GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

HABILITATION THESIS

"The involvement of analytical methods in the evaluation of physiological mechanisms and in the determination of biochemical markers in pathology"

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CONTENTS

Rezumat

Teza de abilitare intitulată "Implicarea metodelor analitice în evaluarea mecanismelor fiziologice și în determinarea markerilor biochimici în patologie" este o sinteză a celor mai reprezentative direcții de cercetare abordate în perioada postdoctorală.

Lucrarea își propune prezentarea activității științifice după susținerea tezei de doctorat (intitulată "Metode analitice de evaluare a disponibilității unor substanțe active legate de suport polimeric"), și a principalelor perspective de dezvoltare a carierei academice, profesionale și de cercetare.

Structura tezei de abilitare este realizată conform recomandărilor Consiliului Național de Atestare a Titlurilor, Diplomelor și Certificatelor Universitare (CNATDCU) și este structurată în trei secțiuni:
Secțiunea I - cuprinde o scurtă prezentare a activității științifice din perioada postdoctorală.
Secțiunea II - prezintă realizările științifice, profesionale și academice, precum și proiectele viitoare legate de acestea.
Secțiunea III - enumeră referințele bibliografice utilizate în teză.

În medicină, atât în cercetare, cât și în practică, evaluarea stării fiziologice se face, printre altele, și în funcție de nivelul diferiților parametri biologici. Deciziile luate in tratamentul medical, fie el chirurgical sau medicamentos, sunt dictate și în funcție de concentrațiile unor parametri biologici. Mai mult, adaptarea tratamentului precum și urmărirea evoluției unei boli presupune determinarea corectă a concentrațiilor markerilor acesteia. Prin urmare, determinarea cât mai exactă a concentrației anumitor compuși, care sunt considerați markeri, este crucială în toate etapele actului medical: diagnostic, tratament, evoluție și vindecare.
De aici și necesitatea dezvoltării unor metode de determinare cât mai specifice și mai sensibile a unor parametri consacrați dar și a unora noi cu potențial de depistare timpurie și precisă a unei afecțiuni. În plus determinările de concentrații în diferite medii biologice (ser, plasmă, urină etc) stau la baza studiilor de biodisponibilitate, farmacocinetică și de cercetare medicală și farmaceutică. Pe baza acestor studii se poate confirma sau infirma un mecanism de acțiune, stabili doza, tipul de formulare, schema de administrare, eficacitatea, și siguranța unui compus propus ca agent terapeutic.
În concluzie tehnicile instrumentale de detecție și cuantificare au un rol decisiv atât în practica medicală cat și în cercetarea medicală și farmaceutică. Pentru a fi aplicate, tehnicile și metodele alese/propuse trebuie să fie supuse procesului de validare. Procesul de validare are ca scop confirmarea utilității, preciziei și reproductibilității metodei de determinare alese.
Pentru a valida o metodă analitică trebuie îndeplinite o serie de criterii de acceptare, cum ar fi: liniaritate, precizie, acurateț̦e („recuperare" sau „efect de matrice"), specificitate și sensibilitate, criterii care sunt stabilite și reglementate de foruri specializate ca Food and Drug Administration (FDA), European Medicine Agency (EMEA) și International Conference of Harmonization (ICH). O metodă validată care îndeplinește criteriile impuse, enunțate mai sus, va oferi rezultatul cel mai apropiat de valoarea reală pentru concentrația unui compus dintr-un mediu sau dintr-o matrice.

Principalele direcții pe care le-am abordat în activitatea de cercetare științifică, desfășurată după obținerea în anul 2000 a titlului de Doctor în domeniul Chimie, au ca numitor comun metodele analitice de cuantificare. Astfel au fost dezvoltate două direcții și anume: pe de o parte identificarea și validarea unor noi metode de determinare pentru diferiți compuși prezenți în medii biologice și pe de altă parte utilizarea acestor metode de analiză în scopul cercetării fundamentale (mecanisme de acțiune) sau aplicative (depistarea potențialului terapeutic al unor noi compuși de sinteză).

Secțiunea I cuprinde patru capitole care descriu direcțiile de cercetare, realizarea acestora, rezultatele obținute și modalitatea de diseminare a rezultatelor.

Prima direcție de cercetare este intitulată " Dezvoltarea și validarea metodelor cromatografice utlizate pentru determinarea unor amine biogene din diferite medii biologice" a avut ca scop validarea unor metode analitice specifice și sensibile pentru cuantificarea aminelor biogene din diferite medii biologice: ser șobolan, medii de cultură, nutrienți de origine animală.
Aminele biogene sunt compuși endogeni care îndeplinesc diferite roluri fiziologice. În concentrații crescute, însă, sunt responsabile de efecte adverse severe cum ar fi creșterea tensiunii arteriale sau chiar șocul anafilactic. Pericolul crescut al prezenței acestor compuși în organism provine din faptul că pot fi simultan generați și introduși, prin alimente procesate precum carne, brânza, vin, putând astfel declanșa reacții adverse grave. Din acest motiv măsurarea lor cat mai exactă impune alegerea unor metode analitice cât mai sensibile și specifice. Astfel au fost selectate o serie metode cromatografice HPLC de separare cuplate cu diferite tipuri de detecție funcție de matricea din care aminele au fost extrase dar și de cantitatea în care ele se găsesc în aceste matrici. Astfel au fost folosite două metode cromatografice de separare HPLC, una cu detecție prin spectrometrie de masa (MS) și cea de a doua cu derivatizare și detecție în fluorescentă.
Metoda HPLC/MS a fost utilizată pentru cuantificarea individuală/simultană a patru amine biogene din două matrici diferite serul animal (șobolani) și mediile de cultură bacteriene (denumite starter, care sunt utilizate în procesarea alimentelor si care generează amine biogene în acest proces). A fost astfel necesară realizarea a două validări de metoda: una pentru cuantificarea aminelor din ser cea de a doua pentru cuantificarea acestora din mediile de cultura (Lactobacillus curvatus și Proteus mirabilis). Rezultatele obținute au indicat posibilitatea determinării simultane a aminelor biogene din medii biologice, cu utilitate practică în determinările toxicologice.
În ceea ce privește utilizarea celor două microorganisme în prelucrarea nutrientilor de origine animală, rezultatele obținute au arătat ca acestea generează cantități moderate de amine biogene în special cadaverină si putresceină și eventual tiramină (doar Lactobacillus curvatus) dar nu generează histamină. Prin urmare ele pot fi utilizate in procesele preparative ca și culturi starter iar controlul aminelor pe care le generează poate fi efectuat prin metoda propusă.
Cea de a doua metodă cromatografică HPLC realizată cu derivatizare și cuplată cu detecția în fluorescență a fost utilizată pentru a determina cantitatea de amine biogene prezentă în nutrienți de origine animală. Și pentru această metoda au fost realizate toate etapele procesului de validare. După validare, metoda a fost utilizată pentru determinarea simultană a aminelor biogene dintr-un număr de 28 de produse diferite (carne proaspătă sau procesată). Rezultatele obținute au confirmat prezența unor cantități crescute de amine biogene în produsele procesate comparativ cu cele proaspete.
În concluzie au fost validate două tipuri de metode cromatografice utile în cuantificarea individuală și simultană a aminelor biogene atât la concentrații relativ mici (ser, medii de cultura) cât și pentru concentrații relativ mari (nutrienți de origine animală). Metodele au aplicabilitate atât în scop

diagnostic în analize toxicologice dar și în depistarea aminelor biogene din culturile starter cât și din nutrienți de origine animală.

A doua direcție de cercetare intitulată ”Evaluarea concentrațiilor homocisteinei în diferite procese patologice și a posibilității utilizării terapeutice a unor produși noi de sinteză” a cumulat mai multe studii care au avut drept scop investigarea implicării homocisteinei in patologie.
Homocisteina (Hcy) este un aminoacid ne-proteinogen generat în cursul metabolizării unui aminoacid proteinogen numit metionina. Pacienții care prezintă niveluri ridicate de Hcy în sânge, situație numită hiperhomocisteinemie (HHcy), pot dezvolta tromboembolism, arterioscleroză prematură, retard mintal, fragilitate osoasă, boli oculare; la femeile gravide poate promova avortul spontan. Deși efectele hyperhomocisteinemiei au fost semnalate încă de acum 70 ani, mecanismul patogen al acesteia nu a fost pe deplin elucidat. În prezent se consideră că hiperhomocisteinemia afectează funcția endoteliului vascular, favorizând tromboza și reprezintă un factor de risc cardiovascular mult mai sever decât hipercolesterolemia. Un nivel crescut al Hcy asociat unei afecțiuni deja instalate, cum ar fi arterioscleroza sau diabetul (afecțiuni endemice in lumea civilizată) este considerat un factor agravant al acestora. Studiul HHcy a cuprins două aspecte: primul a fost crearea unui model de HHcy, model pe care sa poată fi studiate consecințele HHcy asupra unor factori de risc (hipercolesterolemie, hiperglicemie) iar al doilea a fost utilizarea unor compuși de sinteză în vederea evaluării potențialului lor de a diminua concentrațiilor acestor factori.
Astfel a fost realizat, un model experimental de hiperhomocisteinemie, prin administrare de metionină la șobolani, model pe care au fost evaluate nivelurile unor parametri care sunt implicați în bolile cardiovasculare și diabet, colesterolul respectiv glicemia. Pentru a determina nivelele homocisteinei (în vederea verificării instalării statusului hiperhomocisteinemic) a fost selectată o metodă cromatografică HPLC cu derivatizare și cu detecție în UV care a fost supusă procesului de validare. Pe acest model de hiperhomocisteinemie, verificat, au fost evaluate concentrațiile colesterolului și glicemiei precum și efectele HHcy asupra parametrilor stresului oxidativ.
Sistemul antioxidant din organism este unul complex ce conține atât componente enzimatice din care fac parte enzime ca superoxid dismutaza (SOD), glutation peroxidaza (GPx) și catalaza (CAT) dar și molecule simple circulante cum ar fi glutationul, vitamina C, acidul uric, bilirubina care poartă denumirea generică de capacitate antioxidanta totală (TAC). Modificările suferite de acest sistem în context de HHcy au fost investigate utilizând metode standardizate de determinare a activității enzimelor și a concentrațiilor acestor compuși specifici.
Studiul a continuat cu investigarea influenței a doi derivați rutozidici de sinteză asupra nivelurilor tuturor parametrilor menționați anterior în două modele experimentale realizate pe șobolan (modelul de HHcy și cel de diabet). Menționăm că derivații studiați au fost sintetizați la disciplina Chimie Farmaceutică a Facultății de Farmacie, a UMF “Grigore T.Popa” Iași.
Ca și concluzie generală a acestor studii se poate afirma că hyperhomocysteinemia afectează sistemul antioxidant, mai sever pe cel intracelular (în cazul nostru intra-eritrocitar) decât pe cel total (TAC) circulant. Referitor la compușii de sinteză, s-a constatat că aceștia nu determină o scădere a concentrației homocisteinei ci mai curând o acțiune de prevenire a creșterii concentrațiilor ei. În ceea ce privește nivelurile glicemiei și colesterolului a fost înregistrată o acțiune moderată de descreștere a acestora.

A treia direcție de cercetare intitulată “Investigarea statusului antioxidant în inflamația acută și cronică” a urmărit evaluarea sistemului de apărare antioxidant în inflamația acută indusă

experimental la șobolan și în inflamația cronică la subiecți umani în vederea corelării cu o serie de markeri ai inflamației.

Fenomenele oxido-reducatoare stau la baza proceselor vitale din organism, de la generarea de energie pana la procese de sinteză, apărare, detoxificare și comunicare. În ultima vreme sistemul oxido-reducator este considerat a fi implicat în procesul inflamator, speciile reactive ale oxigenului fiind responsabile de eliberarea de citokine pro-inflamatorii ca urmare a activării unui grup de receptori aparținând sistemului imun, numit inflamozom. Prin urmare au fost studiate modificările apărute în sistemul antioxidant și în cel imun prin determinarea concentrațiilor unor markeri specifici pe un model de inflamație acută realizat pe șobolani prin injectarea de carageennan la nivelul labei posterioare a animalelor. Aceeași parametri au fost urmăriți într-un alt studiu efectuat pe subiecți umani cu afecțiuni inflamatorii cronice ale urechii medii. Astfel pentru investigarea statusului antioxidant au fost determinate activitatea enzimelor SOD, GPx, CAT, a concentrațiilor TAS și ale MDA (malondialdehida - marker fidel pentru amploarea distrugerilor cauzate de stresul oxidativ) iar pentru investigarea răspunsului imun au fost determinate concentrațiile interleukinelor IL-1, IL-6 și IL-8.

Studiul realizat pe modelul de inflamație acută a plecat de la considerentele actuale privind natura inflamatorie a arteriosclerozei și utilizarea donorilor de NO în tratament. Literatura menționează o activitate ambivalentă, antiinflamatoare și pro inflamatoare, a donorilor de NO în funcție de condițiile fiziopatologice. Pe modelul inflamator, realizat pe laba de șobolan, a fost investigat potențialul antiinflamator al unor noi donori de NO nebivolol (NEB) și S-nitroso-glutathione (GSNO) asupra statusului oxidativ și inflamator precum și efectele administrării acestora asupra funcției hepatice. Nebivololul este deja introdus în terapie, în timp ce S-nitroso-glutathionul este un donor de NO în faza de testare, similar celor utilizați în terapia bolilor cardiovasculare. Datele obținute sugerează potențialul antiinflamator al donatorilor de oxid nitric ca fiind apropiat de cel al indometacinului (IND). În plus S-nitroso-glutathionul prezintă un beneficiu suplimentar în terapia anti-aterogena, deoarece structura sa ii conferă rolul de depozit de NO.

În concluzie rezultatele obținute au fost mulțumitoare deoarece atât NEB cat și GSNO au manifestat efecte antiinflamatorii certe (deși ceva mai reduse ca intensitate decât cele ale IND) și au diminuat amploarea stresului oxidativ. Cu toate că, per ansamblu, NEB a avut efect mai intens in diminuarea parametrilor inflamatori și oxidativi, S-nitroso-glutathionul a prezentat o toxicitate hepatică și renală mult mai redusă.

Studiul variației markerilor inflamatori și ai stresului oxidativ în inflamația cronică a fost realizat pe subiecți umani cu afecțiuni cronice ale urechii medii și anume: otita cronică supurativă cu sau fără colesteatom respectiv cu recidivă. Investigarea statusului antioxidant a indicat modificări semnificative ale acestuia în toate cele trei tipuri de afecțiuni ale urechii medii. Intensitatea atacului oxidativ a fost confirmată de nivelurile crescute ale malondialdehidei (MDA) produs final al peroxidării lipidice. Investigarea parametrilor inflamației a indicat o creștere semnificativă a nivelurilor acestora la pacienții cu afecțiuni cronice a urechii medii față de normal cu unele variații funcție de stadiul bolii. Analiza statistică a nivelurilor markerilor inflamatori și parametrilor stresului oxidativ a indicat o corelație directă, moderată, intre aceștia și enzimele aparținând sistemului defensiv antioxidant. O corelație indirectă a fost observată intre markerii inflamației și capacitatea totala antioxidantă (TAC). Prin urmare datele obținute confirmă afectarea echilibrului oxido-reducator în procesul inflamator și interconexiunea celor două procese. Astfel datele obținute sugerează posibilitatea de interferare medicamentoasă a acțiunii speciilor oxidante cu efect direct asupra markerilor inflamatori și de stabilire a conduitei terapeutice. Pe de altă parte nivelele interleukinelor IL-6, predictor al proliferării epiteliale ce poate servi la stadializarea

colesteatomului, asociate cu nivelele interleukinelor IL-1, predictor al gradului de distrucție tisulară, pot dicta conduita chirurgicală (tehnica operatorie, gradul de prezervare a funcției auditive, timpanoplastia).
În concluzie parametrii stresului oxidativ corelați cu cei ai inflamației pot fi utili în decizia terapeutică.

