean	100.30
		Min	99.20
		Max	101.88

Discussions

The influence of the concentration of reagents upon maximum absorption was investigated (table 48).

While studying the precision of the method, for all sets of data shown in table 52, the relative standard deviation was lower than 2% (RSD = 0.78), which proved that the proposed method was precise.

It was established that the recovery for the studied concentration range was in between 99.20% and 101.88% and the mean was 100.3%, so the proposed method was accurate (table 53).

After that, a spectrophotometric method for the quantitative determination of bisoprolol was developed by our group, based on the formation of an ion pair complex between bisoprolol and methyl orange, in acidic medium, extractable in dichloroethane, with a maximum absorbance at 427 nm. The LOD was 0.20 μg/mL and the LOQ was 0.66 μg/mL. The elaborated method was validated and applied with good results in pharmaceutical preparations (101).

Later on, a new Vis spectrophotometric method was developed for the assay of bisoprolol in pharmaceutical preparations using bromocresol green in hydrochloric acidic medium. The reaction product showed a maximum absorbance at 402 nm proportional with the concentration of bisoprolol. The optimum conditions for the reaction were established. The developed method was validated. The method showed a good linearity in the concentration range of 7 – 80μg/mL (correlation coefficient r = 0.9998). The LOD was 1.78 μg/mL and the LOQ was 5.41 μg/mL. The precision and the accuracy were determined; mean recovery was 100.11% in the 98.35-101.57% concentration range (102).

On the other hand, a simple, rapid and sensitive method for the spectrophotometric determination, of bisoprolol was developed by other researchers. The proposed methods were based on the charge-transfer reactions of bisoprolol, as n-electron donor, with 7,7,8,8-tetracyanoqumodimethane (TCNQ) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) as π-acceptors to give highly colored complexes. The proposed methods were validated according to the ICH guidelines with respect to linearity, limit of detection, limit of quantification, accuracy, precision, recovery and specificity. Beer's law is obeyed over the concentration ranges of 10-60 and 10-80 μg/mL bisoprolol with TCNQ and DDQ, respectively. The proposed methods were successfully applied to the assay of bisoprolol in pharmaceutical preparations (103). But the reagents used by our team (tropaeolin 00, methyl orange, bromocresol green) are more suitable and easier to find in any laboratories.

Conclusions

This study describes the successful development of a simple and new spectrophotometric method for the determination of bisoprolol fumarate.

The proposed method is based on the formation of a colored complex combination with tropaeolin 00.

The method was validated by determining the following parameters: linearity range 5-30 μg/mL, r = 0.9997, r^2 = 0.9997, LOD = 0.67 μg/mL, LOQ = 2.23 μg/mL, precision (RSD = 0.78 %) and accuracy (100.3 % mean recovery).

All analytical reagents used are inexpensive, quite stable and widely available in analytical laboratory; complex procedures are not required and the method is suitable for routine analysis in quality control laboratories.

I.3. Development of some Atomic Absorption Spectroscopy (AAS) analytical methods

As per United States Federal Food And Drug Administration (USFDA), the pharmaceutical products/compounds need to be completely characterized. The metals were found in all phases of the drug development process, from drug synthesis to quality control, metals are used in the synthesis of drug substances, as an excipients in tablets, capsules, liquids and metal based products are used as imaging agents and the equipment used in the manufacturing of drug substances or compounds can be a source for the trace metals. Various atomic absorption based techniques like AAS are often used to help for fully characterization of the pharmaceutical products (104).

Atomic spectrometry enables the determination of drugs with higher sensitivity and accuracy. The method is free from interference by excipients present in the drug formulations (105). A wide variety of metals and metalloids are used in the manufacture of pharmaceuticals and some are also used as the active pharmaceutical ingredient in drug products. Palladium, platinum are commonly used catalysts in the pharmaceutical industry. Because of the potential routes of entry for metals and metalloids into pharmaceutical products, the pharmaceutical industry is interested in monitoring elements at all stages of the development process (106).

Published papers in the field

♦ Apostu Mihai, Țântaru Gladiola, Vieriu Mădălina, **Bibire Nela**, Panainte Alina Diana. Study of the Presence of Lead in a Series of Foods of Plant Origin. *Rev Chim (Bucharest)* 2018; 69(5): 1223-1225.
♦ Mihai Apostu, Gladiola Țântaru, Mădălina Vieriu, Alina Diana Panainte, **Nela Bibire**, Luminița Agoroaei. Evaluation of in Vitro Reducing Effect of Several Vegetable Extracts on the Digestive Bioavailability of Heavy Metals. *Rev Chim (Bucharest)* 2017; 68(4): 683-687.
♦ Luminița Agoroaei, **Nela Bibire**, Mihai Apostu, Monica Strugaru, Ioana Grigoriu, Elena Butnaru. The Content of Heavy Metals in Tobacco of Commonly Smoked Cigarettes in Romania. *Rev Chim (Bucharest)* 2014; 65(9): 1026-1028.

I.3.1. AAS analysis of some heavy metals from tobacco

Introduction

The composition of tobacco, as well as tobacco smoke, is complex, that gather a large number of substances with major toxicological implications, many incompletely studied.

In 2003, one in 10 deaths was related to tobacco comsumption, while in 2008, according to WHO data, one person loosing life every 6 s due to tobacco related illnesses (107). About 4000 chemicals (inorganic and organic) have been identified in tobacco (107-109). Among the inorganic components, following metals have been detected: Cd, Pb, Cr, Ni, As, Hg, Co, Cu, Zn, Fe, Mn, Bi, Ce (109, 110-112).

Tobacco smoking is an important source of heavy metals in both human body and environment (107). The heavy metals are toxic in low levels, being easily assimilate in the human body during smoking. Exposure to metals through tobacco depends on the amount of metal present in tobacco, a percentage that is transferred to the tobacco smoke and the percentage that is absorbed (107). The heavy metals of tobacco are responsible for carcinogenicity (especially lung cancer) (Cd, Pb, Cr and Ni), peripheral arterial disease, genetic disorders etc. (107, 108). Many researchers demonstrate the role of reactive oxygen species in the toxicity of heavy metal ions (107).

The level of heavy metals in tobacco vary within wide limits and depends on (107, 111, 112):

- ✓ plant variety;
- ✓ development stage of the plant;
- ✓ physico-chemical attributes of soil – influence the mobilization and the transfer of metals from soil into the plant:
    - pH - the acidic pH (5.5-6.5) favors a better absorption of metals in plant (the increase of pH at least one unit by alkalinization of soil with $CaCO_3$ may result in a 50% reduction of Cd level in leaves);

- redox conditions - affects the mobility of oxidised metals;

- ✓ interactions between metals in the absorption from soil (absorption of cadmium is stimulated in the presence of lead);
- ✓ agricultural practices – the use of fertilizers (responsible for large amounts of As, Hg, Pb, Cd, Cr, Po);
- ✓ precipitations;
- ✓ technological processing of tobacco (which enriches the metal content in leaves);
- ✓ type of product (cigarettes, cigars, chewing tobacco, snuff, etc.).

Tobacco plant accumulates heavy metals in the order of leaves > roots > stems (107).

In general, tobacco plants accumulate heavy metals like Cd, Pb, and Zn, preferentially. Cadmium is the most absorbed heavy metal, so it is the most studied metal from tobacco plant, the toxicity of the metal being an important reason too (107, 113).

Materials and methods

Six heavy metals potentially toxic, Cd, Pb, Cr, Ni, Cu and Zn, were determined in tobacco of 15 cigarette varieties (brands) from market, commonly smoked in Romania.

After removing filter and paper, 2.5 g of homogenized tobacco was mineralized with 20 mL extra pure nitric acid (65%, 1.4 g/mL) by heating on a sandbath at 150^{o}C until total digestion. The metals were determined through atomic absorbtion spectrophotometry (Buck Scientific 200A Flame Atomic Absorption Spectrophotometer).

Quality control of determinations was achieved by measuring known standard solutions of heavy metals covering the range of concentrations found in samples. The results were expressed in µg/g tobacco dried at 105^{o}C.

Results

In table 54, the concentrations of heavy metals in tobacco of analyzed cigarettes are presented. Table 55 contains the limits and the mean values of these concentrations. Mean concentrations recorded in the 15 tobacco varieties is 1.06 µg Cd/g tobacco, which is close to the value recorded in Germany in 1998 (1.0 µg/g) (table 56).

Table 54. The content of heavy metals from tobacco of analyzed cigarettes

Cigarette brand (sample)	µg/g tobacco dried at 105^{o}C					
	Cd	Pb	Cr	Ni	Cu	Zn
I	1.00	3.44	3.74	0.79	8.77	1.28
II	1.23	4.60	2.81	0.79	9.89	2.23
III	1.39	5.18	6.33	1.04	11.13	2.70
IV	0.80	4.77	6.81	0.96	7.41	1.91
V	1.55	2.73	2.23	0.94	12.43	3.04
VI	1.58	4.71	2.88	0.81	9.56	2.57
VII	0.71	3.97	3.24	1.07	6.96	1.80
VIII	0.80	4.77	4.86	0.82	7.41	1.21
IX	1.11	4.76	5.82	1.09	14.21	5.47
X	0.94	3.51	3.81	0.94	8.94	2.38
XI	1.16	3.72	6.06	1.00	10.66	3.36
XII	0.72	4.30	7.01	0.74	10.27	2.08
XIII	0.76	2.26	1.85	0.78	9.74	2.08
XIV	1.20	4.81	4.90	0.96	6.90	1.68
XV	0.98	4.01	3.27	0.92	7.67	2.07

Table 55. Minima, maxima, median and mean values of the heavy metals concentrations from tobacco

Value	µg/g tobacco dried at 105°C					
	Cd	Pb	Cr	Ni	Cu	Zn
Minima	0.71	2.26	1.85	0.74	6.90	1.21
Maxima	1.58	5.18	7.01	1.09	14.21	5.47
Median	1.05	4.15	3.81	0.93	9.65	2.15
Mean±SD	1.06±0.28	4.10±0.84	4.37±1.71	0.91±0.11	9.46±2.09	2.39±1.03

SD - standard deviation

Mean concentration of lead, 4.10 µg/g, is close to the value recorded in China in 1998 (4.48 µg/g); it is a high value compared to the concentrations reported by other countries (table 56).

The mean value, 4.37 µg Cr/g tobacco, is higher than the dates identified in the literature (table 56).

Mean concentrations of nickel, 0.91 µg/g, is the lowest average recorded in our study (table 56).

The mean value, 9.46 µg Cu/g tobacco, is close to the concentration level registered in Germany in 1993 (table 56).

The mean value of 2.39 µg Zn/g are much smaller than similar concentrations reported in the literature (table 56).

In table 56, the concentrations of the six metals from tobacco reported in the literature by different country are presented (107, 111).

Table 56. Metal concentration levels from tobacco reported by different country

Country	µg/g tobacco					
	Cd	Pb	Cr	Ni	Cu	Zn
USA (1997)	0.98	-	-	-	-	35.1
Russia (1998)	1.11	1.86	0.88	-	-	-
Germany (1993)	-	-	-	2.4	9.70	-
Germany (1998)	1.00	1.62	1.26	-	-	36.3
Finland (1986)					15.6	50
Italy (1989)	-	7.39	-	-	-	-
Yugoslav (1978)					18.9	-
Hungry (1995)	1.89	1.17	-	5.4	-	57.6
Poland (2008)	0.61	0.56	-	-	-	-
Turkey (2001)	-	-	1.63	0.22	-	-
China (1998)	2.22	4.48	0.46	-	-	-
China (2005)	0.18	0.64	-	2.23	4.13	-
India (2010)	0.45	1.94	4.07	8.79	14	27
Pakistan (2008)	-	14.35	-	-	-	-
Egypt (1999)						76.8-190
Jordan (2005)					12.90	55.62
Mexico (1991)	4.41	-	-	-	-	-

Table 57 presents the distribution of the first three elevated concentrations of metals in tobacco samples. Related to this criteria, we estimate that tobacco sample IX is the most contaminated (recording maximum concentrations for nickel, copper and zinc), followed by tobacco samples III and V.

Table 57. The distribution of the higher concentration levels of heavy metals from tobacco

Cigarette brand (sample)	Cd	Pb	Cr	Ni	Cu	Zn
I						
II						
III	xxx	x	xxx	xxx	xxx	
IV			xx			
V	xx				xx	xxx
VI	x					
VII				xx		
VIII						
IX		xxx		x	x	x
X						
XI						xx
XII			x			
XIII						
XIV		xx				
XV						

the first three elevated values of concentrations (in descending order): x – the maxima value; xx – the second higher value; xxx – the third higher value.

Discussions

Cadmium is an irritant, thiol depletion inducer and carcinogen toxic (104, 114, 115). It is considered that 40-60% of cadmium inhaled from tobacco smoke goes directly into the blood stream (it is volatile at 320°C) (107).

A cigarette contains 1-2 µg Cd, of this 0.1-0.2 µg are inhaled; smoking more than 20 cigarettes daily can increase Cd concentration in body by ten-folds (105). The content of cadmium in fat tissue of male smokers can be higher (four times) than of non-smokers (116).

The lowest level of cadmium was recorded in sample VII (0.71 µg/g), followed closely by sample XII (0.72 µg/g) and sample XIII (0.76 µg/g), while the maximum concentration of cadmium was found in tobacco sample VI (1.58 µg/g), followed by tobacco sample V (1.55 µg/g) and sample III (1.39 µg/g) (table 56).

Lead is a blood toxic (causes inhibition of hemoglobin biosynthesis, anemia), neuronal (produces encephalopathy), vascular, renal, and carcinogen toxic. Smokers have significantly higher blood lead levels than non-smokers (107, 108, 114).

In tobacco brand XIII a concentration of 2.26 µg Pb/g was recorded; this minimum value is followed, in ascending order, by the value 2.73 µg Pb/g (V) and 3.44 µg Pb/g (I). The highest concentration in lead, 5.18 µg/g, was determined in sample III; two other samples with high levels were registered for cigarette brands XIV (4.81 µg/g) and IX (4.76 µg/g) (table 56).

Chromium is a methemoglobin inducer, an irritant, allergenic and carcinogen agent (lung, sinonazal cavity), and genotoxic. In 1979 were reported limits from 0.24 to 14.6 µg Cr/g tobacco (107, 114, 117, 118).

The least contaminated with chromium were tobacco samples XIII (1.85 µg/g), V (2.23 µg/g), and II (2.81 µg/g), while the most contaminated were samples XII (7.01 µg/g), IV (6.81 µg/g) and III (6.33 µg/g) (table 56).

Nickel is a toxic of CNS and myocardium, sensitization and carcinogen (in lungs) agent. It is considered that nickel may be combined with carbon monoxide in tobacco smoke of the primary current, with formation of the $Ni(CO)_4$. The smoke of a cigarette may contain up to 50 µg Ni, of which 1.1% is found in the primary current (107, 114, 117, 119, 120).

In the analyzed samples, the lowest level of nickel was recorded in tobacco sample XII (0.74 µg/g), while highest concentration in sample IX (1.09 µg/g). Other tobacco samples with high levels of nickel are VII (1.07 µg/g) and III (1.04 µg/g); the mean determined in our study (0.91 µg/g) is less than levels found in literature (table 56), except the value of 0.22 µg Ni/g tobacco, reported in Turkey, in 2001.

Copper is a hemolytic and methemoglobin inducer toxic (in high doses) (108). In analyzed tobacco samples, copper concentrations ranged between 6.90 µg/g (XIV) and 14.21 µg/g (IX), being the highest concentrations recorded in our study (table 56).

Zinc is a central nervous system, cardiovascular and muscle toxic, but, as in the case of copper, manifests toxicity in high doses; copper and zinc are both oligoelements and, therefore, normally present in the human body in certain limits (114).

The lowest level of zinc was recorded in tobacco of cigarettes brand VIII (1.21 µg/g), while the highest value was determined in tobacco of sample IX (5.47 µg/g). These values are much smaller than similar concentrations reported in the literature (table 56).

Conclusions

Six heavy metals potentially toxic have been determined in tobacco of 15 varieties (brands) of cigarettes from market, commonly smoked in Romania.

Reported to the mean values of metal concentrations in tobacco of analyzed cigarettes, the highest level was recorded in copper, followed in descending order by chromium, lead, zinc, cadmium, nickel.

The tobaccoes most contaminated with heavy metals (relative to the first three elevated metal concentrations recorded) were registered to the cigarette brands: IX > III > V.

The study confirms the ability of tobacco plant to retain heavy metals into the leaves.

We consider that the evaluation of heavy metal contamination of cigarette tobaccoes smoked in Romania needs more detailed studies.

I.3.2. AAS analysis of lead in some foods of plant origin

Introduction

The toxicity of heavy metals is a less common medical condition, yet clinically relevant, which improperly treated may result in significant morbidity and mortality. Some heavy metals in small quantities are essential for the body in different biochemical processes but become toxic at higher levels (121-123). Heavy metal poisoning may harm almost all organs. The severity of the damage depends on the metal involved, the pathway they enter the body, the age of the individual and the level of toxicity (124). Heavy metals may enter the body orally, through ingestion of contaminated food (125-127) and water (128, 129).

Lead is a heavy metal that produce toxic effects after accumulation of more than 30 µg per deciliter of blood in subjects over age 15 (130, 131). Its accumulation may cause disruption of hemoglobin synthesis (132, 133), kidney, gastrointestinal tract and reproductive system damage (134-137), plus acute or chronic effects on the nervous system (138, 139).

Given the high toxicity of this element, this study aims to estimate the lead content in different types of food of plant origin (140).

Materials and methods

The conducted studies required the development and validation of a method for the quantitative determination of lead using atomic absorption spectroscopy (AAS). The foods analyzed were wheat, wheat flour, onion, corn, corn flour, carrot, beans, potatoes and cabbage grown in three geographical areas (113, 141-144).

The study was carried out on an Analytik Jena ContrAA 300 apparatus equipped with an air-acetylene flame and a high-resolution continuum source. The procedure characteristics were optimized according to the manufacturer's recommendations (table 58).

Table 58. Procedure characteristics

Metal	Pb
λ_{max} (nm)	217.00
Burner (mm)	50
Flame type	C_2H_2/air
Acetylene/air flow (L/h)	65

All reagents used were analytical grade, and the glassware was pretreated with 10% HNO_3 and rinsed with double-distilled water to prevent contamination. All necessary solutions used during determinations were prepared by diluting a 1.0 g/L Pb^{+2} commercial standard solution (Fluka, Germany) with double-distilled water and stored in polypropylene bottles. The most important validation parameters of the AAS analysis method of Pb^{+2} are presented in table 59, and the calibration curve can be observed in figure 44. LOD and LOQ were calculated based on the standard deviation and the slope of the regression line.

Table 59. Validation parameters

Cation	Pb^{+2}
Linearity range	2.0-10.0 µg/mL
Regression equation	$y = 0.0147x + 0.0067$
Correlation coefficient (R^2)	0.9991
Slope	0.0147 µg/mL
Standard deviation (σ)	0.001578
LOD	0.3220 µg/mL
LOQ	1.0734 µg/mL

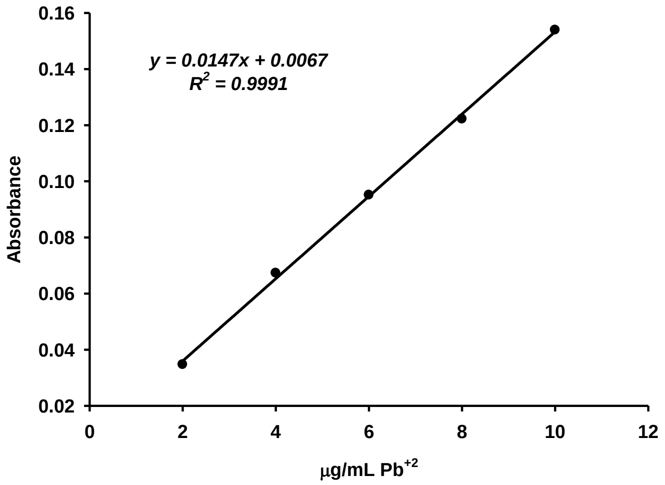

Fig. 44. Calibration curve

Digestion of the vegetal material: 20 g of vegetal material was mixed with 10 mL of concentrated HNO_3 and 2.5 mL of 70% $HClO_4$ and allowed to rest for 24 h. The resulting mixture was heated on a water bath until complete mineralization. After cooling, double-distilled water was added and then it was heated to remove the nitrous vapor and to reach a low volume (0.5-1 mL). When the solution darkened or became yellow during disaggregation, a few drops of HNO_3 or $HClO_4$, respectively, were added. Then 5 mL double-distilled water and 10 mL of 5M HCl solution were added to the solution, and its volume was reduced again to 10 mL. In the end it was filtered and double-distilled water was added up to 50 mL.

Results

The results obtained and the calculated recovery are shown in table 60. The analysis of the results emphasized a recovery in between 97.50% and 103.00%.

Table 60. Accuracy of Pb^{+2} determination in foods

N°	Sample	µg Pb^{+2} added (a)	µg Pb^{+2} found (b)	b - a subtraction	Recovery (%)
1.	wheat	-	2.12	-	-
2.	wheat	1.00	3.14	1.02	102.00
3.	onion	-	2.02	-	-
4.	onion	1.00	3.05	1.03	103.00
5.	corn	-	2.60	-	-
6.	corn	2.00	4.55	1.95	97.50
7.	carrot	-	2.50	-	-
8.	carrot	2.00	4.54	2.04	102.00
9.	beans	-	2.10	-	-
10.	beans	3.00	5.08	2.97	99.00
11.	potatoes	-	2.25	-	-
12.	potatoes	3.00	5.30	3.05	101.66
13.	cabbage	-	2.00	-	-
14.	cabbage	4.00	5.98	3.98	99.50

Table 61. Pb^{+2} levels as µg/1000 g plant products from levigant soil

N°	Sample	Dry product	Fresh product
1.	wheat	90.2	79.0
2.	wheat flour	126.0	114.0
3.	onion	90.0	10.9
4.	corn	135.1	110.0
5.	corn flour	76.2	57.7
6.	carrot	92.5	49.9
7.	beans	102.5	90.0
8.	potatoes	86.6	12.9
9.	cabbage	68.0	57.2

Table 62. Pb^{+2} levels as µg/1000 g plant products from meadow soil

N°	Sample	Dry product	Fresh product
1.	wheat	136.6	134.0
2.	wheat flour	142.3	127.1
3.	onion	45.7	10.2
4.	corn	153.0	123.5
5.	corn flour	146.2	133.4
6.	carrot	109.0	33.5
7.	beans	137.2	104.6
8.	potatoes	63.8	13.9
9.	cabbage	97.5	85.8

Table 63. Pb^{+2} levels as µg/1000 g plant products from forest brown soil

N°	Sample	Dry product	Fresh product
1.	wheat	185.6	154.7
2.	wheat flour	197.2	161.2
3.	onion	59.0	10.2
4.	corn	202.3	164.0
5.	corn flour	184.6	150.0
6.	carrot	115.0	44.7
7.	beans	158.7	130.0
8.	potatoes	72.2	17.2
9.	cabbage	109.0	83.6

The average values of Pb^{+2} content as µg/1000 g of fresh vegetable product, by soil and food type, were plotted in figure 45 (1 - corn, 2 - wheat flour, 3 - wheat, 4 - corn flour , 5 - beans, 6 - cabbage, 7 - carrot, 8 - potatoes, 9 - onion).