A patra direcție de cercetare este intitulată "Determinarea unor parametri biochimici și biomecanici specifici în fractura de fragilitate"
Actualmente in țările dezvoltate arterioscleroza, diabetul și osteoporoza sunt considerate boli endemice. Fractura de fragilitate are o pondere semnificativă în cadrul bolilor osoase la vârstnici. Aceasta afectează în special vertebrele, femurul, humerusul proximal, radiusul distal, pelvisul, împiedicând astfel mobilitatea pacientului. Cea mai debilitantă dintre toate aceste fracturi este fractura de fragilitate a pelvisului (FFP). Alegerea tratamentului potrivit pentru FFP este dificilă deoarece atât terapia conservatoare cât și cea chirurgicală implică riscuri la fel de mari: imobilizare prelungită și/sau riscuri chirurgicale. Prin urmare, terapia farmacologică este o alternativă la cea chirurgicală. Bifosfonații și-au dovedit utilitatea în terapia anti-osteoporotică și aportul lor este recunoscut. În schimb influența calciului și a vitaminei D sunt relativ neglijate fie prin non-complianța pacientului în cazul tratamentului conservator fie prin prioritizarea rezultatului operator în cazul tratamentului chirurgical. Scopul studiului a fost de a evalua rolul calciului și vitaminei D în procesul de vindecare a fracturii la pacienții cu osteoporoză. Rezultatele obținute arată că atât calciul cât și vitamina D exercită o influență pozitivă asupra procesului de vindecare al fracturilor de fragilitate și subliniază semnificativ necesitatea educării pacienților pentru a se conforma prescripției de suplimentare a calciului și a vitaminei D.
În concluzie datele obținute indică beneficiul administrării calciului și a vitaminei D în prevenire și recuperare, mai ales la pacienții cu recidivă a FFP.

Menționez că cercetările prezentate în teza de abilitare au fost efectuate în cadrul realizării unor teze de doctorat cât și a unor proiecte de cercetare obținute prin competiții naționale.

Secțiunea II prezintă o sinteză a realizărilor științifice, profesionale și academice cât și principalele planuri de dezvoltare a carierei pe cele trei direcții: științifică, academică și profesională.
Dezvoltarea carierei științifice include continuarea celor două teme principale și anume studiul implicării homocisteinei și a stresului oxidativ în patologie. Implicarea în patologie a homocisteinei va fi investigată în trei direcții noi și anume in tromboza venoasă profundă, în sarcină și in osteoporoză.
Este general acceptat faptul că homocisteina în concentrații crescute are un efect protrombotic. Toate etapele sarcinii, inclusiv nașterea și perioada post-partum, sunt, de asemenea, cunoscute ca fiind caracterizate de un status protrombotic. În plus, în sarcina contemporană care este asistată și caracterizată de vârsta maternă înaintată, starea protrombotică poate fi exacerbată de concentrații mari de homocisteină. Prezența concentrațiilor mari de homocisteină amplifică riscul trombotic și în alte boli, precum trombofilia ereditară care poate declanșa tromboză venoasă profundă și tromboembolismul sau în diabet și arterioscleroză în contextul infecției cu covid.
De asemenea, în cazul fracturilor de fragilitate care apar în primul rând la vârstnici, riscul trombotic crescut este prezent datorită mobilității reduse sau chiar abolite. Din nou, nivelurile ridicate de homocisteină pot amplifica severitatea acestei boli.

Prin urmare, studiul homocisteinei și implicarea acesteia în patologie este o direcție actuală și necesară de cercetare.

Secțiunea III conține referințele bibliografice aferente studiilor prezentate și încheie lucrarea.

Abstract

The habilitation thesis entitled "Involvement of analytical methods in the evaluation of physiological mechanisms and the determination of biochemical markers in pathology" is a synthesis of the most representative research directions developed during the postdoctoral period.

The paper aims to present the scientific activity after the presentation of my PhD thesis, entitled "Analytical methods for evaluating the bioavailability of active substances bound on a polymeric support", and the main prospects for the development of the academic, professional and research career.

The structure of the habilitation thesis follows the recommendations of the National Council for Attestation of University Titles, Diplomas and Certificates (CNATDCU) and is structured in three sections:
Section I includes a brief presentation of the scientific activity of the postdoctoral period.
Section II presents scientific, professional and academic achievements as well as future projects related to them.
Section III lists the bibliographic references used in the thesis.

In medicine, both in research and in practice, the assessment of the physiological state is done, depending on the levels of different biological parameters. The decisions made in the medical treatment, either it is surgical or medical, are also dictated by the concentrations of some biological parameters. Moreover, the adaptation of a therapy as well as the follow-up of a disease requires the correct determination of the concentrations of its markers. Therefore, the most accurate determination of the concentration of certain compounds, which are considered markers, is crucial in all phases of the medical act: diagnosis, treatment, evolution and healing.
Hence the need to develop more specific and sensitive methods to determine specific parameters as well as finding new ones with potential for early detection of diseases. In addition, the determination of concentrations in different biological environments (serum, plasma, urine, etc.) are the basis of bioavailability, pharmacokinetics and medical or pharmaceutical research studies. Based on pharmacokinetics, the mechanism of action can be confirmed or denied, the dose, the type of formulation, the administration schedule, the effectiveness, and the safety of a compound proposed as a therapeutic agent can be determined.
In conclusion, instrumental detection and quantification techniques have a decisive role in both medical practice as well as medical and pharmaceutical research. In order to be applied, the envisaged techniques and methods must go through the validation procedure. The aim of the validation process is to confirm the usefulness, precision and reproducibility of the method chosen for determination.
To validate an analytical method, a series of acceptance criteria must be fulfilled, such as: linearity, precision, accuracy ("recovery" or "matrix effect"), specificity and sensitivity. These requirements are established by specialized forums such as Food and Drug Administration (FDA), European Medicines Agency (EMEA) and International Conference of Harmonization (ICH).
A validated method that fulfills the stated criteria, will provide the result closest to the real value for the concentration of a compound in an environment/in a matrix.

The main directions that I approached in the scientific research activity, carried out after obtaining in 2000 the title of Doctor in the field of Chemistry, have as a common denominator which is the analytical methods for quantification. Thus, two directions were developed, as follows: first, the development of new methods for the determination of different compounds present in biological environments and second the use of analytical method for fundamental research purpose (mechanisms of action) or for applied research purpose (detection of the therapeutic potential of new synthetic compounds).

Section I includes four chapters that describe the research directions, their implementation, the results obtained and the method of disseminating the results.

The first research direction is entitled " Development and validation of chromatographic methods used for the determination of some biogenic amines from different biological environments" aimed to validate specific and sensitive analytical methods for the quantification of biogenic amines from different biological environments such as: rat serum, culture media, nutrients of animal origin.
Biogenic amines are endogenous compounds that fulfill different physiological roles. In high concentrations, however, they are responsible for severe adverse effects such as increased blood pressure or even anaphylactic shock. The increased danger of the presence of these compounds in the body comes from the fact that they can be simultaneously generated and introduced (through processed foods such as meat, cheese, wine) thus triggering serious adverse reactions. For this reason, their measurement as accurately as possible, requires the choice of analytical methods with the highest degree of sensitivity and specificity. Thus, a series of HPLC separation chromatographic methods coupled with different types of detection were selected depending on the matrix from which the amines were extracted, but also on the quantity in which amines are found in these matrices. Thus, two HPLC separation chromatographic methods were used, first, with mass spectrometry (MS) detection and second, with derivatization and fluorescence detection.
The HPLC/MS method was used for the individual/simultaneous quantification of four biogenic amines from two different matrices, serum taken from animals (rats) and bacterial culture media (called starter, which are used in food processing and which generate biogenic amines in this process). It was thus necessary to carry out two validations procedures: one for the quantification of amines in the serum and the other for their quantification in the culture media (Lactobacillus curvatus and Proteus mirabilis). The obtained results showed that the chosen method allows the simultaneous determination of biogenic amines from biological environments and may have practical use in toxicology.
Regarding the use of the two microorganisms in the processing of meat foodstuff, the obtained results showed that both of them generate moderate amounts of biogenic amines, especially cadaverine and putrescin but do not generate histamine. As a conclusion, the use of these microorganisms as starter cultures in food preparation processes is safe, and the control of the amines they generate can be achieved by the proposed method.
The second HPLC chromatographic method with derivatization and fluorescence coupled detection was used to determine the concentration of biogenic amines generated by the meat foodstuff. For this method, all the stages of the validation process were also completed. Once validated, the method was used for the simultaneous determination of biogenic amines in 28 different fresh or processed meat foodstuff. The obtained results confirmed the presence of increased amounts of biogenic amines in processed products compared to fresh ones.

As a conclusion, two types of chromatographic methods useful in individual or simultaneous quantification of biogenic amines were validated in both low concentrations (serum, culture media) and high concentrations (meat foodstuff samples). The methods are applicable both for diagnostic purposes in toxicological analyzes and in the detection of biogenic amines in starter cultures and meat foodstuffs.

The second research direction is entitled "Evaluation of homocysteine concentrations in different pathological processes and the possibility of therapeutic use of new synthetic products" has summarized several studies that aim to investigate the involvement of homocysteine in pathology. Homocysteine (Hcy) is a non-proteinogenic amino acid generated during the metabolism of a proteinogenic amino acid called methionine. Patients with high levels of Hcy in the blood, a situation called hyperhomocysteinemia (HHcy), develop thromboembolism, premature atherosclerosis, mental retardation, bone fragility, eye diseases; in pregnant women it can cause miscarriage. Although the effects of hyperhomocysteinemia were reported 70 years ago, its pathogenic mechanism has not yet been fully elucidated. Currently, it is considered that hyperhomocysteinemia affects the function of the vascular endothelium favoring thrombosis and it represents a much more severe cardiovascular risk factor compared to hypercholesterolemia. An increased level of Hcy associated with an already installed disease such as atherosclerosis or diabetes (endemic diseases in the civilized world) is considered an aggravating factor. The HHcy research included two aspects: firstly, to develop a model of HHcy on which to study the consequences of HHcy on certain risk factors (hypercholesterolemia, hyperglycemia) and secondly to investigate the potential of new synthetic compounds to reduce the concentrations of risk factors mentioned above. Thus, an experimental model of hyperhomocysteinemia was developed through the administration of methionine, in rats. In order to verify the installing of hyperhomocysteinemic status, homocysteine concentrations were determined through a HPLC chromatographic method with derivatization and UV detection. The selected method was subjected to the validation procedures. On this hyperhomocysteinemic model, cholesterol and blood glucose concentrations were evaluated; supplementary the effects of HHcy on the antioxidant system was assessed.
The antioxidant system in the body contains both enzymes (superoxide dismutase (SOD), glutathione peroxodase (GPx), catalase (CAT)) and circulating small molecules with antioxidant roles (glutathione (GSH), vitamin C, uric acid, bilirubin) that are known as total antioxidant capacity (TAC). The enzymes activity and the TAC concentrations in the context of HHcy were performed using standardized methods (for research use only).
The study has been continued by investigating the influence of two synthetic rutoside derivatives on the levels of all the above-mentioned parameters, in two experimental models made on rats (the HHcy model and the diabetes model). We mention that the studied derivatives were synthesized at the Pharmaceutical Chemistry discipline of the Faculty of Pharmacy, UMF "Grigore T. Popa" Iasi.
As a general conclusion of these studies, we can state that hyperhomocysteinemia affects the antioxidant system, more severely the intracellular one (in our case intra-erythrocytes) than the total circulating one (TAC). Regarding the synthetic compounds, they show an activity of preventing the increase of the homocysteine concentration rather than decrease it and a moderate action in lowering the blood sugar and cholesterol levels.

The third direction of research entitled “Investigation of the oxidative status in acute and chronic inflammation " aimed to assess some markers of inflammation and of antioxidant defense system,

in experimentally induced acute inflammation, in rats and in chronic inflammation, in human subjects in order to correlate them.

Redox phenomena is the basis of vital processes in the body, from energy generation to synthesis, defense, detoxification and communication processes. Recently, the redox system is considered to be involved in the inflammatory process, reactive oxygen species being responsible for the release of pro-inflammatory cytokines as a result of the activation of a group of receptors belonging to the immune system and called the inflammasome. Therefore, the changes in the antioxidant and the immune system were studied by determining the concentrations of specific markers on an acute inflammation model made on rats by injecting carrageenan in the hind paw. The same parameters were followed in another study conducted on human subjects with chronic inflammatory diseases of the middle ear. Thus, to investigate the oxidative status, the activity of SOD, GPx, CAT enzymes, TAS and malondialdehyde (MDA - a faithful marker for the extent of damage caused by oxidative stress) concentrations were determined. To assess the immune system response, the concentrations of some interleukins IL-1, IL- 6 and IL-8 was also determined. Both types of parameters were determined using standardized kits for research use.

The study carried out on the model of acute inflammation started from the current considerations regarding the inflammatory nature of atherosclerosis and the use of NO donors in its therapy. Current literature mentions the ambivalent, anti-inflammatory and pro-inflammatory, activity of NO donors depending on the physio-pathological conditions. The influence of some new NO donors nebivolol (NEB) and S-nitroso-glutathione (GSNO) on the oxidative and inflammatory status as well as the effects of their administration on the liver function was investigated in the inflammatory model, performed on the rat paw. Nebivolol is already introduced in therapy while S-nitroso-glutathione a NO donor, similar to those used in the cardiovascular diseases therapy, is in the testing phase. The data obtained suggest the anti-inflammatory potential of nitric oxide donors as close to that of indomethacin (IND). Moreover, S-nitroso-glutathione presents an additional benefit in anti-atherogenic therapy, because its structure gives it the role of a NO deposit.

As a conclusion, the results obtained were good because both NEB and GSNO showed clear anti-inflammatory effects (although somewhat less intense than those of IND) and also decreased the extent of oxidative stress. Although, overall, NEB had a more intense effect in reducing inflammatory and oxidative parameters, S-nitroso-glutathione showed a much lower liver and kidney toxicity.

The variation of markers of both inflammation and oxidative stress was studied on human subjects in chronic diseases of the middle ear, namely: chronic suppurative otitis with or without cholesteatoma or relapse. The investigation of the oxidative status markers indicated significant changes for all of them in all three types of middle ear diseases. The intensity of the oxidative attack was confirmed by the increased levels of malondialdehyde (MDA), the final product of lipid peroxidation. The investigation of inflammation markers indicated a significant increase in their levels in patients with chronic middle ear disease compared to normal, with some variations depending on the stage of the disease.

The statistical analysis of the levels of inflammatory markers have indicated a moderate, direct correlation with the activity of the enzymes belonging to the antioxidant defense system. An indirect correlation was observed between inflammation markers and total antioxidant capacity (TAC). Therefore, the obtained data confirm the impairment of the redox balance in the inflammation process and the interconnection of the two processes.

The obtained data suggest the possibility of using drugs to interfere the activity of oxidizing species thus influencing the levels of inflammatory markers and implicitly the therapeutic behavior.
Moreover, the level of interleukin IL-6 is a predictor of epithelial proliferation that can serve to identify the stage of cholesteatoma, and the level of IL-1 is a predictor of the degree of tissue destruction. Thus, the associations of these two interleukins can help to choose the surgical approach regarding: the operative technique, the degree of preservation of the auditory function, or the tympanoplasty.
As a conclusion, the parameters of oxidative stress correlated with those of inflammation can be useful in the therapeutic decision.

The fourth research direction entitled "Investigation of calcium and vitamin D status in osteoporotic fracture."
Currently, in developed countries, atherosclerosis, diabetes and osteoporosis are considered the most widespread diseases in adulthood. Fragility fracture is the most common bone disease in the elderly. It mainly affects the vertebrae, femur, proximal humerus, distal radius, pelvis, thus preventing the patient's free movement. The most debilitating of all these fractures is the fragility fracture of the pelvis. Choosing the right treatment for FFP is difficult because both conservative and surgical therapy involve equally high risks: prolonged immobilization and/or surgical risks. Therefore, pharmacological therapy is an alternative to surgery. Bisphosphonates have proven their usefulness in anti-osteoporotic therapy and their contribution is recognized. Instead, the influence of calcium and vitamin D are relatively neglected either by the patient's non-compliance in the case of conservative treatment or by prioritizing the surgical result in the case of the surgical approach. The aim of the study was to evaluate the role of calcium and vitamin D in the fracture healing process in osteoporotic patients. The obtained results show that both calcium and vitamin D exert a positive influence on the healing process of fragility fractures and significantly emphasize the need to educate patients to comply with the prescription to supplement calcium and vitamin D in order to improve FFP healing.
As a conclusion, the obtained data indicate the benefit of calcium and vitamin D administration in prevention and recovery, especially in patients with recurrent FFP.

I would like to mention that the research presented in the habilitation thesis was carried out as part of the realization of doctoral theses as well as research projects obtained through national competitions.

Section II presents a synthesis of scientific, professional and academic achievements as well as the main career development plans in the three directions: scientific, academic and professional.
The development of the scientific career includes the continuation of the two main themes, namely the study of the involvement of homocysteine and oxidative stress in pathology. The involvement of homocysteine in pathology will be investigated in three new directions, namely: in deep venous thrombosis, in pregnancy and in osteoporosis.
It is generally accepted that homocysteine in elevated concentrations has a prothrombotic effect. All stages of pregnancy, including delivery and the postpartum period, are also known to be characterized by a prothrombotic status. Furthermore, in contemporary pregnancy that is assisted and characterized by advanced maternal age, the prothrombotic state may be exacerbated by high homocysteine concentrations. The presence of high concentrations of homocysteine amplifies the thrombotic risk in other diseases such as hereditary thrombophilia that triggers deep vein

thrombosis and thromboembolism or in diabetes and atherosclerosis in the context of covid infection.
Also, in the case of fragility fractures that occur primarily in the elderly, the increased thrombotic risk is present due to reduced or even abolished mobility. Again, high levels of homocysteine can amplify the severity of this disease.
Therefore, the study of homocysteine and its involvement in pathology is a current and necessary direction of research.

Section III contains the bibliographic references related to the presented studies and the conclusion of the paper.

Section I: Scientific achievements in the postdoctoral period (2001-2022)

The habilitation thesis has as central point the involvement of analytical methods used to quantify different compounds present in biological environments. In order for a proposed method to be applied, it must meet certain conditions that certify the correctness of the method. The verification of the fulfillment of these imposed conditions is called the validation process.
During the doctoral thesis work I acquired the expertise in the analytical validation procedure, thus I will make a short presentation (half a page) of the doctoral thesis.
The doctoral thesis has been entitled "Analytical methods for estimating the bioavailability of active substances bound on a polymeric support" and aimed to validate methods for determining the concentration of two active substances: nicotinic acid and diclofenac in order to estimate their bioavailability after oral administration in rats. The two active compounds were chemically bound (by different chemical bonds) to a dextran polymeric support. The purpose of polymer binding was to develop new pharmaceutical formulations in order to improve the pharmacokinetics of these compounds such as: avoiding the fluctuating profile of the active substance concentrations in blood, the prolonged release in the interval of effective therapeutic concentration, the removal of some side effects. Following the study, two polymeric conjugates showed an improved pharmacokinetics and therefore increased therapeutic potential, one for diclofenac and one for nicotinic acid. I mention that the validated method for the determination of diclofenac was implemented, at that moment, at the Antibiotice Iasi pharmaceutical company.
The pharmacokinetics of a drug involve the determination of the following parameters: maximum concentration C max (µg / ml), maximum time Tmax (h), half-life T1/2 (h), area under the AUC curve (µg.h / ml), elimination constant -Ke (h-1). The calculation of all these parameters, which allow the evaluation of pharmacokinetics and therapeutic efficacy, requires the accurate determination of the blood and urinary concentrations of the administered substances. Obtaining precise concentrations involves the use of specific and sensitive, validated analytical methods. Therefore, the aim of the thesis was to standardize methods for determining the two substances in the blood, urine but also in artificial dissolution medium of the polymeric drug system.
To validate an analytical method, a series of acceptance criteria must be met, as follows: linearity, precision, accuracy ("recovery" or "matrix effect"), specificity and sensitivity. In the following, the validation criteria for the chromatographic separation methods according to [1] will be briefly presented.
Linearity: for this parameter the slope, the intercept and the correlation coefficient are required and they must be between 0.98 and 1.00. The regression line represented as an average RSD (relative standard deviation) or CV% (coefficient of variation) must not be greater than 2%. The intercept of the regression line must not differ significantly from zero, ie the value of the intercept must not exceed 2% of the corresponding value of the intercept for 100% substance**.**
Accuracy: requires at least 5 replicates at three concentrations in the target range (extreme and medium concentrations). The mean of the retrieval in the samples, of the amount of analyte added may vary in the investigation phase by ± 20% of the value, and for the preclinical and clinical phase by ± 10% of the value. Alternatively, it is recommended that the average retrieval be within ± 15% of the value, except for the limit of quantification where coefficients of variation of ± 20% are allowed. The accuracy is required intra-day and inter-days as well.
Specificity: techniques using UV detection require that the peak spectrum of the studied substance match the spectrum of the standard. The maximum absorption of the analyte must be the same as that of the reference substance ± 2 nm. The peak spectrum of the substance studied in the sample

must not be visually different from that of the standard on different portions with a relative absorbance greater than 10%. If the absorbance of the peak at two or more wavelengths, determined for the treated and untreated sample, differs by a maximum of 5%, the peak is considered pure. For the mass spectrometry (MS) the specificity is based on the equivalence of the mass-to-charge ratio (m/z) between the sample compared and the pure substance.

Sensitivity: represents the minimum quantifiable quantity or limit of quantitation (LOQ). This is the minimum amount of the substance in a test sample that we can determine with acceptable repeatability and accuracy. It is different from the minimum detectable amount or limit of detection (LOD), which is the lowest concentration of the substance studied in a test sample that we can easily distinguish from zero, considering a signal that is three times higher than the background noise.

Chromatographic methods are characterized by increased sensitivity and specificity. From this reason they are perfectly suitable for determinations in biological environments, which are complex environments and in which a large number of compounds and their metabolites are present simultaneously.

In the research activity, including the postdoctoral one, I used chromatographic methods in identifying and quantifying compounds of interest such as: biogenic amines or homocysteine. The quantification of these compounds requires the selection and validation of the method of analysis as well as the extraction method from the matrix in which the compounds of interest are found. These matrices can be: blood, urine, culture medium or other matrices such as food. In the Chapter I there will be presented the chromatographic methods used to determine the biogenic amines from different matrices and their applications. The chromatographic method used for homocysteine determination will be presented in Chapter II.

Chapter I. Development and validation of chromatographic methods used for the determination of some biogenic amines from different biological environments

I.1. Development and validation of some biogenic amine's determination in rats' serum and culture mediums, using a HPLC/MS method.

I.1.1. Introduction

Biogenic amines are compounds produced by human metabolism [2,3] which, in small concentrations, exert physiological roles such as regulation of body temperature and blood pressure. In high concentrations, biogenic amines can become toxic causing headaches, blood pressure spikes, life-threatening reactions [4] or even death [5,6].

In addition to the in vivo generation, biogenic amines result from technological processes for the preparation and preservation of food products such as: milk, fish, beer, cheese, chocolate, and red wine [7-10]. The alteration of proteins, which are present in food, also generates biogenic amines as a consequence of microorganism's activity. If the total amount of amines presents in the body reach concentrations higher than normal, severe side effects can be triggered. Thus, due to their potential toxicity, there is a constant interest in the monitoring biogenic amines concentrations in booth food and biological environments. Therefore, quantification of biogenic amine in both food and biological fluids is of major interest.

As a consequence, literature contains many methods for determining the biogenic amine concentrations, such as thin layer chromatography, gas chromatography, capillary electrophoresis,

high performance liquid chromatography and immunological assay. HPLC with pre-or post-column derivatization is the most used method for separation and quantification of biogenic amines with high sensibility and specificity [11-18]. At the moment, the most advanced method of separation and quantification of compounds mixtures, including biogenic amines, is represented by high-performance chromatography (HPLC) coupled with mass spectroscopy (MS) detection.
The study on biogenic amines, aimed to identify and quantified them in food, in culture media used in food processing but also in biological fluids. This required the selection and validation of very specific and sensitive methods as biogenic amines may be present in very small quantities. The selected amines most commonly found in foods and culture media are histamine, cadaverine, tyramine and putrescine.
The study aimed to validate the extraction and quantification methods of these four biogenic amines from serum taken from rats, culture media and meat food. In order to determine the amines in these different matrices, it was necessary to select the most appropriate method to be able to separate and quantify the amines that usually are simultaneously present.
Thus, for the determination of biogenic amines in serum and culture media, a method of separation by high performance liquid chromatography (HPLC) followed by mass spectrometry detection was adapted and modified (M.C. Gennaro et all [19].
For the determination the concentration of histamine, cadaverine and tyramine in meat food, a method of chromatographic separation with pre-column derivatization and UV detection was adapted and modified from Lange, Thomas and Wittmann [12].
The procedures for separation and quantification of the studied biogenic amines were validated according to the validation guidelines for the analytical methods of the FDA (Food and Drug Administration, USA) [20]; EMEA (European Agency for the Evaluation of of Medicinal Products on Evaluation of Medicines for Human Use) [21] and the Standard Operating Procedures of the Bioequivalence Laboratory of the Antibiotice Iaşi pharmaceutical company.

I.1.2. Determination of biogenic amines in rat serum by high performance liquid chromatography coupled with mass spectrometric detection (HPLC-MS)

Aim: To develop and validate a sensitive and specific method for the biogenic amine's determination in serum (rat serum).

In order to determine the biogenic amines in rat serum the standard and the calibration curves were performed:

I.1.2.a. standard curves for histamine, cadaverine, tyramine, putrescin individually and in mixture.

I.1.2.b. calibration curves for the determination of the studied amines following their extraction from rat plasma.

I.1.2.a. Plotting the standard curves for histamine, cadaverine, tyramine, putrescin individually and in mixture.

Materials and Methods

Reagents and standard solutions

Cadaverine, histamine, dihydrochloride putrescine, tyramine, formic acid, ammonia (25%), perchloric acid were acquired from Fluka. Methanol and water Chromasolv (HPLC purity),

sodium metabisulphite is purchased from Sigma-Aldrich. Standard solutions of biogenic amines, 1 mg/mL concentration, were prepared in perchloric acid and kept at -20^{o}C for 1week. Working solutions was prepared by dilution in perchloric acid.

Instruments
The analyses were performed on an Agilent 1200 HPLC 6520-Accurate-Mass Q Tof LC/MS with Agilent 1200 Binary Pump and C18 column. For pH measurements a pH-meter Sension 1 was used. An Eppendorf Centrifuge and Ultra-Turrax homogenizer was used for rat plasma preparation.
HPLC-MS conditions
The stationary phase was ZORBAX SB C18 column (150 x 4,6mm, particle size 5µm). The mobile phase was composed of two solutions: solution A methanol and solution B a mixture of formic acid and ammonia (15 mM NH_3, 15 mM HCOOH) in ultra-pure water, bring to a final pH=4. The mobile phase was eluted under gradient conditions: time=0 min 10% solution A, 90% solution B; time=5min 60% solution A, 40% solution B, at a flow rate of 1 mL/min. The injection volume was 10µL. Mass spectrometer was equipped with an electrospray (ESI) ion source, with nitrogen gas (drying gas), temperature of 325^{o}C, flow nitrogen gas 7 L/min, pressure nebulizer gas 30 psi, capillary potential 4500 V, capillary temperature 325^{o}C and range m/z was 50-300. The interface was operated only in positive ion ionization mode.
Produced ions used for quantitative purposes to the following mass/charge ratio:
Histamine m/z = 112
Tyramine m/z = 121
Cadaverine m/z = 103
Putrescine m/z = 89

Standard curves preparation
Stock solutions of the four amines at 1000 mg / L were prepared by dissolving 5.0 mg histamine, 5.0 mg tyramine, 5.0 mg cadaverine and 5.0 mg putrescin in 5 mL trichloroacetic acid.
Successive dilutions of the stock standard solutions for the four amines were made in trichloroacetic acid, thus resulting working standard solutions with the following concentrations: 10 ng / mL, 50 ng / mL, 100 ng / mL, 200 ng / mL, 400 ng / mL. Aliquots of 1 ml of each working standard solution was transferred in glass centrifuge tubes that contain 0,5 mL EDTA-metabisulphite solution (20 g EDTA and 10 g sodium metabisulphite/L) and centrifuged 10 min at 4000 rpm. Half mL was then transferred in Eppendorf tubes which contained 0,5 mL perchloric acid; allowed to rest for 10 min and then centrifuged 20 min at 13000 rpm. Aliquots of 10 µL were injected into the HPLC-MS system.

Results and Discussion
The area of the peaks obtained was used to make the standard curve for each amine. The equation of the regression curve and the correlation coefficient r2 were calculated. The validation parameters, presented below, have been determined.
Retention times (Rt) for the individual biogenic amines are: histamine 1,639 min, tyramine 2.288 min, cadaverine 4.248 min and putrescine 1.607 min. Different and distant retention times allow the simultaneous separation of histamine, tyramine, cadaverine and putrescine without their interference. Therefore, the method is selective.
Chromatograms obtained after determination of the studied amines are shown in figures 1-4.

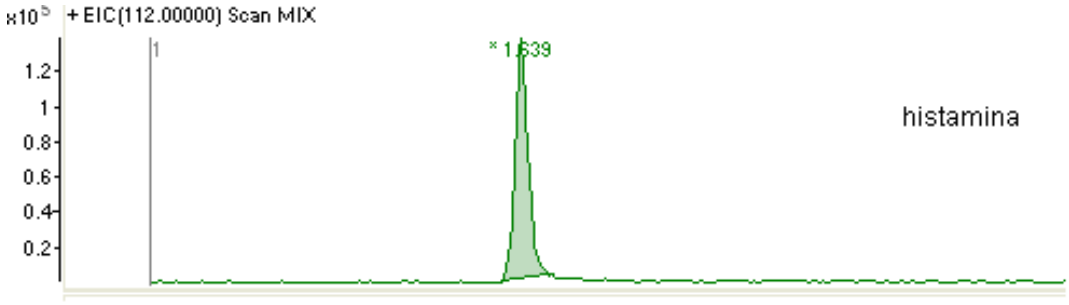


Fig.1. Chromatogram obtained for a histamine sample at a concentration of 100 ng / mL

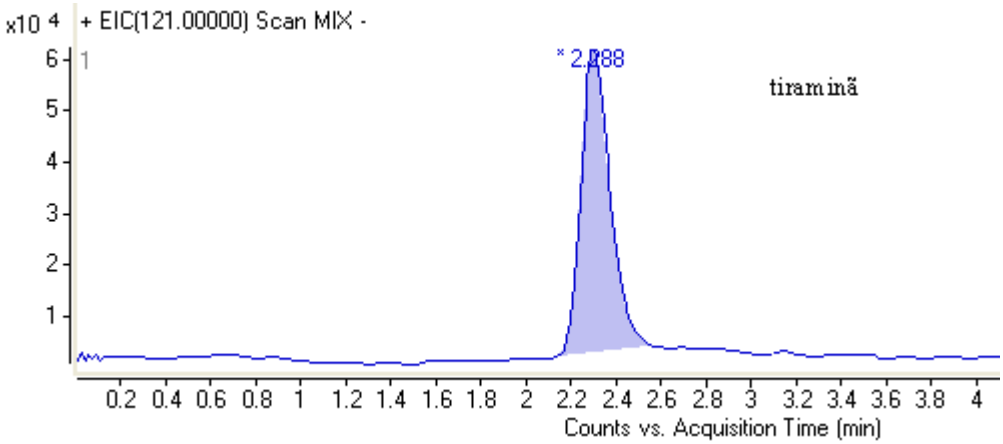


Fig. 2. Chromatogram obtained for a tyramine sample at a concentration of 100 ng / mL

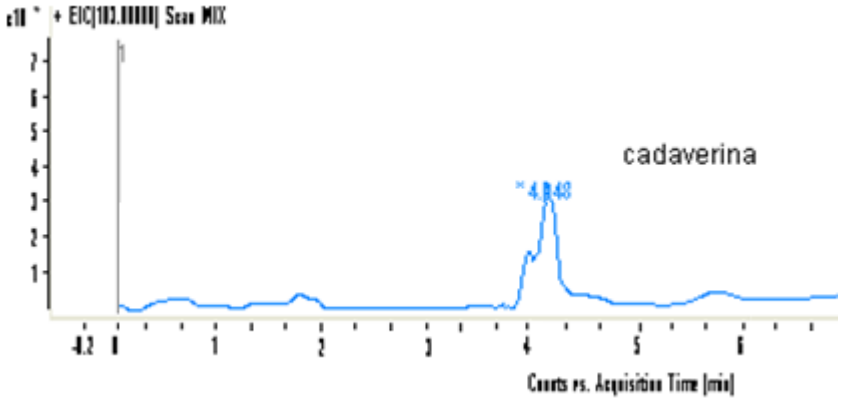


Fig.3. Chromatogram obtained for a cadaverine sample at a concentration of 100 ng / mL

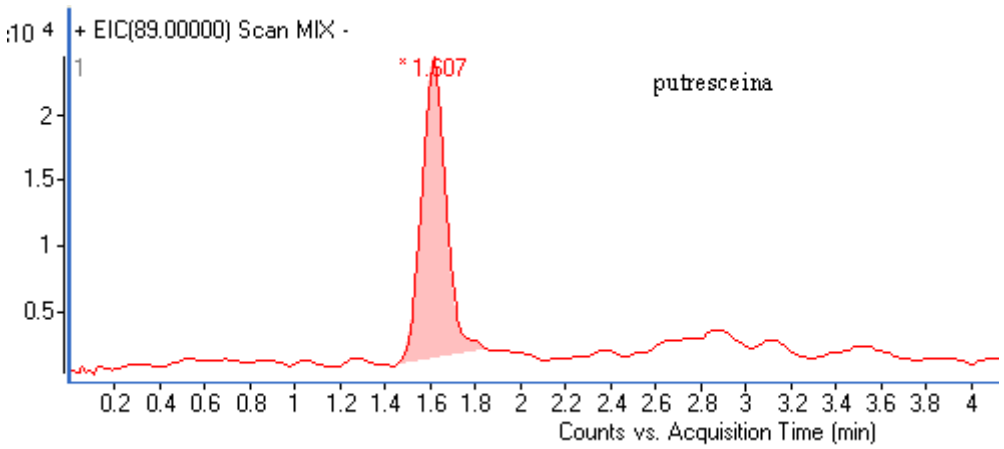


Fig.4. Chromatogram obtained for a putrescine sample at a concentration of 100 ng / mL

*For histamine***:**

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is Y = 890.4x + 10500 with a correlation coefficient r^2 = 0.993. Linearity range is between: 14.5-1000 ng / mL

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 2%, which fits in the range requested for the accuracy of the analytical method.

c) Accuracy: different histamine concentrations of 10, 50, 200 ng / mL were analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤1%, is met for the concentrations used. The proposed method meets the intra-day accuracy criterion, the values obtained for RSD% 0.680463; 0.702518; 0.567549 being less than 1. The inter-day the values obtained for RSD% are 0.803; 0.637 for the first and the second day, respectively also in the range criterion. For retention time the acceptance criterion requires that the relative deviation of RSD% be a value ≤ 2%. The chosen method meets the criterion of intermediate precision and reproducibility for the retention time, the values obtained for RSD% being of 0.639659; 1.201965 for the first and the second day, respectively, both being smaller than 2.

d) Limit of detection: 2,60 ng/mL

e) Limit of quantitation: 5,06 ng/mL

f) Selectivity: retention times (Rt) for histamine is 1,639 min distant from the other tree amine, therefore the method is selective.