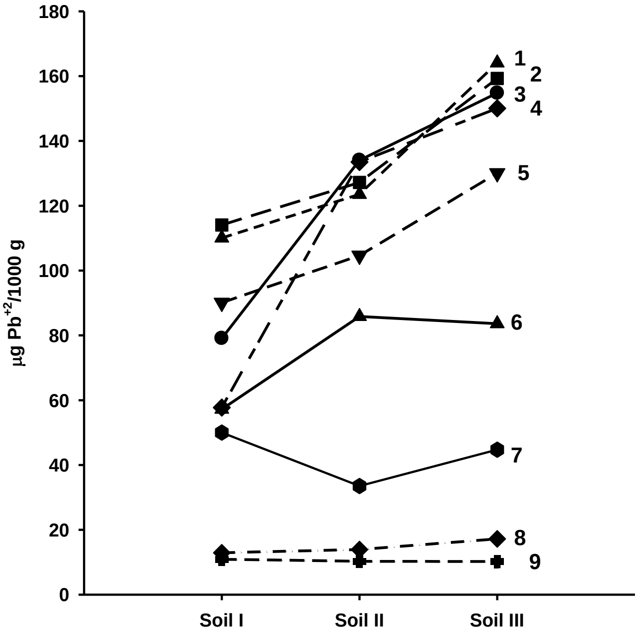

Fig. 45. The average values of Pb^{+2} levels

Discussions

The accuracy of the assays, expressed as the consistency between the real value and the analytical result, was verified using the standard addition method.

For the assay of Pb^{+2} levels in foods, the following types of foods were analyzed: wheat, wheat flour, onion, corn, corn flour, carrot, beans, potatoes, cabbage.

The results of the determinations were expressed as µg Pb^{+2}/1000 g of plant product (weighted arithmetic mean).

Food products were sampled from various localities in three areas, grouped by soil types:

- levigant soil in Moldavian Plain (soil I): Fântânele (Iaşi), Plopeni (Suceava), Havârna (Botoşani), Dorohoi (table 61);
- meadow soil in Siret and Barlad Valley (soil II): Mărășești, Bacău, Pașcani, Focșani, Tecuci, Roman, Siret (table 62);
- forest brown soil in sub-Carpathian area and Suceava Plateau (soil III): Piatra Neamț, Târgu Neamț, Huși, Vlădeni (Iași) (table 63).

Commission Regulation (EC) No 1881/2006 - Consolidated acts, recommends the maximum permitted level of Pb^{+2} in foods: 0.20 mg Pb^{+2}/1000 g fresh beans and cereals (wheat, corn), 0.10 mg Pb^{+2}/1000 g fresh onion, carrot, potatoes and 0.30 mg Pb^{+2}/1000 g fresh vegetables of the *Brassicaceae* Family (cabbage) (145). None of the foods studied exceeded those recommended limits.

Conclusions

In this study it was established that the assimilation of Pb^{+2} by plant foods was very variable, even for the same type of food. Just as it was stated in literature, it varied based on the geochemical conditions, soil pollution, local level of industrialization, etc.

Pb^{2+} levels in fresh product, had the highest values for foods harvested from forest brown soil and meadow soil (except for carrots) while the lowest levels were found in levigant soils.

Out of all the products studied, cereals and beans contained the highest Pb^{+2} levels, unlike carrots, potatoes and onions that assimilated the least amount of cation.

I.4. Development of some Ion-Selective Membrane Electrodes (ISE)

The potentiometric membrane sensors have been widely used in pharmaceutical analysis. This is mainly because of low cost, simple design, wide linear concentration range, low detection limit, adequate selectivity, high accuracy, and applicability of the selective electrodes to colored and turbid solutions. The ion-selective electrodes (ISE) have been widely used in recent years in the analysis of drugs in pure powder form and in their pharmaceutical formulations.

The field of applications was broadened by the introduction of liquid ion-exchanger membranes, membranes containing electroneutral macrocyclic compounds, enzyme electrodes and gas sensors. Such new electrode materials facilitated the development of potentiometric sensors for most of the important inorganic ions and several types of organic compounds, many of which are of ionic character.

The ISE are based on selecting an appropriate matter which works like a sensing part. The sensing materials are the main component of the potentiometric ion sensors. They are responsible for forming a type of discrimination in the electrode behavior toward one ion rather than others. There are several types of the sensing material. They might have host-guest structure, which allows the inclusion of the analyzed ionic material. Another type is able to form a complex with the analyte ion. The third type is based on ion exchange reaction with the ion under investigation (146-148).

Published papers in the field

♦ Apostu M, Hăncianu M, Țântaru G, Vieriu M, **Bibire N**, Panainte ADP. Electrochemical Sensors with Pharmaceutical Applications Based on Polymer Inclusion Membranes Containing Phosphomolybdic Acid Complexes. *Farmacia* 2018; 66(4): 587-591.

♦ Mihai Apostu, Gladiola Țântaru, Mădălina Vieriu, Alina Diana Panainte, Nela Bibire, Luminița Agoroaei. Evaluation of in Vitro Reducing Effect of Several Vegetable Extracts on the Digestive Bioavailability of Heavy Metals. *Rev Chim (Bucharest)* 2017; 68(4): 683-687.

♦ Mihai Apostu, **Nela Bibire**, Gladiola Țântaru, Mădălina Vieriu, Alina Diana Panainte, Luminița Agoroaei. Ion-Selective Membrane Electrodes for the Determination of Heavy Metals. Construction, Characterization and Applications. *Rev Chim (Bucharest)* 2015; 66(5): 657-659.

♦ Mihai Apostu, **Nela Bibire**, Mădălina Vieriu, Alina Diana Panainte, Gladiola Ţântaru. Preparation and Potentiometric Study of Ranitidine Hydrochloride Selective Electrodes and Applications. *Rev Chim (Bucharest)* 2013; 64(7): 781-784.

♦ Mădălina Vieriu, **Nela Bibire**, Gladiola Țântaru, M. Apostu, Mariana Mândrescu, V Dorneanu. Construcția si caracterizarea unui electrod membrană-selectiv pentru determinarea lisinoprilului. *Rev Med Chir Soc Med Nat Iași* 2010; 114(4): 1227-1231.

♦ Mihai Apostu, **Nela Bibire**, Gladiola Ţântaru. Construction and Characterisation of a Membrane Selective for Ranitidine Hydrochloride. *Farmacia* 2008; 56(2): 147-153.

♦ **Nela Bibire**, V. Dorneanu. Electrozi membrană-selectivi în analiza medicamentelor. Nota II. *Rev Med Chir Soc Med Nat Iași* 2005; 109(2): 426-430.

♦ **Nela Bibire**, Gladiola Țântaru, Maria Stan, M. Apostu. Electrod selectiv cu membrană lichidă pentru determinarea cantitativă a atropinei. *Farmacia* 2004; LII(5):

103-109.

♦ **Nela Bibire**, Gladiola Ţântaru,V. Dorneanu, Maria Stan, M. Apostu. Electrod membrană-selectiv pentru determinarea cantitativă a atropinei. *Rev Med Chir Soc Med Nat Iași* 2003; 107(1): 218-222.

♦ V. Dorneanu, **Nela Bibire**, Ursula Helena Stănescu, Maria Stan. Utilizarea electrozilor membrană ion-selectivi în analiza cantitativă a alcaloizilor derivați de tropanol. *Farmacia* 2000; XLVIII(3): 57-64.

♦ **Nela Bibire**, V. Dorneanu, V. Șunel, Ursula Helena Stănescu, M. Apostu. Electrozi membrană ion-selectivi cu matrice PVC pentru determinarea bromurii de N-butilscopolamoniu. *Farmacia* 2000; XLVIII(4): 41-49.

♦ **Nela Bibire**, V. Dorneanu. Electrozi ion-selectivi cu membrană lichidă pentru determinarea bromurii de N-butilscopolamoniu. *Rev Med Chir Soc Med Nat Iași* 1999; 103(1-2): 190-194.

♦ **Nela Bibire**, V. Dorneanu, Maria Stan. Realizarea și caracterizarea unor electrozi membrană-selectivi pentru determinarea atropinei. *Farmacia* 1998; XLVI(5): 29-36.

I.4.1. ISE analysis of ranitidine, famotidine and nizatidine

Introduction

Selective membrane sensors are a system in which a special membrane separates two electrolyte solutions containing the same chemical species in different concentrations, and between which there is a potential difference. As the potential changes because of the variation in concentration of one of the chemical species it comes into contact with, it is considered that such a membrane, specific to a certain chemical species, functions as a transducer whose potential is a measure of the activity of that particular chemical species (149-152).

The literature on the use of specific electrochemical sensors in the analysis of pharmaceutical products is very up-to-date (153, 154). Selective membrane sensors based on electroactive material incorporated in vinyl polychloride (PVC) matrix have the advantage of design, long life and saves active material. They have been used to determine galantamine (155), umeclidinium (156), tetracaine (157), azithromycin (158), ranitidine (159), oxomemazine (160), phenobarbital (161), oxalate (162), heavy metals (163) and so on.

Heteropolyacids precipitate with some organic substances with basic groups, forming ion-pair complexes, that are crystallized substances, with a set composition, which can be used to obtain polyvinyl chloride (PVC) matrix membranes, used when constructing sensors for oseltamivir phosphate (164), promethazine (151), amitriptyline (150) or midodrine (165).

The present study describes the construction and characterization of ion selective membrane sensors (ion selective electrodes - ISE) with PVC matrix for the determination of ranitidine (ISE-R), famotidine (ISE-F), and nizatidine (ISE-N) using as electroactive material their respective complexes with phosphomolybdic acid (166).

Materials and methods

Potentiometric measurements were carried out using a 301 digital Hanna pH/millivoltmeter. The ion-selective membrane electrode was used as indicator electrode in conjunction with a OP-0830P Radelkis saturated calomel electrode (SCE) as reference electrode.

Reagents used while preparing the membranes were produced by Fluka or Aldrich: polyvinyl chloride (PVC), phosphomolybdic acid (PMA), o-nitrophenyloctyleter (o-

NPOE), di(butyl)butylphosphonate (DBBP), dioctylphthalate (DOP), sodium tetraphenylborate (NaTPB) and tetrahydrofuran (THF). All chemicals were of analytical-reagent grade.