For tyramine:

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is Y= 443,6x+2192, with a correlation coefficient r^2 = 0.997. Linearity range is between: 6,023-1000 ng/mL

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 2%, which fits in the range requested for the accuracy of the analytical method.

c) Accuracy: different tyramine concentrations of 10, 50, 200 ng / mL were analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤1%, is met for the concentrations used. The proposed method meets the intra-day accuracy criterion, the values obtained for RSD % 0,896326; 0,618504; 0,380889 being less than 1. The inter-day the

values obtained for RSD % 0,804396; 0,72903 for the first and the second day, respectively also in the range criterion. For retention time the acceptance criterion requires that the relative deviation of RSD% be a value ≤ 2%. The chosen method meets the criterion of intermediate precision and reproducibility for the retention time, the values obtained for RSD% being of 1.344425; 1.096625 for the first and the second day, respectively, both being smaller than 2.

d) Limit of detection: 0,26 ng/mL

e) Limit of quantitation: 0, 50 ng/mL

f) Selectivity: retention times (Rt) for tyramine is 2.288 min min distant from the other tree amine, therefore the method is selective.

*For cadaverine***:**

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is Y = 36.64x+1183 with a correlation coefficient r^2 = 0.992. Linearity range is between: 40,70 – 1000 ng/mL

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 2%, which fits in the range requested for the accuracy of the analytical method.

c) Accuracy: different cadaverine concentrations of 10, 50, 200 ng / mL were analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤1%, is met for the concentrations used. The proposed method meets the intra-day accuracy criterion, the values obtained for RSD % are 0,907304; 0,556672; 0,644441 being less than 1. The inter-day the values obtained for RSD% are 0,933812; 0,85009 for the first and the second day, respectively also in the range criterion. For retention time the acceptance criterion requires that the relative deviation of RSD% be a value ≤ 2%. The chosen method meets the criterion of intermediate precision and reproducibility for the retention time, the values obtained for RSD% being of 0.57127; 0.901978 for the first and the second day, respectively, both being smaller than 2.

d) Limit of detection: 0, 34 ng/mL

e) Limit of quantitation: 0,51 ng/mL

f) Selectivity: retention times (Rt) for cadaverine is 4.248 min distant from the other three amine, therefore the method is selective.

For putrescine:

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is Y = 156.8x+7261 cu un coeficient de corelaţie R^2=0.9907. Linearity range is between: 51.78-1000 ng/mL

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 2%, which fits in the range requested for the accuracy of the analytical method.

c) Accuracy: different putrescine concentrations of 10, 50, 200 ng / mL were analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤1%, is met for the concentrations used. The proposed method meets the intra-day accuracy criterion, the values obtained for RSD% 0,694; 0,694; 0,532 being less than 1. The inter-day the values obtained for RSD% are 0,483412; 0,438595 for the first and the second day, respectively also in the range criterion. For retention time the acceptance criterion requires that the relative deviation of RSD% be a value ≤ 2%. The chosen method meets the criterion of intermediate precision and

reproducibility for the retention time, the values obtained for RSD% being of 1.625877; 1.375309for the first and the second day, respectively, both being smaller than 2.

d) Limit of detection: 1,02 ng/mL

e) Limit of quantitation: 2,05 ng/mL

f) Selectivity: retention times (Rt) for putrescine is 1.607 9min distant from the other tree amine, therefore the method is selective.

The chromatograms obtained after the injection of the eluent and of the the biogenic amines, the amines do not interfere either with the solvent or with each other, which proves that the separation is efficient and selective. Every biogenic amine is determined distant from both the solvent front and the three other amines areas.
The chosen method is selective, allowing the simultaneous separation of all biogenic amines without coelution.

I.1.2.b. Plotting the calibration curves to determine the four studied amines following their extraction from rat plasma.

Aim: To develop and validate a sensitive and specific method for the biogenic amines determination after being extracted from rat serum.

Materials and Methods
For the calibration curves of histamine, tyramine, cadaverine and putrescine, increasing standard concentrations for each of them were added to serum samples taken from rats and then prepared in a similar way to the standard aliquots used for standard curves.
Adult Wistar male rats (120-200 g) were used and treated according to the standards of the European Legislation. All procedures were performed according to the European legislation concerning the care and use of animals for scientific purposes (Directive 86/609/EEC). Whole blood was collected in vacutainer tubes containing heparin and aliquots of 1 mL was immediately transferred in glass centrifuge tubes with 0,5 mL EDTA-metabisulphite solution (20 g EDTA and 10 g sodium metabisulphite/L) to be centrifuged 10 min at 4000 rpm. Half mL of plasma was transferred in Eppendorf tubes which contained 0,5 mL perchloric acid; allowed to rest for 10 min and then centrifuged 20 min at 13000 rpm. The supernatant was removed and stored at -70^{0}C until analyzed.
Calibration curves
To draw the calibration curves, to 1 ml of blood collected from rats, increasing concentrations of each of the amines were added. Amine's concentration was identical to those used for the standard curves. A blood sample to which no amines were added was the control. The samples together with the control were then processed according to the procedure presented above. Then aliquots of 10µL were taken and injected into the HPLC-MS system. The area of the peaks obtained was used to draw the calibration curve for each amine alone and also in mixture. The equation of the regression curve and the correlation coefficient R^2 were calculated. The validation parameters, which are presented below, have been determined.

Results and Discussion

Mass spectrum obtained from free (a) and spiked rat plasma with putrescine (b), histamine (c), tyramine (d) and cadaverine (e) respectively, are presented in figures 5 (a, b, c, d, e).

Biogenic amines identification was made based on mass / load ratio (m / z).:

Histamine m/z = 112

Tyramine m/z = 121

Cadaverine m/z = 103

Putrescine m/z = 89

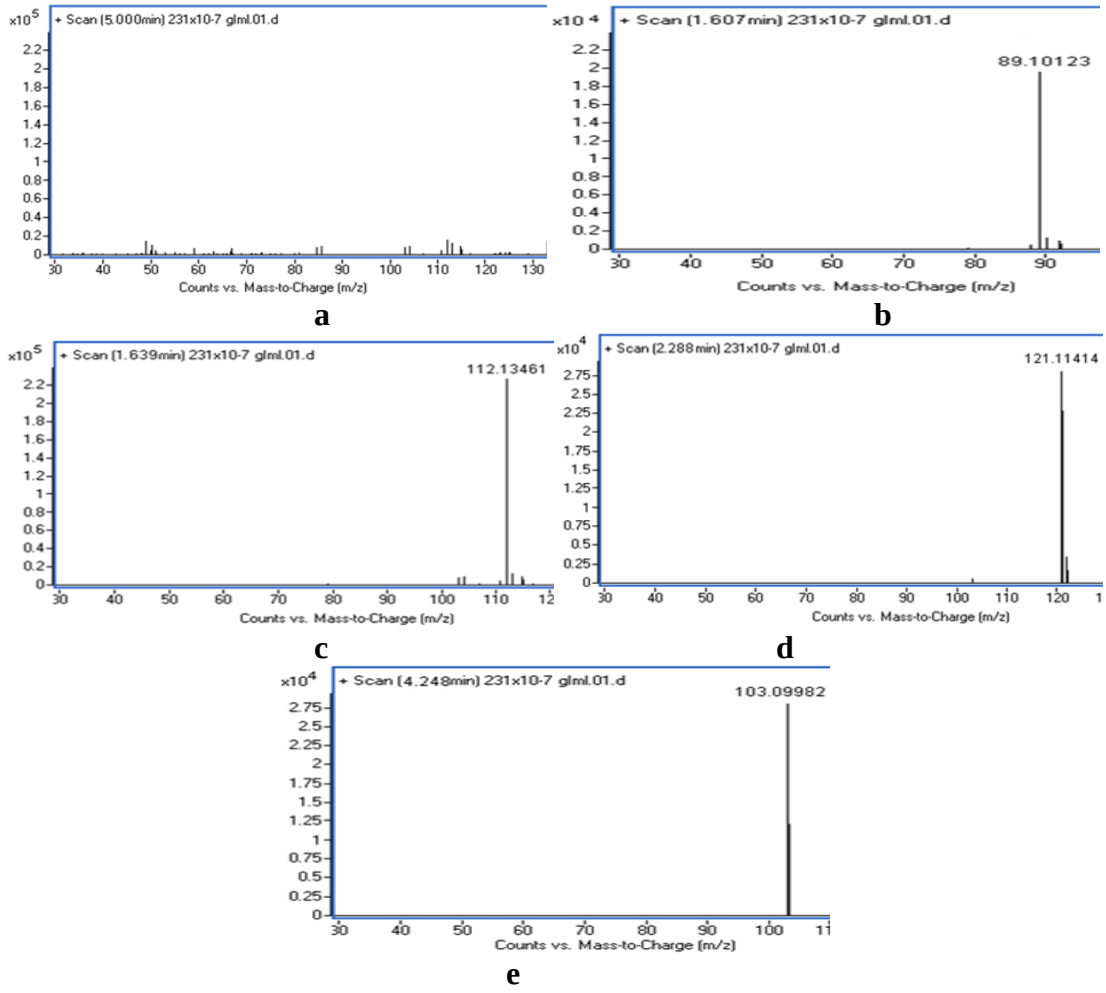


Fig.5. Mass spectrum for free plasma (a) and plasma spiked with biogenic amines: putrescine (b), histamine (c), tyramine (d) and cadaverine (e).

No detectible signals were found at the m/z ratio of the corresponding amine in the free rats' plasma. The retention times for individual amines from the rats' plasma spiked with amine mixture are presented in figure 6. Individual amines are well separated as they eluted at different retention time (RT) in minutes as follows:1.607 for putrescine, 1.639 for histamine, 2.288 for tyramine, 4.248 for cadaverine.

The amines mixture is eluted over a period of five minutes which means a reasonable time measurement per sample. The reproducibility for the retention times and for the peak areas was 96.55% and 92% respectively.

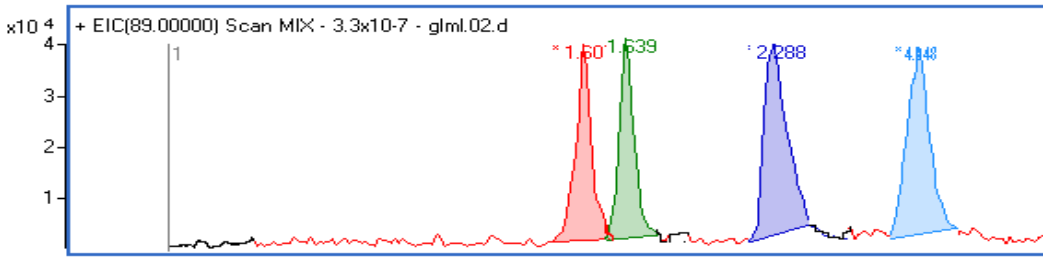


Figure 6. Chromatogram obtained from rats' serum spiked with the amine mixture.

The calibration curves for individual amines were realized by plotting each amines peak area versus concentrations in ng/mL. The linear regression equations, coefficient of determination (r^2), linearity range, detection limits (LOD) and quantification limits (LOQ) for each amine are presented in table I.

Table I
The linear regression equations, coefficient of determination (r^2), linearity range, detection limits (LOD) and quantification limits (LOQ) for individual amines

Eluted compound	Quantifier transition (m/z)	Linear regression calibration curve	r^2	Linearity range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
P	89-72	Y=142.6 X+ 6546.8	0.9995	2-50	1.5	2
H	112-95	Y= 870.8 X- 4341.6	0.9992	5-100	3	5
T	138-121	Y=370.2 X-74.62	0.9985	0.5-10	0.2	0.5
C	103-86	Y=28.85 X+8.186	0.9994	0.5-20	0.2	0.5

To evaluate the intra-day and inter-days accuracy, precision and recovery (R), the amine mixture was eluted in four replicates for low, medium and high concentrations added to plasma samples from the standard working solution. The accuracy was expressed as Bias% (relative difference between found and spiked concentration) and precision as CV% (coefficient of variation). The intra-day and inter-days run accuracy, precision and recovery for putrescine, histamine, tyramine and cadaverine are presented in table II.

Table II
Intra-day and inter-days runs accuracy, precision and recovery for amines

	Intra-day runs				Inter-days runs			
Added conc.	Mean found conc. (ng/mL ± SD)	Bias (%)	CV (%)	Mean Recov. (% ± SD)	Mean found conc. (ng/mL ± SD)	Bias (%)	CV (%)	Mean recov. (% ± SD)

				Putrescine				
2	2.1±0.06	7.1	2.8	107.1±3.0	2.17±0.01	8.8	0.8	108.8±0.8
5	5.2±0.11	5.0	2.1	105.1±2.2	5.31±0.02	6.2	0.4	106.3±0.4
50	50.6±0.7	1.5	1.4	101.5±1.4	51.7±0.83	3.4	1.6	103.4±1.7
				Histamine				
5	5.2±0.16	4.9	3.2	104.9±3.3	5.3±0.10	6.8	1.9	106.8±2.3
50	51.2±1.12	2.5	2.2	102.5±2.2	52.7±1.9	5.4	3.7	105.4±4.5
100	102.4±2.3	2.4	2.2	102.4±2.3	104.6±2.7	4.6	2.6	104.6±3.1
				Tyramine				
0.5	0.55±0.01	10.4	3.0	110.4±3.4	0.54±0.04	9.8	7.4	109.8±8.2
1	1.08±0.02	7.9	1.7	107.9±1.8	1.06±0.02	5.8	2.4	105.8±2.6
10	10.6±0.17	5.8	1.6	105.8±1.7	10.3±0.17	3.4	1.6	103.4±1.7
				Cadaverine				
1	1.07±0.02	7.8	2.2	107.8±2.4	1.10±0.03	10.5	2.3	110.5±2.5
10	10.39±0.2	4.0	1.8	103.9±1.9	10.75±0.3	7.5	3.2	107.5±3.5
20	20.60±0.8	3.0	3.7	103.0±3.9	21.39±0.6	7.0	2.7	107.0±2.9

After being validated the method was used to determine the levels of biogenic amines in biological samples from **rats' serum** as well as in **bacterial culture media**.

Conclusions

Determination of biogenic amines is of real interest due to their involvement in numerous diseases. The analytical proposed method is very useful as it allows to simultaneously determinate histamine, cadaverine, tyramine and putrescine in biological fluids in our case rat plasma. The modified HPLC/MS method for the separation and quantification of histamine, tyramine, cadaverine and putrescine from plasma have the advantage of avoiding the time-consuming derivatization step. This technique also offers the advantage of a reduced retention times compared to other analytic methods used for the determination of the biogenic amines. Thus, this method ensures a reasonable analysis time.

The validated method was published in Farmacia, 2018;66(3):548-52 (the title of the work, the authors and the complete data are presented at the end of the chapter, pg 48).

I.1.3. Determination of histamine and tyramine levels following their administration in rats.

The previous analytical method was used to assess the levels of some biogenic amines after their administration in rats.

Aim: The study aimed to evaluate the level of histamine and tyramine following their administration in rats in order to corelate it with the concentrations of reactive oxygen species (ROS). The levels of intra-cellular (GPx, SOD) and extracellular (TAS) antioxidant defence systems were investigated and will be presented in paragraph III.4

Material and Method

Wistar white rats weighing 220-250 g were used. The animals were purchased from the Cantacuzino Institute Bucurest, and kept under standard laboratory conditions. The animals were kept in cages of appropriate size according to current rules; ventilation conditions were also ensured. The amines histamine and tyrosine that trigger the strongest documented adverse reactions in the human body were selected for study and were acquired from Fluka. For the chromatographic determination the previously present method (and which was the object of validation) was used.

Rats were randomly divided into 3 groups (of 10 animals each). Each group was its own witness and they received histamine and tyramine as follows:

- group I - 10 mg / kg histamine, i.p., single administration
- group II - 10 mg / kg tyramine, i.p., single administration
- group III - 5 mg / kg histamine, 5 mg / kg tyramine, single administration

The experiment complied with the current legislation regarding the working with experimental animals and obtained the agreement of the Ethics Commission of the University of Medicine and Pharmacy "Gr.T.Popa" Iasi.

Animals were anesthetized with ketamine (75 mg / kg i.p.) and blood samples were collected from the retroorbital plexus initial, 24 and 72 hours after amine administration under anesthesia. The concentrations of histamine and tyramine in rat serum sample were determined. The total antioxidant activity in the serum were also determined.

To determine the histamine and tyramine concentration the previously validated HPLC-MS / MS method was used.

To assesse the response of the antioxydative defence system standardised Kits for research were used. The obtained results are presented in the paragraph III.4.

The ANOVA one way test and the Tukey test for post-hoc analysis were used for the statistical interpretation of the results. Results were expressed as mean ± standard deviation. The value of $p < 0.05$ was considered significant.

Results and Discussion

Chromatograms obtained after HPLC-MS / MS analysis obtained from rats serum are shown in figures 7-10.

Following the HPLC-MS / MS analysis of the serum samples, only the histamine and tyramine concentrations were identified and determined, cadaverine and putrescine were not found.

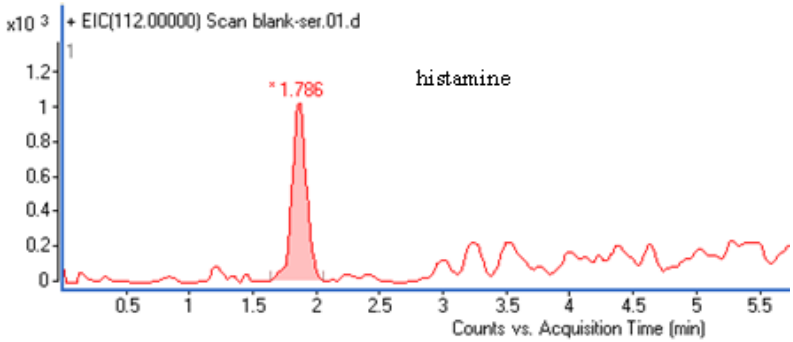


Fig.7. Chromatogram obtained from naive rat that did not receive any amine.

At initial moment, before receiving any amines only histamine was identified; it represents the physiological concentration present in the blood in very small amounts. At the initial moment, tyramine, putrescine and cadaverine were not detected in both group I and groups II and III because in the physiological state all three amines are not present in detectable amounts.
Groups I received only histamine; at 72 hours after histamine administration cromatogram obtained from group I is presented in figure 8. Tyramine, putrescine and cadaverine were again not detected.

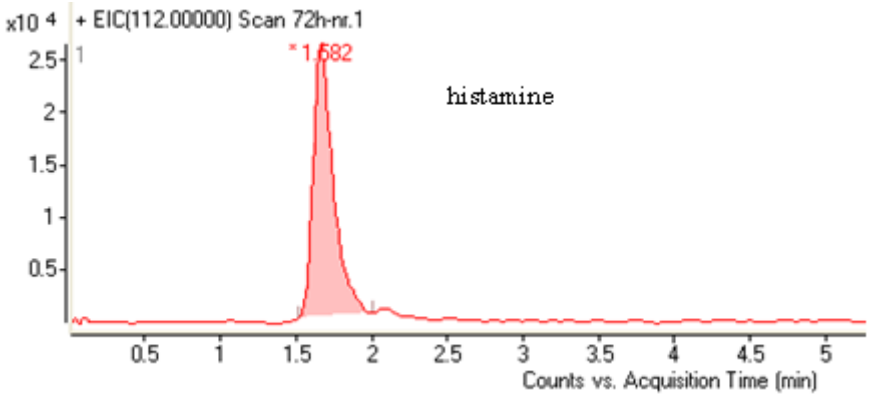


Fig.8. Chromatogram obtained for serum sample collected from group I, 72 hours after histamine administration (10 mg / kg body weight).

Groups II received only tyramine; chromatograms obtained 72 hours after tyramine administration is presented in figure 9. As can be seen together with tyramine, histamine is also detected due to its physiological concentration. Thus chromatogram indicates the simultaneous detection of histamine and tyramine.

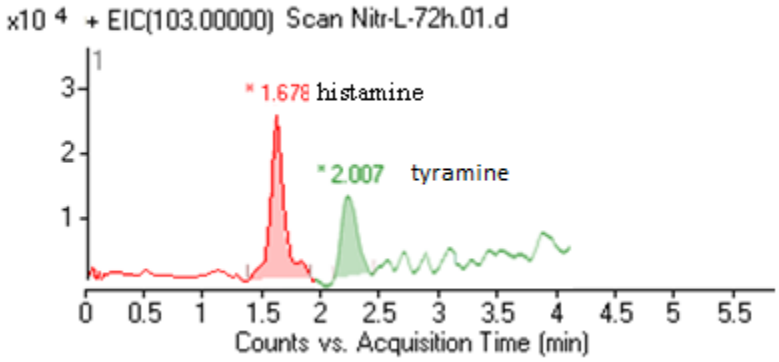


Fig. 9. Chromatogram obtained for a serum sample collected from rats in group II, 72 hours after tyramine administration (10 mg / kg body weight).

Groups III received a mixture of histamine and tyramine; chromatograms obtained 72 hours after mixture adminstration is presented in figure 10.

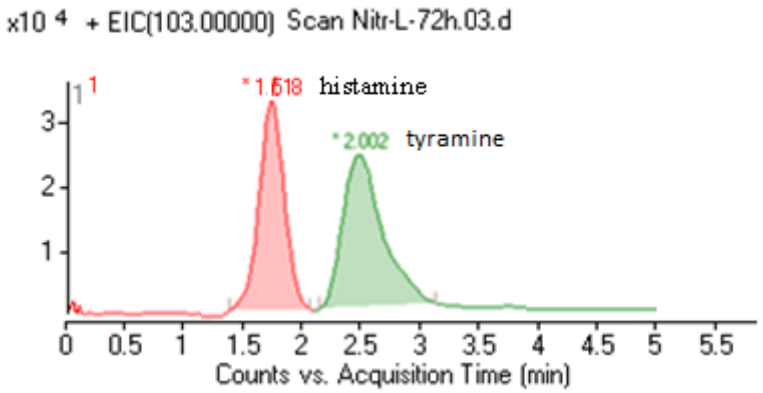


Fig. 10. Chromatogram obtained for a serum sample collected from rats in group III, 72 hours after mixture administration (5 mg / kg body weight, each).

The histamine and tyrosine concentrations (ng / ml) following administration of histidine, tyramine and mixture, initil, 1 hour and 72 hours after administration are shown in tables III, IV, V.

Tab.III. Plasma concentrations (ng / ml) of biogenic amines in the histamine receiving group (10 mg / kg body weight)

Lot I - received Histamine (10mg / kg body weight)	Initial	La 1h	La 72 ore
Histamine concentration (ng / ml)	19.03±0,52	41.4±1,34	20.64±2,37
Tyramine concentration (ng/ml)	ND	ND	ND

Tab. IV. Plasma concentrations (ng / ml) of biogenic amines in the group receiving tyramine (10 mg / kg body weight)

Lotul II received Tyramine (10mg / kg body weight)	Initial	La 1 h	La 24 ore
Histamine concentration (ng / ml)	19.83±0,51	18.81±0,65	18.52±0,58
Tyramine concentration (ng/ml)	ND	5±1,47	1.44±0.88

Tab. V. Plasma concentrations (ng / ml) of biogenic amines in the group receiving histamine, tyramine and cadaverine (5 mg / kg body weight, each)

Lotul III - received Histamine, Tyramine (5mg/kg body weight each)	Initial	La 1h	La 24 ore
Histamine concentration (ng / ml)	18.67±0,44	32.33±0,72	19.07±0.59
Tyramine concentration (ng/ml)	ND	3.55±1.12	2.13±0,95

Legend: ND-undetected concentration

For biogenic amines present in the plasma of experimental animals (rats), relatively little data are available in literature. Those few found in the literature indicate the following concentration values

for the amines selected in the present study as follows: histamine: 5 - 100 ng/mL [22] and tyramine: 0.5 - 10 ng/mL [23]
Obtained data show that histamine is the only biogenic amine present in very small quantities in naive rats. Instead putrescine and cadaverine were not detectable in any of the studied groups.
For group I, in which only histamine was administered at a dose of 10 mg / kg, the plasma histamine concentration showed a significant increase compared to the initial time. However, the increase does not exceed the upper limit of the normal range values. Tiramine was not detected in this group.
For group II, in which only tyramine 10 mg / kg body weight was administered, a significant increase in its concentration was also observed. Neither here the limit of normal values is not exceeded. The presence of histamine was identified similar to those in group I at very low concentrations .
For group III in which the mixture of histamine and tyramine was administered 5 mg / kg body weight each, the plasma concentrations of histamine and tyramine are increased compared to the initial time. For the tyrozine is important to noticed that it can accumulate to some extent.
The data in the literature show a very wide range for normal values [22,23]. For this reason in the statistical analysis we took into account for comparison the initial values considered as a control. Thus we can consider that the levels of biogenic amines have increased significantly following their individual administration as well as in the mixture.

Conclusions
Even if biogenic amines are compounds with short half-lives (histidine maximum one hour, tyramine maximum 3 hours) we consider, based on the obtained data, that the administration of biogenic amines can be used as an experimental acute model.

The study based was published in Rev.Med. Chir, 2009, 113(2):502-504 (the title of the work, the authors and the complete data are presented at the end of the chapter, pg 48).

I.1.4. Determination of biogenic amines from cellular mediums by high performance liquid chromatography coupled with mass spectrometric detection (HPLC-MS)

Biogenic amines are involved in intoxications, which can be severe or even lethal, caused by the consumption of fermented products such as meat, fish, cheese, wine [24-28]. Intoxications are caused by the generation of biogenic amines in the manufacturing process of raw products as a result of the activity of the so-called starter cultures [29-31].
Starter cultures are cultures of microorganisms that imprint a series of characteristics of food products such as: flavor, color, consistency and at the same time limit their alteration. These cultures can act on different amino acids generating biogenic amines [32-39] with potentially toxic effects as follows:

- histidine generates histamine,
- tyrosine generates tyramine,
- lysine generates cadaverine
- ornithine generates putrescine.

For this reason, the accurate determination of their quantity in food is mandatory.

Aim: The study investigates the potential of two microorganisms: Lactobacillus curvatus and Proteus mirabilis (used as starter cultures) to generate histamine, tyramine, cadaverine and putrescine in two culture medium.

Material and Method

The microorganisms selected for the study were: Lactobacillus curvatus and Proteus mirabilis. Lactobacillus curvatus was purchased from the food industry where it is found under the name PROCULTOR GE 35 and is used in the processing of raw meat products. Proteus mirabilis was obtained from the collection of microorganisms of the Microbiology Laboratory of the Faculty of Pharmacy "Gr.T.Popa" Iasi, isolated and identified by conventional laboratory methods.

The culture medium for these microorganisms were: liquid Man Rogosa Sharpe (MRS) and nutrient broth (BN). Both culture media was supplemented with biogenic amines precursor amino acids: histidine, tyrosine, lysine and ornithine.

The study was performed on 10 series, each containing 6 replicates as follows:

- Series I-V contained the Lactobacillus curvatus strain on MRS culture medium. Series I was control and series II-V had been supplemented with 1% of the following amino acids which were added in order: histidine, tyrosine, lysine and ornithine.
- Series VI - X contained the strain Proteus mirabilis cultured liquid culture medium BN. Series VI was controlled and series VII-X had been supplemented with1% of the following amino acids which were added in order: histidine, tyrosine, lysine and ornithine.

The culture series were incubated in a thermostat at 37^0 C and samples corresponding to each series were taken 72 h after the inoculation moment. To determine the concentration of biogenic amines, 2 ml of culture medium were taken and processed according to the same working protocol used for the determination in rat plasma.

Quantitative determination of amines generated in culture medium was performed by previously validated HPLC-MS / MS method.

Results and Discussion

Following the action of the two microorganisms on the specific amino acids added in the culture medium, the biogenic amines histamine, tyramine, cadaverine and putrescine were identified and quantified. Chromatograms obtained from the two cultures medium MRS and BN that contain the four biogenic amines are shown in figures 11-18.

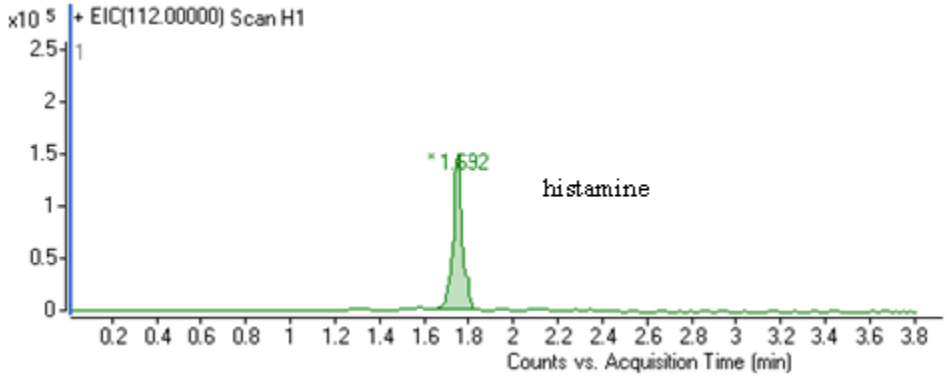


Fig. 11. Chromatogram obtained for MRS culture media with the addition of histidine at a concentration of 100 μg / L.

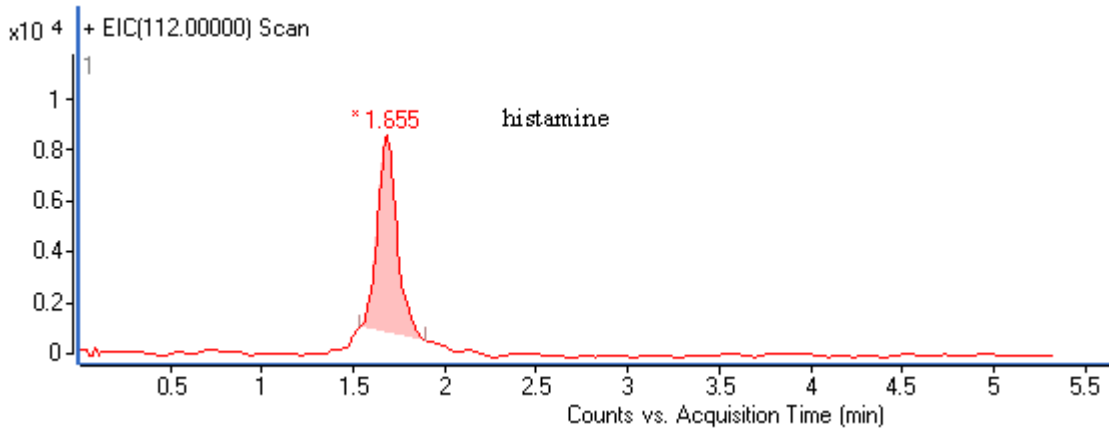


Fig.12. Chromatogram obtained for BN culture media with the addition of histidine at a concentration of 100 µg / L.