Synthesis of complexes

Ranitidine, famotidine, and nizatidine (figure 46) produced during the reaction with phosphomolybdic acid, crystalline yellowish-green crystals.

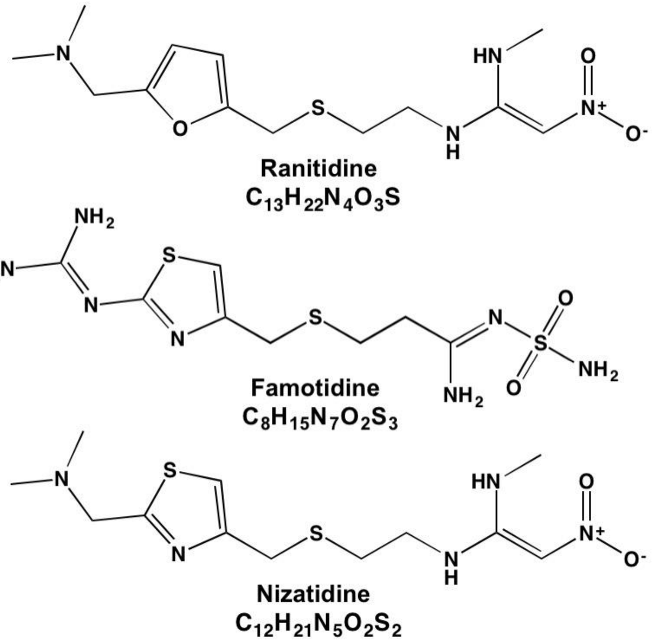

Fig. 46. The chemical structures of ranitidine, famotidine, and nizatidine

Precipitation took place at pH 1.0 and ~50°C using 1% phosphomolybdic acid solution. After 60 minutes of rest at room temperature, the complexes were separated through filtration, washed with a saturated solution of the precipitate and then with distilled water and dried to constant weight at room temperature in a vacuum desiccator.

The insoluble complexes were characterized by UV spectra (*HP 8453 - diode-array spectrophotometer*), specific absorbance ($\mathbf{A}^{1\%}_{\mathrm{cm}}$), solubility (**S**), and melting points (*MEL-TEMP II - capillary melting point apparatus*) (table 64).

Table 64. Specific absorbance, solubility and melting point of the complexes

Complex	$\mathbf{A}^{1\%}_{\mathrm{cm}}$	S (g/L)	Melting point
R-PMA (*ranitidine phosphomolybdate*)	136.11 (λ = 312 nm)	$5.3392\cdot10^{-3}$	> 350°C with decomposition
F-PMA (*famotidine phosphomolybdate*)	147.84 (λ = 209 nm)	$8.1495\cdot10^{-2}$	> 350°C with decomposition
N-PMA (*nizatidine phosphomolybdate*)	157.25 (λ = 309 nm)	$5.0999\cdot10^{-3}$	> 350°C with decomposition

Elemental analysis (*CE 440 Elemental Analyzer*) confirmed the formation 3:1 complexes of R-PMA or F-PMA or N-PMA, respectively, as shown in table 65.

Table 65. Elemental analysis of complexes

Element %	R-PMA $[C_{13}H_{23}N_4O_3S]_3\cdot[PMo_{12}O_{40}]$		F-PMA $[C_8H_{16}N_7O_2S_3]_3\cdot[PMo_{12}O_{40}]$		N-PMA $[C_{12}H_{22}N_5O_2S_2]_3\cdot[PMo_{12}O_{40}]$	
	Found	Calculated	Found	Calculated	Found	Calculated
C	16.88	16.92	10.19	10.16	15.41	15.33
H	2.54	2.51	1.73	1.70	2.39	2.36
N	6.12	6.07	10.30	10.37	7.62	7.45

Electrode construction

The selective membrane was obtained after evaporation of THF out of 2 mL solution that had been continuously poured as a thin layer inside a glass ring, 30 mm in diameter mounted, on a glass plate. The solution contained various proportions of an electroactive compound (ionophore), plasticizer, PVC powder, and additives.

Discs of suitable diameter had been cut from the membrane in order to be applied at the end of a PVC tube with 10 mm inner diameter using a PVC and THF mixture as a binder and it was allowed to dry for 24 hours. The body of the electrode that had the selective membrane attached to, was filled with the internal reference solution, within which the Ag/AgCl internal reference electrode was immersed. A solution of analyte salt that the electrode was selective to, was used as internal reference solution in a concentration of

10^{-3} mol/L in AgCl saturated solution. In order to obtain the Ag/AgCl electrode, a silver wire 1 mm in diameter and 50 mm long was used after it had previously been cleaned with concentrated HNO_3 and then rinsed with distilled water. The silver wire constituted the anode of an electrolysis cell, while the cathode was a platinum plate. The electrode assembly was placed in an AgCl saturated solution and then it was connected to a 9 V battery with a 1 Mohm resistance to provide a 10 μA current. Thus a compact and uniform layer of AgCl was deposited on the surface of the silver wire. Before the measurements, the selective electrode membrane had been conditioned by immersion in a 10^{-5} mol/L analyte salt solution for 120 minutes after which it was rinsed thoroughly with distilled water.

Results

Optimization of PVC membrane sensor composition

Table 66 shows the slope of the electrodes (mV/decade) depending on the plasticizer used and its proportion (weight percentage - %wt).

Table 66. Optimization of membranes composition - slope (mV/decade)

Plasticizer (%wt)	DOP			DBBF			o-NPOE		
	ISE-R	ISE-F	ISE-N	ISE-R	ISE-F	ISE-N	ISE-R	ISE-F	ISE-N
63	53.81	50.14	49.77	52.88	49.89	48.65	47.70	44.02	42.66
64	54.90	51.30	50.82	53.01	50.77	49.13	48.55	44.81	43.55
65	55.30	52.40	51.80	54.85	51.69	50.01	49.12	46.21	44.87
66	55.01	52.00	51.23	54.17	51.01	49.72	48.70	45.78	44.11
67	54.11	51.68	50.71	53.69	50.13	49.00	48.11	45.00	43.17

The optimal composition for obtaining homogeneous, thin, elastic, mechanically resistant and best-response membranes is shown in table 67.

Table 67. Composition of PVC matrix selective membranes (%wt)

Electrode	Ionophore (%wt)	Plasticizer (%wt)	Additive (%wt)	Matrix (%wt)
ISE-R	R-PMA (3)	DOP (65)	NaTPB (1)	PVC (31)
ISE-F	F-PMA (3)	DOP (65)	NaTPB (1)	PVC (31)
ISE-N	N-PMA (3)	DOP (65)	NaTPB (1)	PVC (31)

Effect of pH

Figure 47 shows the response of electrodes for 10^{-3} mol/L concentration in the pH range 1.0-10.0.

The electrode response had the same profile for three concentration levels, and the optimal pH range was found to be in between 2.0 and 6.0. The potential measurements were conducted at pH 4.0 maintained using acetate buffer solution.

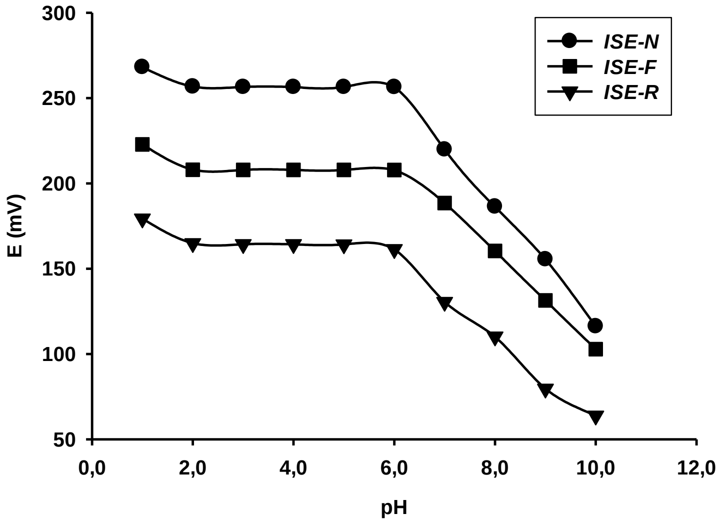

Fig. 47. Effect of pH on electrode response

Total ionic strength

A 0.1 value for the ionic strength was found to be optimal for samples with the concentration below 10^{-2} mol/L, and it was obtained using 1mol/L KNO_3.

Response time

The response time varied depending on the analyte concentration. For lower concentrations the response time was within 55 seconds while the electrodes response to higher concentrations was virtually instantaneous in all cases.

Experimental data obtained were subjected to statistical processing, establishing for each constructed electrode its linear response range, limit of quantification, precision, accuracy, selectivity, and robustness of the method.

Linearity

The response of the electrodes was studied in the 10^{-7}-10^{-1} mol/L concentration range at pH 4.0 and at 0.1 ionic strength (table 68).

An example for the limit of quantification (LOQ) is shown in figure 48 for ISE-R (E = mV, C = mol/L, pC = - log C).

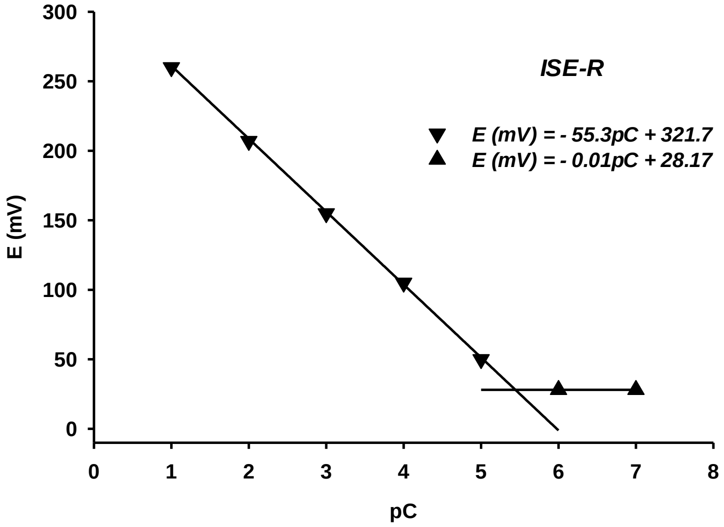

Fig. 48. LOQ and calibration curves for ISE-R

Precision

The precision of the method was studied in terms of repeatability and reproducibility. There have been two series of measurements in various days for three different concentrations of analyte 10^{-4}, 10^{-3} and 10^{-2} mol/L. For each concentration level three determinations series were carried out (table 68).

Accuracy

The accuracy of the electrodes was assessed by analyzing three standard solutions of 10^{-4}, 10^{-3} and 10^{-2} mol/L respectively, following the correspondence between the real and the analytical result obtained from measurements by calculating the relative error, Xd (%), using the equation (1) (167):

$$Xd(\%) = \frac{|Xr - Xa|}{Xa} \cdot 100 \quad (1)$$

where Xr was the value calculated from the calibration curve for the theoretical value Xa (table 68).

Table 68. Validation parameters of potentiometric determination methods

Electrode		ISE-R	ISE-F	ISE-N
Linearity range		10^{-1}-10^{-5} mol/L	10^{-1}-10^{-5} mol/L	10^{-1}-10^{-5} mol/L
Regression		E = -55.3·pC+321.7	E = -52.4·pC+324.7	E = -1.8·pC+382.2
Correlation coefficient (r^2)		0.9986	0.9981	0.9973
Slope		55.3 mV/decade	52.4 mV/decade	51.8 mV/decade
Standard deviation (σ)		0.6239	0.5300	0.4393
LOQ		$5.76 \cdot 10^{-6}$ mol/L	$2.65 \cdot 10^{-6}$ mol/L	$3.32 \cdot 10^{-6}$ mol/L
Repeatability Ist Series	SD	2.04	1.71	1.05
	RSD	2.05%	1.75%	1.06%
Repeatability IInd Series	SD	2.07	1.57	0.76
	RSD	2.08%	1.60%	0.77%
Reproducibility	SD	2.00	1.62	0.90
	RSD	2.01%	1.66%	0.92%
Accuracy	$\overline{Xd}$	1.91%	2.17%	1.57%

Robustness

The robustness of the methods was assessed by comparison of the intraday and interdays assay results measured by two analysts under a variety of conditions such as small changes of laboratory temperature and provenience of chemicals. The percent recoveries were good.

Electrode selectivity

The selectivity of electrodes (table 69) was investigated by the separate solution method and the potentiometric selective coefficients (K) were calculated using equations (2) and (3) (168):

$$\log K = \frac{E_{(II)} - E_{(I)}}{P} + \log[A^{y+}] - \log[I^{z+}] \quad (2), \quad K = 10^{\frac{\Delta E}{P}} \cdot \frac{[A^{y+}]}{[I^{z+}]} \quad (3)$$

Two separate solutions of the same 10^{-3} mol/L concentration were prepared for the primary ion (A^{y+}) and the interfering secondary ion (I^{z+}). Their potentials E_I (for A^{y+}) and E_{II} (for I^{z+}) were measured (P = slope of the calibration curve).

Table 69. Selectivity coefficient (K)

Interferer	ISE-R	ISE-F	ISE-N
NH_4^+	$1.27 \cdot 10^{-3}$	$5.30 \cdot 10^{-3}$	$1.12 \cdot 10^{-3}$
Na^+	$3.70 \cdot 10^{-3}$	$4.30 \cdot 10^{-3}$	$1.26 \cdot 10^{-3}$
Ca^{+2}	$1.90 \cdot 10^{-3}$	$3.44 \cdot 10^{-3}$	$4.19 \cdot 10^{-3}$
Mg^{+2}	$2.54 \cdot 10^{-3}$	$9.21 \cdot 10^{-4}$	$2.36 \cdot 10^{-4}$
Al^{+3}	$8.96 \cdot 10^{-4}$	$8.19 \cdot 10^{-4}$	$3.35 \cdot 10^{-4}$
Ranitidine	-	$1.11 \cdot 10^{-1}$	$1.60 \cdot 10^{-1}$
Famotidine	$1.23 \cdot 10^{-1}$	-	$1.32 \cdot 10^{-1}$
Nizatidine	$1.78 \cdot 10^{-1}$	$1.90 \cdot 10^{-1}$	-

Lifetime of electrodes

The electrodes used constantly during the experiment had an average lifetime of 4-5 weeks.

Analytical applications

The constructed and characterized electrodes have been used to quantify through direct potentiometry the analytes from some pharmaceutical products such as injectable solutions, tablets, and capsules. The results obtained were within the limits set by the Romanian Pharmacopoeia Xth Edition (FRX) regarding the accepted variation of content for the active substance when compared to the labeled value (table 70).

Table 70. Direct potentiometric quantitative determination

Active Substance	Tablets/capsules			Injectable solution		
	Labeled	Quantified	FRX	Labeled	Quantified	FRX
Ranitidine (n = 6)	75 mg	74.70 ± 0.24mg	75 ± 5.62 mg	50 mg/2 mL	49.79 ± 0.13 mg/2 mL	50 ± 2.50 mg
Famotidine (n = 6)	40 mg	39.52 ± 0.12mg	40 ± 3.00 mg	20 mg/5 mL	19.56 ± 0.08 mg/5 mL	20 ± 1.00 mg
Nizatidine (n = 6)	300 mg	298.60 ± 0.27 Mg	300 ± 15.0 mg	100 mg/4 mL	98.54 ± 0.17 mg/4 mL	100 ± 5.00 mg

Discussions

Various selective membrane compositions have been evaluated. According to literature, the plasticizer solubilizes the ion pairs complex, and its proportion regulates both membrane permeability and ions mobility to obtain the most selective and sensitive response (169, 170) (table 66).

The effect of pH on electrode response was examined by measuring the variation of the potential of the cell for three different concentration levels (10^{-4}, 10^{-3} and 10^{-2} mol/L).

The optimal pH range was found to be in between 2.0 and 6.0. At lower pH levels, there is a slight interference from the hydrogen ions, while in alkaline media, the membrane potential decreases due to the gradual precipitation of ranitidine, famotidine or nizatidine as free bases (figure 47).

Because the electrode response depended on ionic activity, it was important to keep the activity coefficient constant for all solutions. It was necessary to achieve a relatively constant concentration of a highly pure electrolyte to which the selective membrane did not respond. The measured potential was uninfluenced by ionic strength for 10^{-2} and 10^{-1} mol/L sample solutions.

For each electrode the concentration range for linear response of the measured potential correlated to the concentration of the analyzed ion was determined (table 68).

A graphical method was applied for calculating the LOQ defined as the intersection of the regression line for the linear domain with the range when the electrode response is relatively constant (figure 48).

The separate solution method is one of the methods recommended by IUPAC for study of electrode selectivity (171).

Because of their similar structures, molecular weights and molecule dimensions, ranitidine, famotidine, and nizatidine interfered with each other slightly as far as the response of electrodes. Since there are no pharmaceutical products containing any association of those three active ingredients, it is unlikely that these interferences will actually occur. The cations usually present in the excipients used in the formulation of tablets/capsules did not exhibit any interference.

Determinations are non-destructive, and they can be done even by direct analysis of turbid or viscous solutions with a sensitivity superior to classic titrimetric and spectrophotometric methods, but inferior to that of high performance liquid chromatography.

The main disadvantages are the short life of the sensor membrane and the need for its periodic renewal.

Conclusions

A series of selective membrane potentiometric sensors have been constructed based on electro-active material embedded in the PVC matrix.

The sensors were evaluated as far as their functional characteristics and then they were used for the quantitative determination of ranitidine, famotidine, and nizatidine within 10^{-1}-10^{-5} mol/L concentration range.

The proposed methods can be used for routine analysis in drug quality control laboratories providing a quick, simple, accurate and inexpensive solution considering the necessary equipment involved and the consumption of reagents.

I.4.2. ISE analysis of some heavy metals' ions

Introduction

Heavy metal toxicity is a clinically significant medical condition that improperly treated may result in significant morbidity and mortality. Heavy metals may enter the body orally, through inhalation, or skin absorption. Body part and severity of damage varies with the metal involved, the route it has entered the body, patient's age and level of toxicity.

Ion-selective membrane electrodes (ISE) play an important part in chemical analysis due to their simplicity, speed and accuracy when compared to other analytical methods. High selectivity allows their use for the determination of numerous organic and inorganic compounds without prior separation.

Review of the literature discloses the use with good results of membrane ion selective electrodes for the determination of heavy metals from various media (172-176).

In this study ion-selective membrane electrodes with PVC matrix were constructed and characterized for the determination of cations such as Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2} and Hg^{+2} (163).

Materials and methods

Potentiometric measurements were carried out using a 301 digital Hanna pH/millivoltmeter. The ion-selective membrane electrode was used as indicator electrode in conjunction with a OP-0830P Radelkis saturated calomel electrode (ESC) as reference electrode.

All reagents used while preparing the membranes were produced by Fluka or Aldrich: polyvinyl chloride (PVC), acetoacetanilide (AAA), dicyclohexane-24-crown-8 (DCH24C8), poly-(4-vinyl pyridine) (P4VP), dibenzo-18-crown-6 (DB18C6), dicyclohexyl-18-crown-6 (DC18C6), o-nitrophenyloctyleter (o-NPOE), di(butyl)butylphosphonate (DBBP), dioctylphthalate (DOP), sodium tetraphenylborate (NaTPB) and tetrahydrofuran (THF).

The construction of the electrodes

Selective membrane was obtained after evaporation of THF out of 2 mL solution that had been continuously poured into a thin layer inside a 30 mm in diameter glass ring mounted on a glass plate (177-180) (table 71).

Discs of suitable diameter had been cut from the obtained membrane in order to be applied at the end of a PVC tube of 10 mm inner diameter using a PVC and THF mixture as a binder and it was allowed to dry for 24 hours.

Table 71. Mass percentage composition of PVC matrix ion-selective membranes

Electrode	Electroactive compound	Plasticizer	Additive	Matrix
Cu^{+2} ISE	AAA (1)	*o*-NPOE (67)	NaTPB (1)	PVC (31)
Cd^{+2} ISE	DCH24C8 (1)	DBBP (67)	NaTPB (1)	PVC (31)
Ni^{+2} ISE	DB18C6 (1)	DOP (67)	NaTPB (1)	PVC (31)
Pb^{+2} ISE	DC18C6 (1)	*o*-NPOE (67)	NaTPB (1)	PVC (31)
Hg^{+2} ISE	P4VP (1)	DOP (67)	NaTPB (1)	PVC (31)

The body of the electrode that had the selective membrane attached to, was filled with internal reference solution and then the Ag/AgCl internal reference electrode was immersed. A solution of analyte salt that the electrode was selective to, was used as internal reference solution in a concentration of 10^{-3} mol/L in AgCl saturated solution.

In order to obtain the Ag/AgCl electrode, a 1 mm in diameter and 50 mm long silver wire was used after it had previously been cleaned with concentrated HNO_3 and then rinsed with distilled water. The silver wire constituted the anode of an electrolysis cell, while the cathode was a platinum plate. The electrode assembly was placed in a AgCl saturated solution and then it was connected to a 9V battery with a 1Mohm resistance to give a 10µA current. Thus a compact and uniform layer of AgCl was deposited on the surface of the silver wire.

Before the measurements, the selective electrode membrane had been conditioned by immersion in a 10^{-5} mol/L analyte salt solution for 120 minutes, after which it was rinsed thoroughly with distilled water. When not in use the electrode was stored dry.

The electrochemical cell included as external reference electrode a saturated calomel electrode (SCE).

Results

pH effect

The effect of pH on electrode response was examined by measuring the potential variation of the electrochemical cell with three solutions of various concentrations (10^{-4}, 10^{-3} and 10^{-2} mol/L) (figure 49).

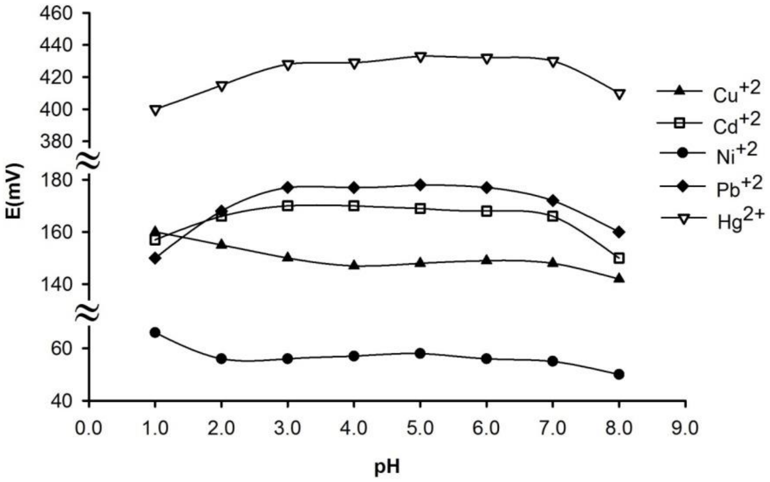

Fig. 49. The effect of pH on electrode response

The working technique for the *in vitro* determination of trace heavy metals in aqueous extracts orally administered for therapeutic purposes has imposed the need to do all determinations at pH 7.0.