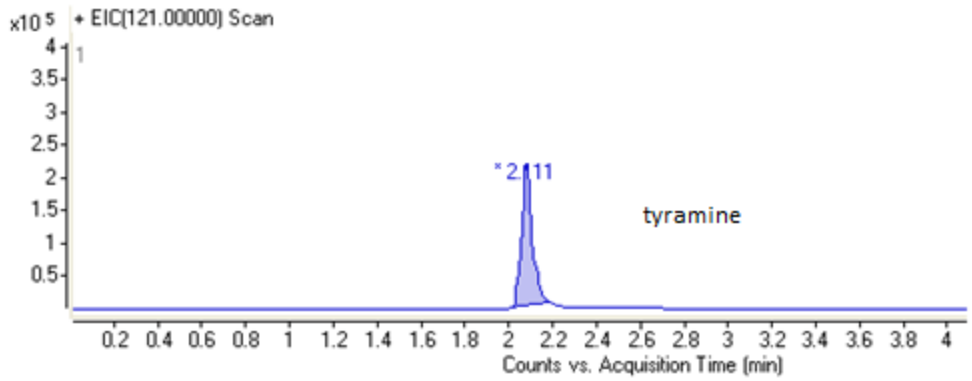


Fig.13. Chromatogram obtained for culture media MRS with the addition of tyrosine in a concentration of 100 µg / L.

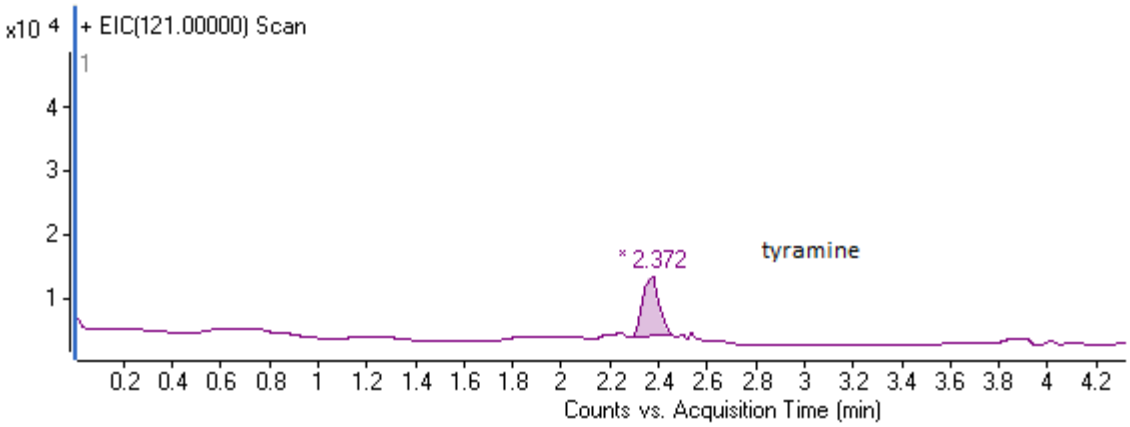


Fig.14. Chromatogram obtained for culture media BN with the addition of tyrosine in a concentration of 100 µg / L.

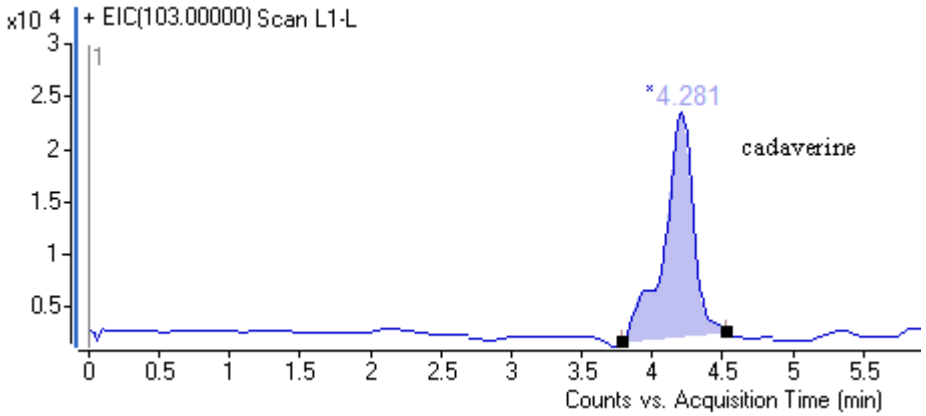


Fig.15. Chromatogram obtained for culture media MRS with the addition of lysine in a concentration of 100 µg / L.

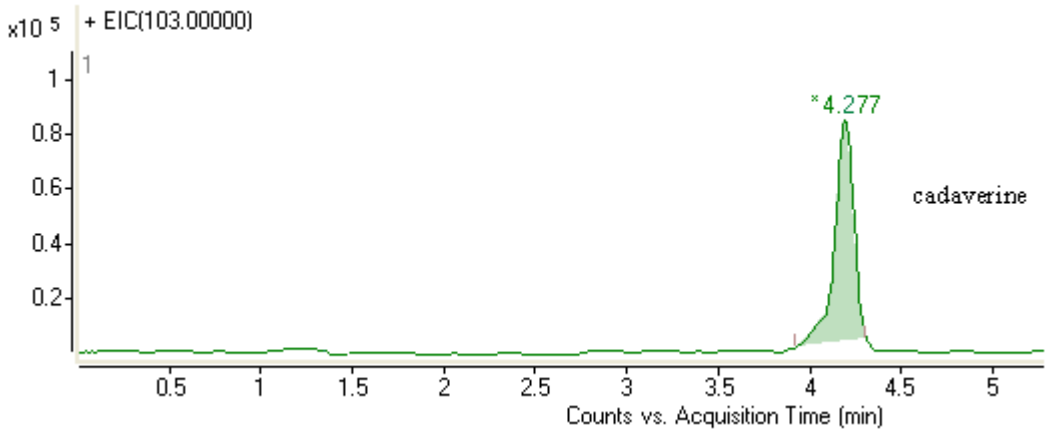


Fig. 16. Chromatogram obtained for culture media BN with the addition of lysine in a concentration of 100 µg / L.

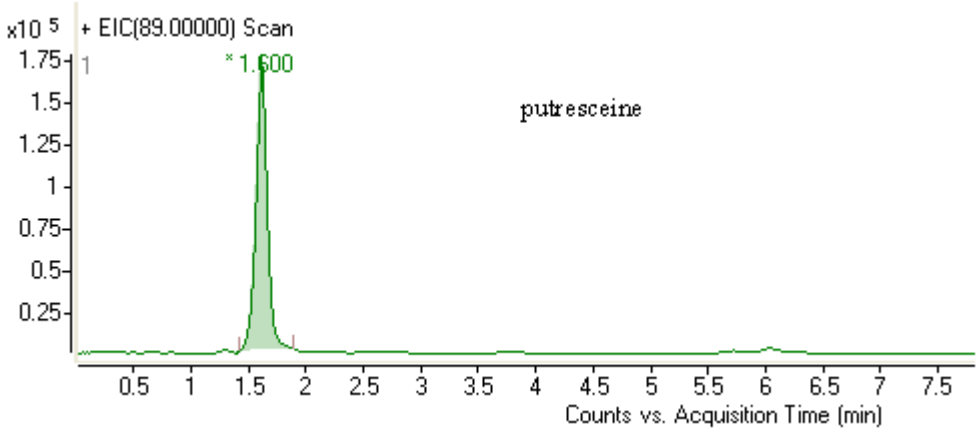


Fig.17. Chromatogram obtained for culture media MRS with the addition of ornithine in a concentration of 100 µg / L.

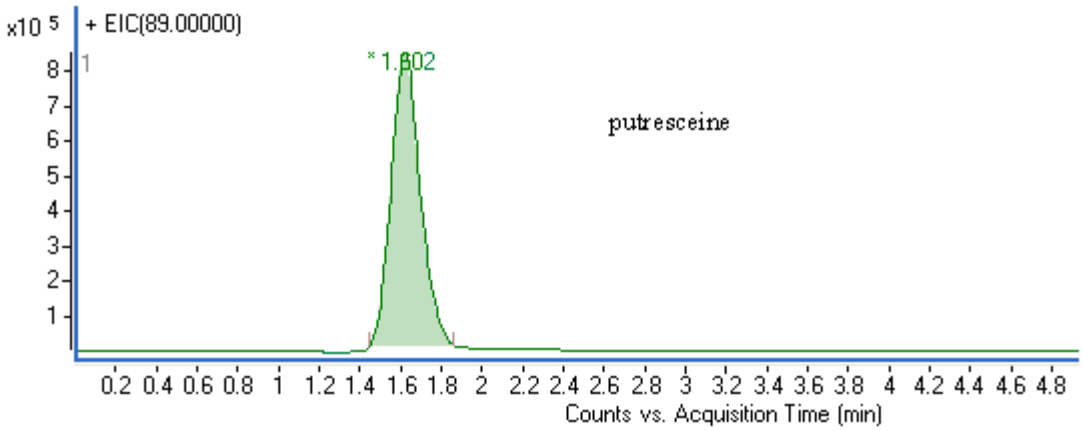


Fig. 18. Chromatogram obtained for culture BN with the addition of ornithine in a concentration of 100 µg / L.

The concentrations of biogenic amines generated by Lactobacillus curvatus and Proteus mirabilis in culture medium (µg / ml medium) at 72 h after inoculation, calculated as the average of 6 replicates, are shown in table VI.

Tab. VI: Concentrations for histamine, tyramine, cadaverine and putrescine that have been generated in the culture medium as a result of Lactobacillus Curvatus and Proteus mirabilis acivities, 72 h after inoculation.

Microorganisme / Amine	Histamine (µg/l mediu) (Medie ± SD)	Tyramine (µg/l mediu) (Medie ± SD)	Cadaverine (µg/l mediu) (Medie ± SD)	Putrescine (µg/l mediu) (Medie ± SD)
Lactobacillus curvatus	28 ± 1,3	254±12,3	9,21±1,1	342±14,8
Proteus mirabilis	7,11±1,1	2,45±0,9	115±9,7	67,2±7,3

Considerable differences are observed in the metabolic activity of biogenic amine formation by the two studied microorganisms.
In the MRS culture medium, the obtained results show that the Lactobacillus curvatus strain forms important amounts of tyramine and putrescein, from the precursor specific amino acids, suggesting an increased activity of the enzymes tyrosine and ornithine decarboxylase respectively.
Histamine was detected in identical concentration to the control culture sample, and cadaverine was found in a low concentration compared to tyramine and putrescein. Therefore, the activity of histidine decarboxylase recorded no activity and lysine decarboxylase show very a low activity.
In conclusion, the obtained data show that the presence of the microorganism Lactobacillus curvatus, tyramine, cadaverine and putrescein are generated in different concentrations while histamine is not generate, data similar to the literature [40,41].
In the samples obtained from the BN culture medium, the results show that the Proteus mirabilis strain generates histamine and tyramine, cadaverine and putrescein, the last two in higher concentrations, from specific precursor amino acids.
In conclusion, the obtained data show that the Proteus mirabilis strain is involved in the generation of biogenic amines, especially cadaverine and putrescein, similar data to the literature [42,43].

Conclusions

As a conclusion, the data obtained indicate that the Lactobacillus curvatus strain generates high concentrations of tyramine and putrescein, and the Proteus mirabilis strain forms high concentrations of cadaverine.

Accurate knowledge of amines types being generated by various microorganisms allows their efficient and targeted quantification in foods that use these microorganisms.

The proposed HPLC/MS method for the separation and quantification of histamine, tyramine, cadaverine and putrescine from plasma presents a significant advantage due to the simultaneously separation of all four amines. Another significant advantage is the elimination of the time-consuming derivatization step thus shortening the time for each analysis.

The methos is versatile as it is can be used for different type of sample such as: serum/plasma as culture media for bacteria or forensic biological liquid analysis.

I.2. Development and validation of biogenic amines determination in nutrients of animal origin using a HPLC with fluorescence detection

I.2.1 Introduction

Nutrients of animal origin such as meat food can contain high levels of biogenic amines due to either proteins alteration as a result of microorganism activity or to the technological procedure of food preparation and conservation [8,2]. Ingestion of such product can cause life-threatening reaction [44]. For this reason, it becomes necessary to determine the biogenic amines levels in meat food. In this study we investigated the presence of the selected four biogenic amines in different type of fresh or processed meat food. To identify and quantify the histamine, tyramine, cadaverine and putrescene we use a chromatographic method adapted from (Lange, Thomas şi Wittmann [12] and modified to be suite for our goal. The modified method was submitted to validation procedure.

I.2.2. Determination of some biogenic amines in meat stuffs by high performance liquid chromatography coupled with fluorescence detection.

Aim: To develop a sensitive and specific method for the biogenic amine determination appliable to different meat food.

In order to develop and validate a specific method useful for the determination of the biogenic amines in meat food, the etalon and calibration curves were performed:

- I.2.2.a. standard curves for the following amines: histamine, cadaverine, tyramine individually and in mixture
- I.2.2.b. calibration curves for the determination of amines studied following their extraction from meat food.

Material and Method

I.2.2.a. Plotting the standard curves for the following amines: histamine, cadaverine, tyramine individually and in mixture.

To determine the concentration of histamine, cadaverine, and tyramine in meat foods, the chromatographic method with pre-column derivatization was choose. Detection was achieved in fluorescence at 350 nm excitation and 460 nm emission. Putrescin could not be determined by this method. This chromatographic determination particularly takes place in two stages:

- the pre-column stage in which the derivatization is performed in order to improve the signal
- the column stage where the separation takes place followed by the fluorescence detection

Although it is a laborious method, this type of determination significantly improves the specificity of the analysis

Reagents

Reagents and chemicals were supplied by Fluka and were of HPLC purity: histamine (HI); tyramine (TYR); cadaverine (CAD); putrescine (PU); trichloracetic acid (TCA); mercaptoethanol (2-ME); o-phtalaldehide (OPA); methanol; boric acid; potassium hydroxide 45%; triethylamine 0.01%; sodium acetate 0,02M and 0.1M ; acetic acid 2% ; tetrahidrofurane 0.3%; acetonitrile ; 1.7 diaminoheptan.

Chromatographic conditions

The HPLC system was on 1200 series model (Agilent Technologies).

The liquid chromatograph consisted of:

- column Lichrosorb RP 18 (250mm x 4.6 mm. i.d.; 5μm);
- mobile phase A: a mixture of 0,1 ml triethylamine and 800 ml sodium acetate 0,02M brought at pH 7.2 with acetic acid 2% at which 3 ml tetrahidrofuran were added and all brought at a final volume of 1000 ml distillated water;
- mobile phase B: sodium acetate 0.1M (brought at pH=7.2 with acetic acid 2%) : acetonitrile : methanol: 200:400:400 (v/v/v);
- mobile phase contains a mixture with gradient volume of phase A and phase B. The flow of the mobile phase is 0.9 ml/min;
- the detection was in fluorescence at 350 nm excitation and 460 nm emission.

Preparation of standard solutions:

Standard stock solutions for each of the three amines were prepared of 1 mg / ml concentration by dissolving 5.0 mg of histamine, 5.0 mg of tyramine and 5.0 mg of cadaverine in 5 mL of trichloroacetic acid.

Preparation of working solutions:

Working solutions for each of the three amines in the following concentrations 10, 25, 50, 100, 200, 500, 600 ng / mL were obtained by successive dilutions in trichloroacetic acid of the individual stock solutions.

Derivatization stage

A solution containing 100 mg orthophthalaldehyde, 10 mL boric acid solution (2.47 g boric acid with 75 mL distilled water adjusted to pH 10.4 with 45% potassium hydroxide), 0.03 mL mercaptoethanol and 0.1 mL methanol was used for derivatization reaction.

Derivatization was performed by adding 0.2 mL of derivatization solution to 1 mL of working solution of concentrations: 10, 25, 50, 100, 200, 500, 600 ng / mL. The mixture was stirred and an aliquot of 20 µl inject into the column.

Results and Discussion

The area of the peaks obtained was used to draw the standard curve for each amine separately and in mixture. The equation of the regression curve and the correlation coefficient r^2 were calculated. The validation parameters, below, have been determined.

Chromatograms obtained after elution of the mobile phase (solvent mixture), of the individual amines and in the mixture are shown in the figures 19-23.

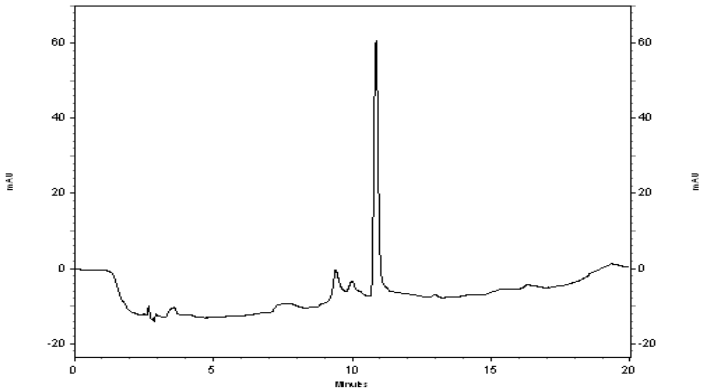


Fig. 19. Chromatogram obtained after the elution of the mobile phase

The chromatogram obtained highlights a peak at the retention time of 10 minutes which represents the solvent front. Within 10-20 minutes no compounds are detected that could interfere with the studied amines.

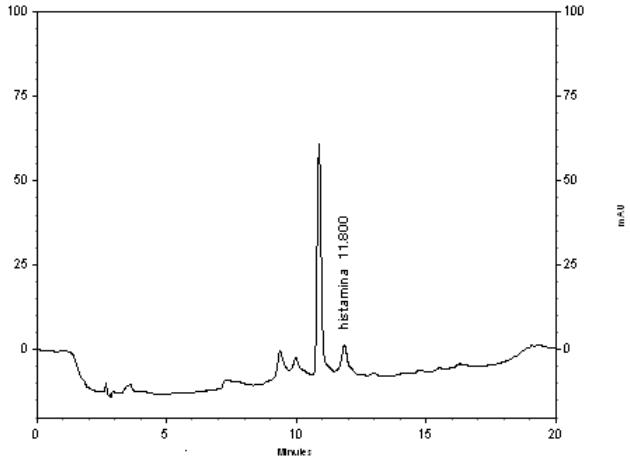


Fig. 20. Chromatogram obtained for a histamine sample at a concentration of 100 µg / mL

The chromatogram shows the peak of histamine obtained at the retention time of 11.80 minutes.

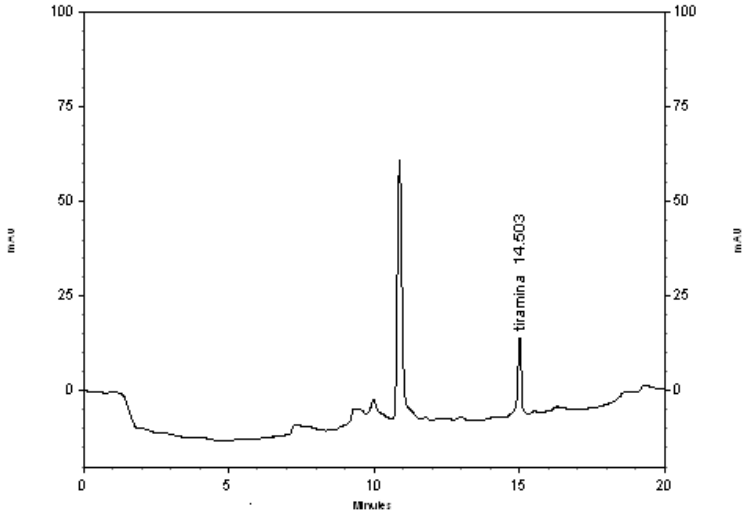


Fig. 21. Chromatogram obtained for a tyramine sample at a concentration of 100 /g / mL

The chromatogram shows the peak of tyramine obtained at the retention time of 14.503 minutes.

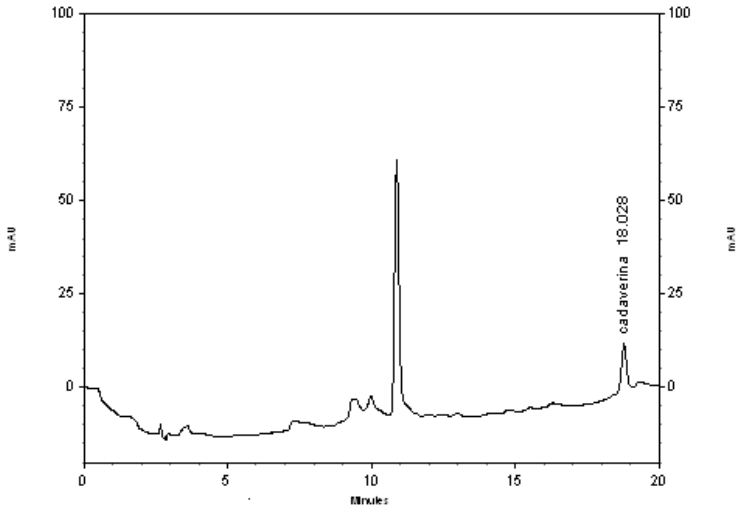


Fig. 22. Chromatogram obtained for a cadaverine sample at a concentration of 100 µg / mL

The chromatogram shows a peak generated by cadaverine obtained at the retention time of 18.028 minutes.

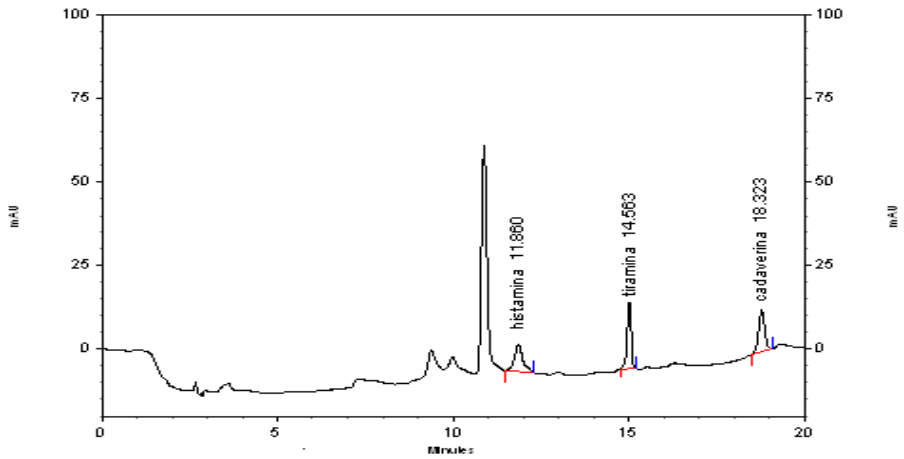


Fig. 23. Chromatogram obtained for a sample containing the mixture of histamine, tyramine, cadaverine at a concentration of 100 µg / mL each

The chromatogram shows the peaks obtained for the three amines well spaced at different retention times. Retention times (Rt) for the individual biogenic amines are:

- histamine Rt = 11,880 min
- tyramine Rt = 14,553 min.
- cadaverine Rt = 18,323min

As for putrescine, it could not be separated and therefore unidentified and unquantified using this method.

Different and spaced retention times allow the simultaneous separation of histamine, tyramine, cadaverine and putrescine without their interference. Therefore, the method is selective and also specific.

The individual standard curves for each of the three amines were plotted. The curves were achieved by eluting a series of increasing concentrations established for each amine. Based on the obtained data, the criteria for validation were determined.

*For Histamine***:**

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is Y = 190,3489x – 327.073 with a correlation coefficient r^2 =0.9966. Linearity range is between: 5,7711 – 600 µg/ml

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 2%, which fits in the range requested for the accuracy of the analytical method.

c) Accuracy: different histamine concentrations of 400, 500, 600 µg/ml. were analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤1%, is met for the concentrations used. The proposed method meets the intra-day accuracy criterion, the values obtained for RSD% 0,949, 0,798, 0,552, being less than 1. The inter-day the values obtained for RSD% are 0,264, 0,140 for the first and the second day, respectively also in the range criterion. For retention time the acceptance criterion requires that the relative deviation of RSD% be a value ≤ 2%. The chosen method meets the criterion of intermediate precision and

reproducibility for the retention time, the values obtained for RSD% being of 0,142, 0,118 for the first and the second day, respectively, both being smaller than 2.

d) Limit of detection: 2,9341 μg/ml

e) Limit of quantitation: 5,7711 μg/ml

f) Selectivity: retention times (Rt) for histamine is 11,880 min distant from the other tree amine, therefore the method is selective.

*For Tyramine***:**

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is 399.1586x-2786.99, with a correlation coefficient r^2=0.9978.. Domeniu de liniaritate: 8,92 – 600 μg/ml

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 2%, which fits in the range requested for the accuracy of the analytical method.

c Accuracy: different histamine concentrations of 400, 500, 600 μg/mlwere analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤1%, is met for the concentrations used. The proposed method meets the intra-day accuracy criterion, the values obtained for RSD % 0,452, 0,414, 0,686 being less than 1. The inter-day the values obtained for RSD % 0,768, 0,970 for the first and the second day, respectively also in the range criterion. For retention time the acceptance criterion requires that the relative deviation of RSD% be a value ≤ 2%. The chosen method meets the criterion of intermediate precision and reproducibility for the retention time, the values obtained for RSD% being of 0,258, 0,151 for the first and the second day, respectively, both being smaller than 2.

d) Limit of detection: 7,56 μg/ml

e) Limit of quantitation: 8,92 μg/ml

f) Selectivity: retention times (Rt) for tyramine is 14,553 min min min distant from the other tree amine, therefore the method is selective.

In the chromatogram obtained after injection of the eluent, no peaks are recorded to interfere with the chromatogram obtained with the solution to be analyzed, which demonstrates that the solvent does not interfere with the analysis. Tyramine is determined distant from both the solvent front and the three other amine areas.

The chosen method is selective, allowing the simultaneous separation of tyramine from other amines without co-eluting them.

*For cadaverine***:**

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is 137,835x –634,258 with a correlation coefficient de corelaţie r^2=0.9946. Linearity range is between: 16,15 – 600 μg/ml

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 2%, which fits in the range requested for the accuracy of the analytical method.

c) Accuracy: different histamine concentrations of 400, 500, 600 μg/ml were analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤1%, is met for the concentrations used. The proposed method meets the intra-day accuracy criterion, the values obtained for RSD % are 0,694, 0,694, 0,532, being less than 1. The inter-day the values obtained for RSD% are 0,769, 0,440 for the first and the second day, respectively also in the range

criterion. For retention time the acceptance criterion requires that the relative deviation of RSD% be a value ≤ 2%. The chosen method meets the criterion of intermediate precision and reproducibility for the retention time, the values obtained for RSD% being of 0,130, 0,068 for the first and the second day, respectively, both being smaller than 2.

d) Limit of detection: 8,13 μg/ml

e) Limit of quantitation: 16,15μg/ml

f) Selectivity: retention times (Rt) for cadaverine is 18,323min min distant from the other three amine, therefore the method is selective.

The chromatograms obtained after the injection of the eluent and of the the biogenic amines, the amines do not interfere either with the solvent or with each other, which proves that the separation is efficient and selective. Every biogenic amine is determined distant from both the solvent front and the two other amines areas.
The chosen method is selective, allowing the simultaneous separation of all biogenic amines without coelution.

I.2.2.b. Plotting the calibration curves to determine the four studied amines following their extraction from meat food

Aim: To develop a specific method to individually quantify the three biogenic amines in meat food.

Material and Method
In order to achieve the calibration curves for the determination of the studied amines in meat food, an additional preliminary stage of sample preparation is required, namely the extraction of biogenic amines from the meat food sample.
Therefore, the determination of biogenic amines in this matrix (meat) is performed in three stages as follows:

- extraction stage
- derivatization stage
- elution step followed by detection

The extractive stage of amines from meat
The extraction of biogenic amines has been realized under the guide for analytical methods accepted by FDA (Food and Drug Administration, SUA); EMEA (European Agency for the Evaluation of Medicinal Products on Evaluation of Medicines for Human Use) [20, 21]. The procedure performs as follows:

- 25 ml of TCA 5%, were added to 10g meat product
- the mixture was mixed for 3 minutes, and then centrifuged at 4000 rpm, and the supernatant separated
- the procedure was applied twice
- the supernatant collected was eluted on a solid cartridge extraction [11].
- the extracted liquid was submitted to the following derivatization and elution steps

The derivatization stage
For derivatization at 1 ml of extracted fluid were added 0.2 ml of derivatization mixture:100 mg O-phtalaldehide, 10 ml boric acid (2.47g boric acid in 75 ml distilled water brought at pH 10.4

with potassium hydroxide 45%), 0.03 ml mercaptoethanol and 0.1 ml methanol. The mixture was allowed to rest for 1 minute.

The elution stage
For elution an aliquot of 20 µl were injected on the chromatograph and then detected with fluorescence detector.

To achieve the calibration curves, increasing concentrations of each of the three amines were added over 10g of meat product and processed according to the above procedures. A meat sample without added amines was processed identically and was the control sample. After the preparative stage had been ended aliquots of 20 µl were injected into the column.

Results and discussions
The area of the peaks obtained was used to draw the calibrations curve for each amine separately and in mixture. The equation of the regression curve and the correlation coefficient r2 were calculated. The validation parameters, below, have been determined.
The chromatograms obtained after elution of the extract from the meat sample without the addition of amines are shown in figure 24. Chromatograms obtained after elution of the extract from meat samples to which both individual amines (50 µg/ml each) and a mixture of amines (in the same concentrations) were added, are shown in figures 25-28.

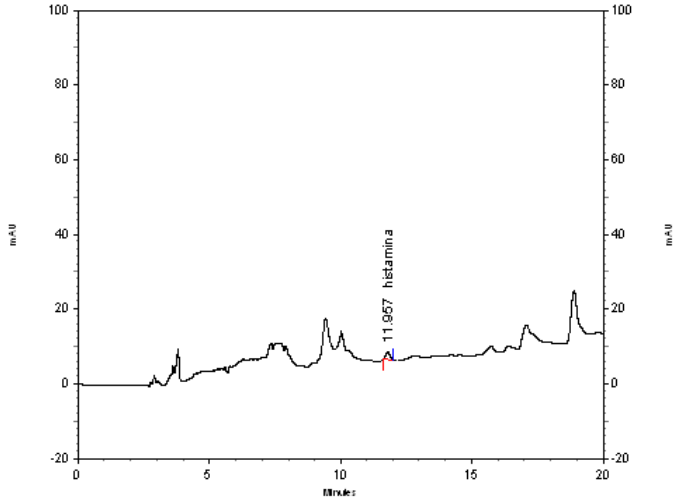


Fig. 24. Chromatogram obtained for a meat sample without amines addition

The chromatogram shows the peak obtained for histamine at the retention time of 11,957 minutes. The presence of histamine in the meat sample, although the amine has not been added, is explained by the fact that a certain amount of histamine is physiologically found in both meat and biological fluids. The amount of amine found in the sample is lower than the limit of quantification, tyramine and cadaverine being absent.

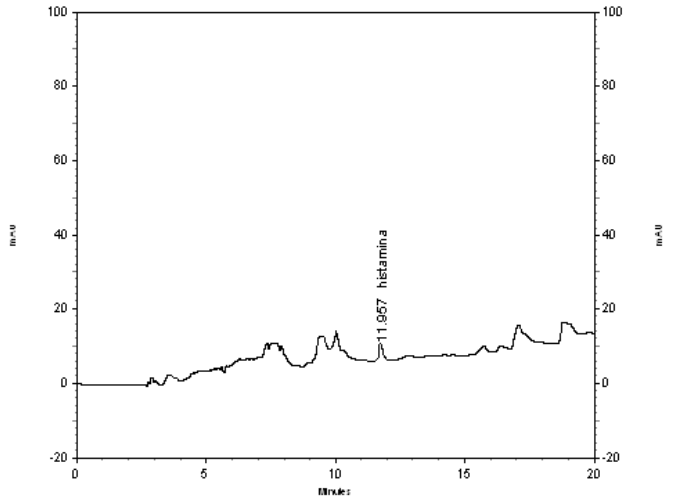


Fig. 25. Chromatogram obtained for a meat sample to which histamine was added at a concentration of 50 µg / ml.

The chromatogram obtained shows the peak generated by histamine at the specific retention time. The area of the peak area includes the amount of endogenous histamine belonging to the meat sample.

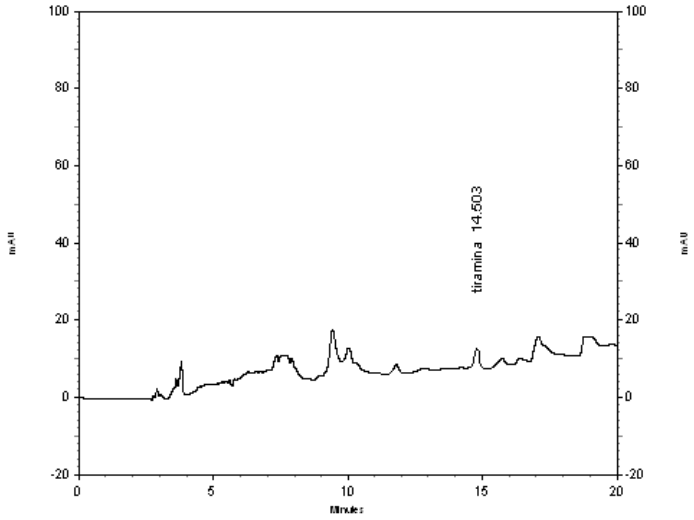


Fig. 26. Chromatogram obtained for a meat sample to which tyramine was added at a concentration of 50 µg / ml.