Total ionic strength

A 0.1 value for the ionic strength was found to be optimum for samples with concentrations below 10^{-1} mol/L and it was obtained by dilution using 1 mol/L KNO_3.

Response time

For low concentrations the response time was within 45 seconds while the electrodes response to high concentrations was virtually instantaneous in all cases.

Experimental data obtained were subjected to statistical processing, establishing for each constructed electrode its linear response range, limit of quantification, precision, accuracy, selectivity and robustness of the method (93, 181).

Linearity

The electrodes response was studied in the concentration range between 10^{-7}-10^{-1} mol/L at pH 7.0 and 0.1 ionic strength (E = mV, C = mol/L, pC = - log C) (table 72). A graphical method was applied to calculate the LOQ (182). An example is shown in figure 50 for Cd^{+2} ISE.

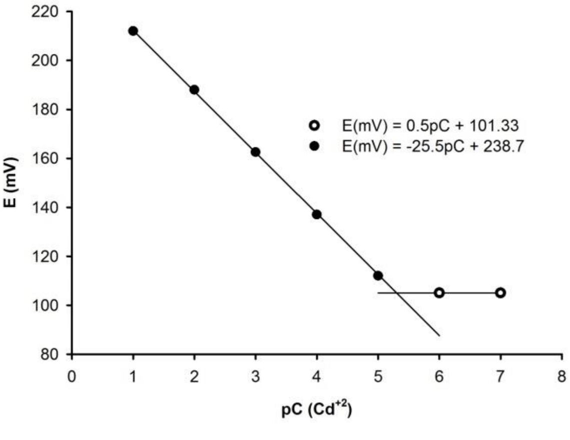

Fig. 50. LOQ and calibration curves for Cd^{+2} ISE

Precision

There have been two series of measurements in various days for three different concentrations of analyte 10^{-4}, 10^{-3} and 10^{-2} mol/L. For each concentration level three determinations series were carried out (table 72).

Table 72. Validation parameters of potentiometric determination methods for Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2} and Hg^{+2} using ISE

Electrode		Cu^{+2} ISE	Cd^{+2} ISE	Ni^{+2} ISE	Pb^{+2} ISE	Hg^{+2} ISE
Linearity range		10^{-2}-10^{-6}mol/L	10^{-1}-10^{-5}mol/L	10^{-1}-10^{-5}mol/L	10^{-2}-10^{-6}mol/L	10^{-2}-10^{-6}mol/L
Regression		E = 0.3·pC+ 208.4	E = -25.5·pC+ 238.7	E = 23.8·pC+ 13.6	E = -19.1·pC+ 233.6	E = 32.6·pC+ 328.8
Correlation coefficient (r^2)		0.9986	0.9948	0.9982	0.9982	0.9939
Slope		20.3mV/decade	25.5mV/decade	23.8mV/decade	19.1mV/decade	32.6mV/decade
Standard deviation (σ)		0.4676	0.9295	0.4393	0.1516	0.3386
LOQ		$3.63\cdot10^{-7}$mol/L	$5.62\cdot10^{-6}$mol/L	$4.57\cdot10^{-6}$mol/L	$3.16\cdot10^{-7}$mol/L	$7.08\cdot10^{-7}$mol/L
Repeatability Ist Series	SD	3.11	3.89	2.04	2.51	2.07
	RSD	3.17%	3.90%	2.05%	2.48%	2.08%
Repeatability IInd Series	SD	3.31	3.62	2.07	1.58	2.23
	RSD	3.36%	3.63%	2.08%	1.57%	2.24%
Reproducibility	SD	3.20	3.65	2.00	2.06	2.09
	RSD	3.25%	3.64%	2.01%	2.04%	2.10%
Accuracy	$\overline{Xd}$	3.26%	3.18%	1.91%	1.69%	2.06%

Accuracy

The accuracy of the electrodes was assessed by analyzing three standard solutions of 10^{-4}, 10^{-3} and 10^{-2} mol/L respectively, following the correspondence between the real and the analytical result obtained from measurements by calculating the relative error, Xd (%), using the equation:

$$Xd(\%) = \frac{|Xr - Xa|}{Xa}\cdot 100 \quad (1)$$

where Xr was the value calculated from the calibration curve for the theoretical value Xa (table 72).

Robustness

The robustness of the methods was assessed by comparison of the intra and inter-day assay results measured by two analysts under a variety of conditions such as small changes of laboratory temperature and provenience of chemicals. The percent recoveries were good.

Electrode selectivity

The selectivity of electrodes (table 73) was investigated by the separate solution method and the potentiometric selective coefficients (K), were calculated by equations (2) and (3) (168):

$$\log K = \frac{E_{(II)} - E_{(I)}}{P} + \log[A^{y+}] - \log[I^{z+}] \ (2),\quad K = 10^{\frac{\Delta E}{P}} \cdot \frac{[A^{y+}]}{[I^{z+}]} \ (3)$$

Two 10^{-3} mol/L separate solutions were prepared for the primary ion (A^{y+}) and the interfering secondary ion (I^{z+}). Their potentials E_I (for A^{y+}) and E_{II} (for I^{z+}) were measured (P - slope of the calibration curve).

Table 73. Selectivity coefficient (K)

Interferer	Cu^{+2} ISE	Cd^{+2} ISE	Ni^{+2} ISE	Pb^{+2} ISE	Hg^{+2} ISE
Cu^{+2}	-	$2.2\cdot10^{-2}$	$2.1\cdot10^{-3}$	$1.4\cdot10^{-3}$	$4.5\cdot10^{-3}$
Cd^{+2}	$1.4\cdot10^{-2}$	-	$3.2\cdot10^{-2}$	$1.0\cdot10^{-3}$	$4.4\cdot10^{-3}$
Ni^{+2}	$3.0\cdot10^{-2}$	$2.0\cdot10^{-2}$	-	$4.8\cdot10^{-4}$	$1.5\cdot10^{-3}$
Pb^{+2}	$3.6\cdot10^{-2}$	$3.1\cdot10^{-2}$	$2.6\cdot10^{-3}$	-	$5.7\cdot10^{-3}$
Hg^{+2}	$3.1\cdot10^{-2}$	$3.5\cdot10^{-2}$	$3.1\cdot10^{-3}$	$7.8\cdot10^{-3}$	-
Zn^{+2}	$7.8\cdot10^{-3}$	$2.7\cdot10^{-2}$	$7.1\cdot10^{-3}$	$3.9\cdot10^{-4}$	$3.5\cdot10^{-3}$
Al^{+3}	$3.9\cdot10^{-3}$	$3.9\cdot10^{-2}$	$2.1\cdot10^{-3}$	$3.1\cdot10^{-3}$	$7.1\cdot10^{-3}$
Co^{+2}	$2.1\cdot10^{-2}$	$2.1\cdot10^{-2}$	$9.1\cdot10^{-3}$	$1.1\cdot10^{-3}$	$4.5\cdot10^{-4}$
Cr^{+3}	$4.2\cdot10^{-3}$	$3.5\cdot10^{-2}$	$1.8\cdot10^{-3}$	$5.1\cdot10^{-3}$	$1.2\cdot10^{-4}$
Fe^{+3}	$1.1\cdot10^{-3}$	$5.7\cdot10^{-2}$	$1.1\cdot10^{-3}$	$6.2\cdot10^{-3}$	$4.5\cdot10^{-4}$
Mn^{+2}	$2.2\cdot10^{-3}$	$3.9\cdot10^{-2}$	$3.6\cdot10^{-3}$	$3.3\cdot10^{-4}$	$6.5\cdot10^{-4}$
Ca^{+2}	$2.3\cdot10^{-3}$	$6.1\cdot10^{-2}$	$1.0\cdot10^{-2}$	$5.2\cdot10^{-3}$	$2.5\cdot10^{-4}$
Mg^{+2}	$2.2\cdot10^{-3}$	$1.1\cdot10^{-2}$	$9.0\cdot10^{-3}$	$3.0\cdot10^{-3}$	$5.3\cdot10^{-4}$
Na^{+}	$4.4\cdot10^{-2}$	$2.2\cdot10^{-1}$	$6.0\cdot10^{-3}$	$4.0\cdot10^{-3}$	$3.5\cdot10^{-3}$
K^{+}	$3.3\cdot10^{-2}$	$2.4\cdot10^{-1}$	$4.4\cdot10^{-3}$	$3.1\cdot10^{-2}$	$4.5\cdot10^{-3}$

Life span of the electrodes

The electrodes used constantly during the experiment had an average duration of use of approximately 5-6 weeks.

Analytical applications

The constructed electrodes were used to quantifying the bioavailability of heavy metals in some aqueous extracts in a simulated digestive system (183). To that end the plant extract was adjusted to pH 2.0 with HCl, swine pepsin was added and incubated for 1 hour at 37°C. It was then brought to pH 5.6 with $NaHCO_3$, a mixture of pancreatin, porcine bile extract and lipase was added, the pH was adjusted to 7.0 using NaOH and the mixture was incubated for 2 hours at 37°C. The amount of free metal was determined using the standard addition method. The results will be the subject of a future scientific report.

Discussions

The solution contained various proportions of electroactive compound, plasticizer, PVC powder and additive. Optimal proportions used to obtain homogeneous, thin, elastic and with good mechanical strength membranes are shown in table 71.

The response of the electrodes to 10^{-3} mol/L solution in the 1.0-9.0 pH range is shown in figure 49.

The measured potential was not influenced by ionic strength for 10^{-2} and 10^{-1} mol/L solutions.

The response time varied depending on the analyte concentration and it did not depend on whether the potentials were recorded from low to high concentrations or vice versa.

For each electrode the concentration range of linear relationship between the measured potential and the concentration of the analyzed ion was determined (table 72).

The precision of the method was studied in terms of repeatability and reproducibility.

Conclusions

A series of ion-selective PVC matrix membrane electrodes for the determination of Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2} and Hg^{+2} was built.

The electrodes were studied from the point of view of main functional characteristics and they were used for the determination of trace of heavy metals from aqueous extracts.

The proposed methods were simple, fast and accurate.

II. PROFESSIONAL, SCIENTIFIC AND ACADEMIC ACHIEVEMENTS

I graduated from the Pharmacy Faculty of ”Grigore T. Popa” University of Medicine and Pharmacy from Iasi in 1988 (University Degree no. 98/01.11.1988).

My postdoctoral evolution involved three interconnected directions: professional, scientific and academic, as long as teaching and pharmaceutical practice are in direct relationship with research.

II.1. Professional achievements

After graduation from the Faculty of Pharmacy in 1988, I was appointed by governmental distribution in the network of the Pharmaceutical Office in Iasi in order to discharge my probation period, covering the following professionall stages throughout the years to come:

- ✓ Pharmacist Apprentice for the Pharmaceutical Office from Iasi by Governmental Distribution (no. 440997/1988);
- ✓ Pharmacist for the Pharmaceutical Office from Iasi by competitive examination confirmed by the Order of the Minister of Health (no.1401/04.12.1990);
- ✓ Resident Pharmacist specialized in General Pharmacy confirmed by the Order of the Minister of Health (no.1512/22.11.1993);
- ✓ Specialist Pharmacist confirmed by the Order of the Minister of Health (no. 2393/18.12.1995) as a result of the competitive exam held on 9.10.1995;
- ✓ Senior Pharmacist confirmed by the Order of the Minister of Health (no. 727/7.09.2000) as a result of the competitive exam held on 20.06.2000.

As pharmacist and member of the university teaching staff, I was on the examining boards approved by the Minister of Health for the posts of pharmacists at the Pharmacy of “St. Pantelimon” County Emergency Hospital from Focsani in 2011, at the Pharmacy of C.F. Clinical Hospital from Iasi in 2018 (head of the board) and of head pharmacist at the Military Hospital from Iasi in 2017.

Throughout this period I have steadily improved my expertise by documentation and by attending training courses/workshops which have broadened the extent of my knowledge and enhanced both my personal and professional development:

- ✓ Workshop: “Microparticles by Spray Drying Process” (held by Professor PhD Jose E. Peris Ribera, Head of Department of Pharmacy and Pharmaceutical Technology, University of Valencia, Spain), Iasi, 2009;
- ✓ Post-university training course: “In vitro Dissolution of Ointments, Creams and Transdermals” (held by Dr. Charles C. Collins, Assistant Dean and Head of the Department of Pharmaceutical Sciences “Lloyd I. Gregory”, School of Pharmacy, Palm Beach Atlantic University, USA), Iasi, 2004.

I am a member of several professional and scientific organizations:

- ✓ The Society of Medical Sciences, the Pharmacy section, Iasi branch;
- ✓ The Pharmacists’College from Romania;
- ✓ The Society of Pharmaceutical Sciences of Romania;
- ✓ The National Society for the History of Pharmacy;
- ✓ The International Society for the History of Pharmacy;
- ✓ Sociedad de Amigos de la Historia de la Ciencia Farmaceutica Catalana.

II.2. Scientific achievements

In 1992 I was admitted as a candidate for a doctor's degree in Analytical Chemistry at the Pharmacy Faculty under the guidance of Professor Vasile Dorneanu and in May 1999 I delivered the doctorate thesis entitled "Contributions to the Analysis of Tropane Alkaloids using Ion-Selective Electrodes" under the guidance of Professor Ursula Helena Stănescu and, consequently, I got the title of Doctor in Pharmacy, specialization Pharmacognosy (Diploma series R, no. 00004945).

The guidance of research toward the great group of instrumental methods of analysis resulted from the requirements of quality control of pharmaceutical products, acknowledging the need to find new methods as sensitive and accurate as possible for their identification, purity and quantitative assay. In this context, Ion-Selective Membrane Electrodes (ISE) currently cause a real interest and offer new perspectives for the analysis and control of pharmaceutical products.

The study undertaken and the results achieved, have brought a real contribution to the analysis of the four tropanol-derived alkaloids (atropine, homatropine, scopolamine and N-butylscopolammonium bromide), with the possibility of extending the scope of potentiometric methods applying ISE to other classes of alkaloids or pharmaceutical substances.

Together with the discipline colleagues and my collaborators from the faculty, university and other research centres, my postdoctoral research has developed in several areas:

- ✓ The achievement and utilization of ISE for the quantitative measurement of some H2 antihistamines (ranitidine, nizatidine, famotidine) and some angiotensin converting enzyme (ACE) inhibitors (lisinopril, ramipril) from diverse pharmaceutical forms (by the direct potentiometric method or by potentiometric titration) as well as the determination of some ions of the heavy metals, a research approach that resumed my initial research objectives naturally and which was also the subject of my doctorate thesis, namely the use of membrane-selective electrodes in the quantitative measurement of tropane alkaloids, an approach that has been one of my steady preoccupations.
- ✓ The elaboration of new methods of spectrophotometric UV-Vis for some drugs belonging to different therapeutic classes (cimetidine, ranitidine, nizatidine, lisinopril, ramipril, bisoprolol), one of which being a "first-time" according to the speciality reference materials studied (e.g. visible spectrophotometric determination of bisoprolol using tropeoline 00 as colour reagent).
- ✓ The development and validation of some methods of chromatographic analysis on a closed column (HPLC with UV detection or HPLC with mass spectrometry detection and GC-MS, respectively) for lisinopril, ramipril, bisoprolol, ciprofloxacin in pharmaceutical products, but also in plasma (alprazolam, clopidogrel, bisoprolol) and the determination of some micotoxins in bee pollen, respectively.
- ✓ The elaboration of analysis methods by spectroscopy of atomic absorption for the determination of some ions of heavy metals in the cigarette smoke and lead in vegetal foods (wheat, beans, carrot, cabbage, onion).
- ✓ The introduction of new analytical reactives (Schiff bases and derivates from the aminoacids'class) for the determination of ions Co(II), Ni(II), Mg(II), Cu(II), Bi(II) and Fe(II), respectively, or of some drugs, as well as the evaluation of the biological action of many of the complex compounds formed with a view to a potential usage in therapeutics.
- ✓ The results of the researches have materialized in scientific papers published in ISI journals, BDI journals or ISI and BDI indexed supplements. Of these, 38 publications

are indexed and quoted ISI (cumulative impact factor 19,278 / 17 as main author works, 21 as co-author works, Hirsch index 7).

All through these years I contributed scientific papers to scientific events (congresses, conferences, symposia, seminars and sessions) both in Romania and abroad, either as posters or oral presentations.

The capacity of working in a team and the efficiency of the above-mentioned scientific collaboration has been demonstrated by the activity done within the research contracts both as a coordinator and as collaborator:

- ✓ COST Action TD1004 as IPC (International Partner Country) 2011-2015 – ”Theragnostics Imaging and Therapy: An Action to Develop Novel Nanosized Systems for Imaging - Guided Drug Delivery” - **National Coordinator**;
- ✓ COST Action CM1403 as IPC (International Partner Country) 2014-2018 - ”The European Upconversion Network - from the Design of Photon-Upconverting Nanomaterials to Biomedical Applications” - **National Coordinator**;
- ✓ COST Action CM1307 as IPC (International Partner Country) 2014-2018 - ”Targeted Chemotherapy Towards Diseases Caused by Endoparasites” - **Collaborator** (Coordinator Prof. Ionel Mangalagiu);
- ✓ Project Partnerships, research contract no. 41017/2007 - ”New Controlled Release Nitric Oxide Donors Therapeutic Systems” 2007-2010; Coordinator ”Grigore T. Popa” University of Medicine and Pharmacy Iași - **Collaborator** (Project Manager Prof. Lenuța Profire);
- ✓ Grant with Romanian Academy, research contract no. 161/2007 - ”New Non-Steroidal Anti-inflammatory Nitric Oxide Donors: Synthesis, Physico-Chemical and Pharmacotoxicological Characterization” 2007-2008; Coordinator ”Grigore T. Popa” University of Medicine and Pharmacy Iași - **Collaborator** (Project Manager Prof. Lenuța Profire);
- ✓ Internal Grant ”Grigore T. Popa” University of Medicine and Pharmacy, research contract no. 30878/2014 - ”Synthesis and Pharmacotoxicity Study of New Derivatives of Sulfonamides with 5-nitroindazol as Support, with Potential Antimicrobial Activity” 2015-2016; Coordinator ”Grigore T. Popa” University of Medicine and Pharmacy Iași - **Collaborator** (Project Manager Lecturer Corina Cheptea).

I participated as member of the Scientific Committee or as one of the organizers of scientific events regarding the field of pharmaceutical sciences:

- ✓ The 15th National Congress of Pharmacy, Iasi, 24-27 September 2014 (member of the Scientific Committee);
- ✓ The 2nd National Symposium - “The Medicament from Conception to Utilization”, Iasi, 20-22 May 2009 (organizer);
- ✓ The 4th National Conference of Fitotherapy, Iasi, 14-16 May 2008 (organizer);
- ✓ The 1st National Symposium - “The Medicament from Conception to Utilization”, 30 May-1 June 2007 (organizer).

II.3. Academic achievements

I have been a member of the academic staff of the University of Medicine and Pharmacy “Grigore T. Popa” Iași since graduation from Pharmacy School, in 1988, occupying the following positions:

- ✓ 1991-1993 - Junior Assistant - Discipline of Analytical Chemistry, Faculty of Pharmacy, ”Grigore T. Popa” University of Medicine and Pharmacy Iași;

- ✓ 1993-2000 - Assistant Professor - Discipline of Analytical Chemistry, Faculty of Pharmacy, ”Grigore T. Popa” University of Medicine and Pharmacy Iași;
- ✓ 2000-2004 - Lecturer - Discipline of Analytical Chemistry, Faculty of Pharmacy, ”Grigore T. Popa” University of Medicine and Pharmacy Iași;
- ✓ 2004-2016 - Associate Professor - Discipline of Analytical Chemistry, Faculty of Pharmacy, ”Grigore T. Popa” University of Medicine and Pharmacy Iași;
- ✓ since 2016 - Professor - Discipline of Analytical Chemistry, Faculty of Pharmacy, ”Grigore T. Popa” University of Medicine and Pharmacy Iași.

Between 1991-2000 I was entrusted with the guidance of Analytical Chemistry practical work of 2nd year students (1st and 2nd semesters), and in 1994-1995, at the same time with the introduction of Instrumental Analysis, I started preparing and supervising the practical work of 3rd year students (1st semester). In this way I contributed to the introduction of some new practice to the Analytical Chemistry Discipline, particularly in the field of physical-chemistry analysis by documentation and paper drawing up, in order to make students familiar with the latest methods that lie at the basis of physical chemistry checking of medicines (optical, electrochemical, thermal, cromatographic methods of analysis).

Between 1994-1997, under the guidance of my mentors, Professor Vasile Dorneanu and Professor Maria Stan, I delivered several lectures to 2nd and 3rd year students. Alongside the continual documentation from reference materials, to be able to teach a high-quality course, I followed the advice and permanent guidance of the discipline professors. I owe them my formation as a researcher and as a teacher. I learnt from them how to be a good analyst and a good teacher, how to plan and conduct research, how to write a specific paper, how to deliver a lecture, how to select the information I offer to my students.

Attending the courses of “Al. I. Cuza” University Pedagogic Seminar (modules on: Scholar Pedagogy, Pedagogy, The Methodology of the Speciality, Specialized Pedagogical Practice) between 1995-1996 also helped me improve my teaching performance.

Starting from 2000, as lecturer, senior lecturer from 2004 and then as a professor from 2016, I have delivered lectures to 1st year (second semester) and 2nd year (first and second semesters) Pharmacy students, as well as to 2nd year (second semester) and 3rd year (second semester) students from the Study Direction in English starting from 2013. With a view to adopting and adapting to the new educational commandments, in 2015 I got the Council of Europe Performance Certificate, level B2 for English, from the Faculty of Letters of ‘Al. I. Cuza” University.

Since 2014-2015 I have conducted an optional course for the 2nd year of study (second term) entitled “Microanalysis of Ions with Biological and Pharmaceutical Importance” introduced for the students to realize the correlation between the necessity of the study of ions not only from the point of view of the micromethods of quantitative and qualitative analysis, but also due to their biological importance in itself in various combinations.

In all these years, I have attended a series of training and specialization courses:

- ✓ Flow Chemistry, Iași, 2016;
- ✓ Pro Analysis Systems Seminar ”Chromatography and Mass Spectrometry", Iași, 2015;
- ✓ Postgraduate Course ”Analytical Applications of GC and HPLC", Faculty of Pharmacy, ”Grigore T. Popa” University of Medicine and Pharmacy Iași, 2011;
- ✓ Laboratory Seminar I organized by ABL & E - JASCO - România “Laboratory Accreditation Aspects, Chyroptic Detection in Liquid Chromatography, Chromatography

and Extraction with Supercritical Fluids, Use of Circular Dicroscopy Spectroscopy in the Study of Molecular Interactions, Purification by Flash Chromatography", Iași, 2009;

- ✓ Postgraduate Course "Instrumental Analysis", "Al. I. Cuza" University Iași, Faculty of Chemistry, 2002-2003;
- ✓ Postgraduate Course "Using the Computer for Information Processing in the Pharmaceutical Sciences", "Grigore T. Popa" University of Medicine and Pharmacy Iași, 1996.

I have guided the scientific research activities in the discipline scientific club and my students have contributed scientific papers to local and national congresses and similar events of the Pharmacy students.

I have also co-ordinated the elaboration of the graduation papers of more than 100 students from both The Study Direction in Romanian and in English as they focused on subjects related to the analysis and checking of some medicinal substances from diverse pharmaceutical forms and products as well as from biological media or types of modern analytical methods and their applications in the pharmaceutical field.

As lecturer and guide of practical tasks, I have got involved in three Master's degree stages carried on in our faculty:

- ✓ Master "The Herbal Drug from Production to Use" - Chemical and Instrumental Methods Applied in the Physico-Chemical Control of the Active Principles of Products of Plant Origin module - 2006-2007;
- ✓ Master "Quality of the Environment and Food Security" - Chemical and Instrumental Methods Applied in the Physico-Chemical Control of Food and Environmental Elements module - 2006-2007;
- ✓ Master "Products of Plant Origin: Medicine, Nutritional Supplement, Food" - modul Chemical and Instrumental Methods Applied in the Physico-Chemical Control of Medicinal Products and Food of Plant Origin - 2008-2012.

I have also got involved in the organization and co-ordination of post-university courses for specialists in the medication field (pharmacists, chemists, biologists):

- ✓ Complexes - Biological and Analytical Implications - 2006;
- ✓ High Performance Liquid Chromatography (Tg. Neamț) - 2005;
- ✓ Good Laboratory Practice (GLP) - 2004;
- ✓ Chromatographic Methods of Analysis - 2002;
- ✓ Electroanalytical Methods of Analysis - 2000.

Teaching and research experience allowed me to write 7 books for students:

- ✓ **Nela Bibire**, Gladiola Țântaru, Mihai Apostu, Mădălina Vieriu, Alina Diana Panainte. *Methods of Quantitative Determination Applied in the Analysis of Drug Substances*. „Gr. T. Popa" Ed., U.M.F. Iași, 2015, ISBN 978-606-544-352-5;
- ✓ Alina Diana Panainte, Mădălina Vieriu, **Nela Bibire**, Mihai Apostu, Gladiola Țântaru. *Applications of Instrumental Methods Used for the Analysis of Pharmaceutical Substances*. „Gr. T. Popa" Ed., U.M.F. Iași, 2015, ISBN 978-606-544-327-3;
- ✓ Țântaru Gladiola, Apostu Mihai, **Bibire Nela**, Vieriu Mădălina, Panainte Alina Diana. *Qualitative Analysis of Drug Substances. Applications*. „Gr. T. Popa" Ed., U.M.F. Iași, 2012, ISBN 978-606-544-096-8;
- ✓ **Nela Bibire**, Gladiola Țântaru, Mihai Apostu. *Analytical Chemistry - 2nd vol.: Quantitative Chemical Analysis of Drug Substances*. Performantica Ed., Iași, 2009, ISBN 978-973-730-621-0;

✓ Mihai Apostu, **Nela Bibire**, Gladiola Țântaru. *Analytical Chemistry - 3rd vol.*: *Instrumental Methods for the Analysis of Drug Substances*. Performantica Ed., Iași, 2009, ISBN 978-973-730-618-0;
✓ Gladiola Țântaru, Mihai Apostu, **Nela Bibire**. *Analytical Chemistry - 1st vol.*: *Theoretical and Practical Basics in the Qualitative Analysis of Drug Substances*. Performantica Ed., Iași, 2009, ISBN 978-973-730-700-2;
✓ Vasile Dorneanu, Maria Stan, **Nela Bibire**, Gladiola Tântaru, Mihai Apostu, Mădălina Vieriu. *Chemical and Instrumental Analysis Methods*. „Gr. T. Popa” Ed., U.M.F. Iași, 2007, ISBN 978-973-7682-26-0.

Occasionally, I was invited as lecturer at national scientific events:

✓ 2011 - Symposium Houston Farma Practic, 8th edition, Gura Humorului (Invited speaker)

Throughout my career I have taken active part in the life of the academic community I belong to:

✓ member of the University Senate of ”Grigore T. Popa” University of Medicine and Pharmacy Iași: 2008-2016;
✓ member of the Department Council of Pharmacy Faculty: 2012-2016;
✓ member of the Committee of Communication, Image and Academic Information of ”Grigore T. Popa” University of Medicine and Pharmacy Iași: 2008-2016;
✓ member of the Scientific Committee of Pharmacy Faculty: 2000-2016;
✓ member of the Pharmacy Faculty Council: 2000-2004 și 2016-present;
✓ teacher for the Record and Reporting of the Scientific Activity of the Faculty of Pharmacy at M.E.C.: 2000-2004.

I was also a member of doctoral commissions (12 commissions, 2 of them in București) and doctoral advisory commissions, a member of numerous commissions for exams or presentations of research reports during doctoral training and a member of the committee for graduate papers presentations (2005 - 2014), being chair of this committee between 2015 and 2018.

I was a member of appointment commissions for the positions of Associate Professor (Discipline of Analytical Chemistry Iasi - 1 and Constanța - 1), Lecturer (Discipline of Analytical Chemistry - 1 and Discipline of Chemistry of Environment and Food - 1), Assistant (Discipline of Analytical Chemistry - 3 and Discipline of Toxicology - 3) or Junior Assistant (Discipline of Analytical Chemistry - 2).

III. FUTURE DIRECTIONS

My future career development will cover the same three directions: professional, research and academic, which cannot be separated. All three directions will contribute to my steady improvement in the pharmaceutical sciences field.

In terms of career development as part of academia, I have to permanently keep in touch with novel discoveries that have to be immediately absorbed and integrated into pharmaceutical practice and research.

Teamwork and collaboration with specialists from other pharmaceutical, chemical, biological and medical fields and even from other institutions are essential in order to improve pharmaceutical care. As they say, career development is based on: professional skills, academic and scientific experience, openness to novelty, desire for constant improvement, team spirit, readiness to help and the ability to collaborate with national and international specialists. In the academic area, I will keep improving my teaching graduate students, by bringing forward more examples from pharmaceutical practice and up-to-date information from scientific literature.

My future research directions will continue the research themes I have been approaching by now, focusing on expanding and deepening my studies, but I will also concentrate my efforts on setting new objectives in correlation with the current scientific context, appropriate to present social and economic realities.

With regard to the development and validation of new methods of analysis for pharmaceutical substances and biological samples, I will continue, together with my collaborators, to develop new HPLC and UV-Vis Spectrophotometric methods for other pharmaceutical substances from the therapeutic classes already studied and I will also expand my area of interest to other classes of therapeutic substances; we will direct our efforts towards designing methods that will be relatively easy to apply in drug analysis laboratories, with reliable and reproducible results.

In the same context, I also intend to work together with colleagues from our faculty and from other research centers to analyze environmental elements, in addition to medicinal substances. This research direction is already launched, taking into consideration the AAS and chromatographic methods developed so far; a Gas Chromatographic method (GC-MS/MS) was developed in collaboration with colleagues from the faculty to quantify beet pollen mycotoxins, knowing that GC-MS is an analytical method which combines the features of gas chromatography and mass spectrometry to identify different substances within a test sample. GC and GC-MS, more recently, are routinely used for the analysis of volatile impurities in active pharmaceutical substances.

The use of small dedicated mass spectrometers as sophisticated detection systems increases the specificity and, therefore, the correct assignment of solvents to their corresponding chromatographic peaks**.** The major success associated with the application of GC methods in pharmaceutical quantitative analysis is firstly due to very high efficiency and separation power, secondly, to the extreme sensitivity of detection of even very small amounts of separated species and, finally, to the precision and accuracy of findings from quantitative analyses of very complex mixtures.

It is our intention to take the research topic regarding the introduction of new analytical reagents for the determination of some ions and drug substances to a higher level, particularly focusing on biological, microbiological and pharmacological investigation of the numerous resulting complexes, which have clear advantages, cumulative of beneficial properties of the ligands, on the one hand, and of the central ion that generated the complex, on the other hand, thus targeting possible applications in therapeutics, as has already been prefigured by several papers published by our team on this subject.

The modelling and correlation of physico-chemical parameters with the chemical structure of newly synthesized chemical entities, in order to identify their utility in therapeutics, represen another important research direction, closely connected to the field of synthesis and biosynthesis of new compounds with pharmacological potential, which require physicochemical characterization and structure confirmation, which are performed by spectral methods of analysis. Some results obtained during this study have already been published in papers written in collaboration with acknowledged teams in this research field and this direction of study will certainly continue.

Throughout the research activity carried out within the Analytical Chemistry Department, I have always had in mind a continuous collaboration with colleagues from other departments within the faculty or the university, but also with research teams from other universities or research centres in Iasi or other cities in Romania, which allowed me to acquire new skills and gain experience mainly in the field of drug analysis, but also regarding other aspects associated with medicinal substances.

The development and validation of drug analysis methods for prolonged-release pharmaceutical formulations, microparticles and nanoparticles occupy a special place in recent and future-oriented research topics of interest, this field already representing the present and especially the future of pharmaceutical technology and of the pharmacy profession in general, pharmacists being deeply involved, both theoretically and practically, in all stages of the drug development process, regardless of the pharmaceutical form.

Together with a research team from Constanta, we will initiate a scientific collaboration to characterize some extracts from marine macroalgae along the Romanian Black Sea coast, as algae are considered a future source of food and raw materials for humanity and their constituents can play an important role in the medical and pharmaceutical field. Therefore, green, brown and red algae have been extensively studied for their antibacterial and antifungal activity, and sterols found in marine algae have the property of lowering blood cholesterol levels, as well as reducing the predisposition to atherosclerosis. In terms of economic relevance, the most important aspect is related to the extraction of polysaccharides from algae, which are widely used in medicine and food industry. In addition, it is known that algae play a major role in maintaining biological balance, by contributing to water purification, and, due to their ability to retain elements in the aquatic environment, they can be used as decontaminating agents for some radioactive substances.

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