The chromatogram obtained shows the peak generated by tyramine at the specific retention time 14,503 minutes. The peak for endogenous histamine is also present similar to the control at a retention time of around 11 minutes.

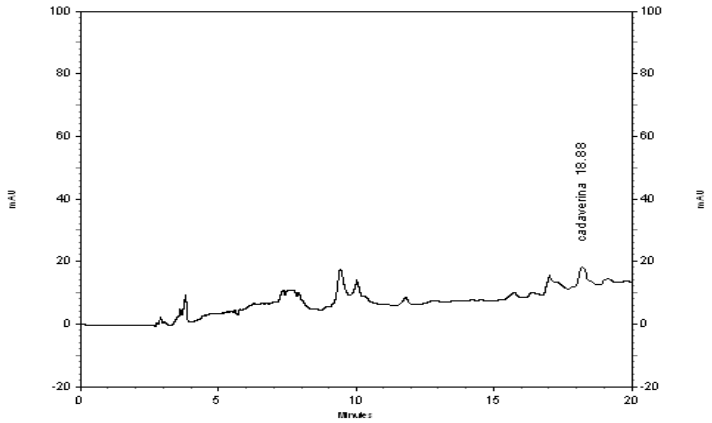


Fig.27. Chromatogram obtained for a meat sample to which cadaverine was added at a concentration of 50 µg / ml.

The chromatogram obtained shows the peak generated by cadaverine at the specific retention time 18.88 minutes. The peak for endogenous histamine is also present similar to the control at a retention time of around 11 minutes.

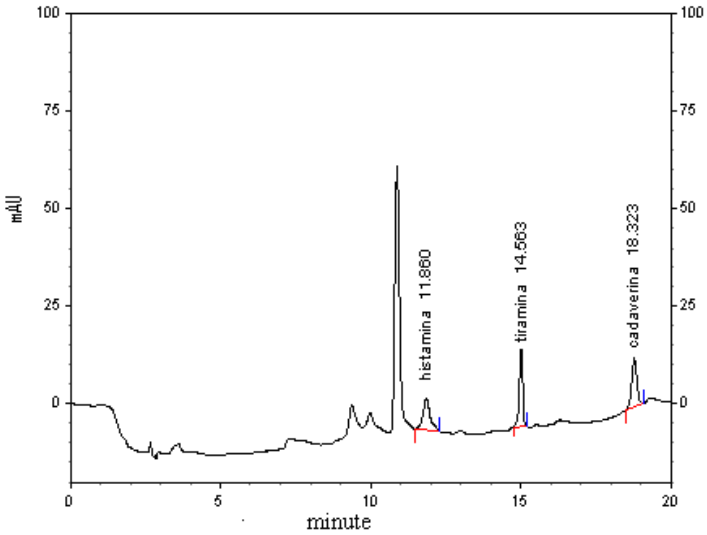


Fig.28. Chromatogram obtained for a meat sample to which the amines mixture in 50 µg / ml concentration each were added.

The extraction method used to determine the amines in the meat sample does not change the retention times found for the three amines when they were eluted as standards thus sugesting an improved specificity. The three amines are eluted well spaced from each other and all from the solvent thus avoiding coelution.
In conclusion, the extraction method allows the simultaneous determination of amines, avoids interference and present a higher specificity.
The individual calibration curves for all three amines were plotted and the criteria for validation were determined.

*For Histamine***:**

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is 162.745x-3118.64with a correlation coefficient r^2 = 0.9966. Linearity range is between: 20,85– 600 μg/ml

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 5%, which fits in the range requested for the accuracy of the analytical method.

c) Accuracy: different histamine concentrations of 25, 200, 400 μg/ml. were analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤5%, is met for the concentrations used. The reproducibility of the histamine extraction method from sausage samples was verified by applying the extraction procedure for three concentrations on the standard curve, 25, 200, 400 /g / ml on intra-day and on inter-days. The data obtained indicate that the method is reproducible, the accuracy being higher than 95.69%.

The average recovery of 84% indicates a good yield for the separation process. The coefficient of variation of 4.94% indicates a good accuracy of the method.

d) Limit of detection: 19,67 μg/ml

e) Limit of quantitation: 20,85 μg/ml

f) Selectiviy: retention times (Rt) for histamine remains 11,880 min distant from the other tree amine, therefore the method is selective.

*For Tyramine***:**

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is 378.17x -5745.5, with a correlation coefficient r^2=0.9964. Domeniu de liniaritate: 17.19– 600 μg/ml

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 5%, which fits in the range requested for the accuracy of the analytical method.

c) Accuracy: different histamine concentrations of 25, 200, 400 μg/ml / ml were analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤1%, is met for the concentrations used. The reproducibility of the method of extracting tyramine from sausage samples was verified by applying the extraction procedure for three concentrations on the standard curve, 25, 200, 400 μg/ml intra-day and inter-days. The data obtained indicate that the method is reproducible, the accuracy being greater than 95.45%. The average recovery of 94.33% indicates a good yield for the separation process. The coefficient of variation of 5.00% indicates a good accuracy of the method.

d) Limit of detection: 15.79 μg/ml

e) Limit of quantitation: 17.19 μg/ml

f) Selectivity: retention times (Rt) for tyramine remains 14,553 min distant from the other tree amine, therefore the method is selective.

*For cadaverine***:**

a) Linearity and linearity domain: the standard curve for the area of the peaks as a function of concentration was drawn. The equation of the regression line is 130.08x -2134.5 with a correlation coefficient de corelaţie R^2=0.9979. Linearity range is between: 20,85– 600 μg/ml

b) Precission: the values obtained for the relative standard deviation (RSD) are less than ≤ 5%, which fits in the range requested for the accuracy of the analytical method.

c) Accuracy: different histamine concentrations of 25, 200, 400 µg/ml were analyzed for accuracy. For each concentration, three injections were made into the chromatographic system. The acceptance criterion for accuracy, which requires that the relative deviation of RSD% be ≤1%, is met for the concentrations used. The reproducibility of the method of extracting cadaverine from sausage samples was verified by applying the extraction procedure for three concentrations on the standard curve, 25, 200, 400 /g / ml on the same day and on different days. The data obtained indicate that the method is reproducible, the accuracy being higher than 99.72%.
The average recovery of 99.52% indicates a good yield for the separation process. The coefficient of variation of 5.00% indicates a good accuracy of the method.

d) Limit of detection: 19,67 µg/ml

e) Limit of quantitation: 20,85 µg/ml

f) Selectivity: retention times (Rt) for cadaverine is 18,323min min distant from the other three amine, therefore the method is selective.

The chosen method is selective, allowing the simultaneous separation of the three amines located at a distance from each other and at a distance from the solvent, thus avoiding co-elution.

I.2.3. Biogenic amines determination in nutrient of animal origin (meat food).

Using the validated methods, concentration of biogenic amines: histamine, tyramine, cadaverine have been determined in 25 different types of fresh and processed meat, purchased from the Romania markets.

Specific chromatogram obtained after elution of extracted sample 11 prepared under the procedure presented above, is showed in figure 29.

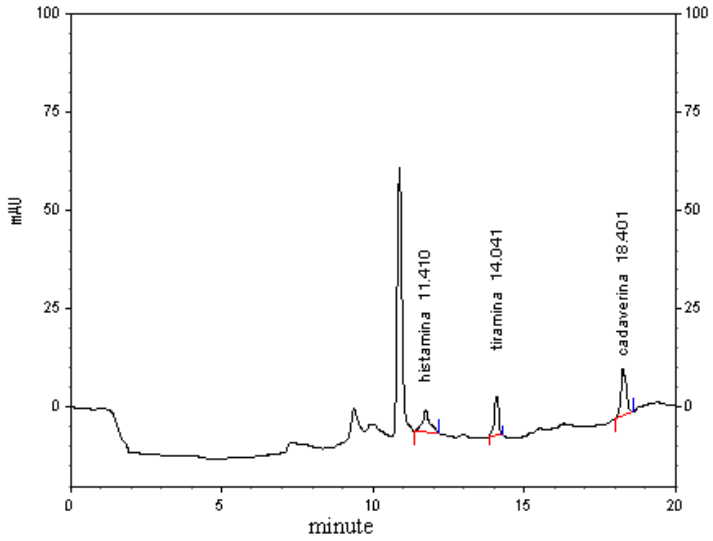


Fig. 29. Chromatogram obtained for sample no. 11

The amount of biogenic amines expressed as (mg/kg) found in different type of fresh meat and meat products purchased from the Romanian market are presented in table VII.

Tab. VII. Quantity of histidine, tyrosine and cadaverine found in fresh meat and meat products purchased from different suppliers on the Romanian market (mg / kg)

No.	Sample type	HISTAMINE mg/kg	CADAVERINE mg/kg	TYRAMINE mg/kg
1	fresh chicken meat	ND	0,8	8,9
2	fresh fat pork meat	2,7	1,3	4,8
3	fresh lean pork meat	2	3,5	2,1
4	bacon meat	1,8	5,8	7,3
5	fresh beef mea	2,4	7,4	6,2
6	spicy sausages	1,4	1,8	58,4
7	Sibiu salami	64,7	125	111
8	Sinaia Salami	52	98	65
9	pork ham Premium	71	34	73,8
10	Polish sausage	1,6	18	57,6
11	Sibiu salami	21	14	79,8
12	smoked loin	6	24	44
13	fermented pork sausage	2,9	6,8	67,3
14	traditional fermented pork ham	4,2	44	162
15	traditional smoked pork	14	67,8	105,6
16	pastrami smoked pork	32,4	98,3	78,2
17	fermented sausage Baciu	2,8	12,5	34,2
18	fermented sausage Luca	8,2	18,5	12,4
19	fermented sausage Chorizo	3,6	5,9	37,4
20	sausage Victoria	2	4,6	21,2
21	home sausage	2,9	ND	33,5
22	pork ham	7	ND	4,3
23	French sausage	2,1	1,3	1,8
24	chicken ham	3,2	9,3	5
25	pastrami smoked chicke	ND	1,7	31,5

*ND- not detected

As expected significant differences regarding the amount of biogenic amine in meat products were found. The three amines are found in almost all sample symultaneously. Their presence is consider to be a direct consequence of either the technological procedure that uses starter cultures for fermentation stage or by other factors as conservation or store conditions.
The simultaneous presence of all these biogenic amines in the samples is very likely to increase the harmful effect on human health [13, 37] .
Moreover, the threat to human health is much greater for people who are on therapy with inhibitors of monoamine oxidases [45-48]. In this regard people who are under medical treatment must be aware about the risk of consuming technological prepared meat products.

Conclusions

The proposed chromatographic method for the simultaneous separation of biogenic amines meets the requirements of the validation rules and allow the simultaneously determination of histamine,

tyramine and cadaverine in meat and meat food. The retention times obtained for the three studied amines are well spaced each other, thus allowing a good separation on the chromatographic column.The method has a good reproducibility and an optimal recovery.
The study confirmed the presence of higher concentrations of biogenic amines in meat preparations compared to fresh meat. Even it is a time consuming methos the specificity is higher due to derivatization stage.

The study was published in Rev. Med. Chir. Soc. Med. Nat. Iasi, 2009; 113(4): 1195-1199. (the title of the work, the authors and the complete data are presented at the end of the chapter, pg 48).

General conclusions on the chapter
The chapter has presented the methods chosen to determine compounds produced in living organisms (biogenic amines) but which can also be introduced into the body through food. The proposed methods have been validated and adapted to allow the simultaneous determination of 4 biogenic amines in different mediums such as: blood, culture medium and food.
In the current context in which food is intensively processed, the possibility of the appearance of toxic compounds is very high. Therefore, it is very important to determine these products with increased toxic potential in the most diverse matrices, whether they are food, blood, water, culture medium, etc. Therefore, the proposed and validated methods are useful in a large number of fields besides the medical one.

The results of all these studies were published in 3 scientific papers presented below:

The results of the studies presented in Chapter I were published in the following publications:

Full articles in ISI journals

1. N Filip, C Mircea, C Iancu, M Hancianu, E Cojocaru, B Stoica, **C Filip**. Determination of Some Biogenic Amines in Rat Plasma Using High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC/MS) Method, Farmacia, 2018, 66 (3), 548-552

Full articles in BDI journals

1. Zamosteanu N., **Filip C.**, Jerca L., Albut I., Cuciureanu R. Biogenic amines determination in some Romanian meat products. Rev. Med. Chir. Soc. Med. Nat. Iasi, 2009; 113(4): 1195-1199.
2. Zamosteanu N., **Filip C.**, Albu E., Ungureanu D., Cuciureanu R. Total antioxidant status in the blood serum of rats after biogenic amines administration. Rev. Med. Chir. Soc. Med. Nat. Iasi, 2009; 113(2): 502-504**.**

Chapter II. Evaluation of homocysteine concentrations in different pathological processes and the possibility of therapeutic use of new synthetic products

II.1. Introduction

Homocysteine metabolism

Homocysteine (Hcy) is an amino acid generated by the human body in the methionine metabolism. It doesn't participate to the proteins' synthesis, but it is an important intermediate in major processes such as transmethylation, trans-sulphuration, cysteine (Cys) formation etc. In the transmethylation process homocysteine participates in the formation of adrenaline, melatonin, lecithin, creatine, etc. The trans-sulphuration pathway leads to cysteine synthesis, amino acid that play a key role in the spatial conformations of protein and particularly in glutathione generation, the most important antioxidant agent in the body (Figure 30).

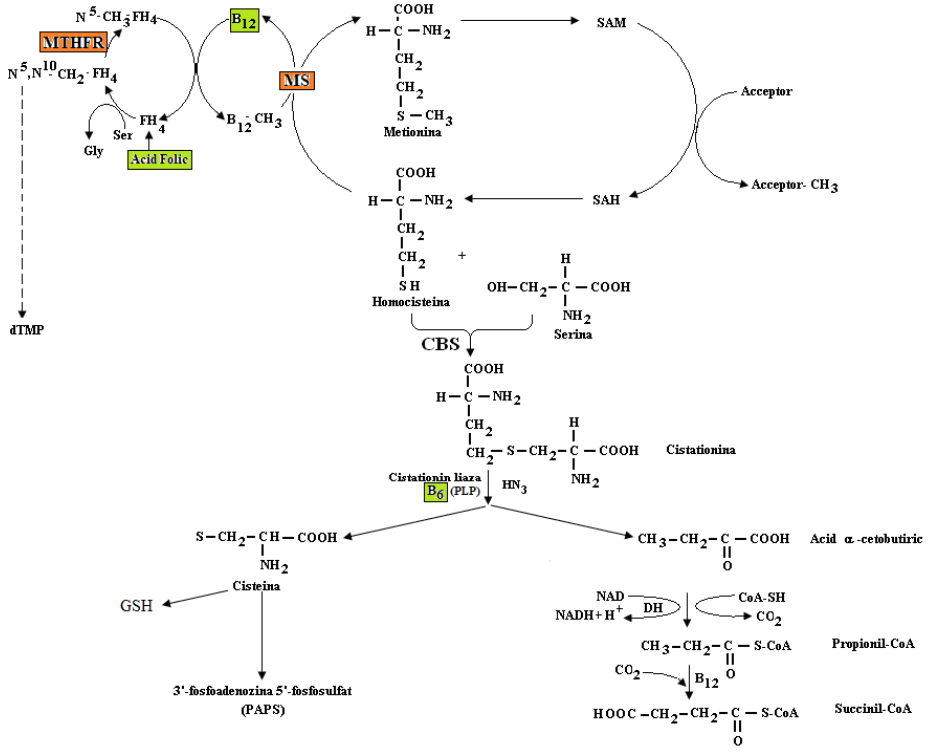


Fig. 30. Homocysteine metabolism.

Homocysteine metabolism presented in Figure 30 highlight the involvement of vitamins (folic acid, vitamin B12, vitamin B6 in its active form of pyridoxal phosphate or PLP) and two important enzymes methylene-tetrahydrofolate reductase (MTHFR) respectively methionine synthase (MS). The absence of these vitamins as well as the enzymes deficiencies trigger the increase of Hcy levels. The normal concentration of homocysteine in human blood is 5-15 μM. Over the past forty years homocysteine has been noticed in various pathologies when its concentrations exceed the above range. This condition is known as hyperhomocysteinemia, abbreviated form HHcy . High homocysteine concentrations are classified according to clinical consequences as being moderate at 16-30 μM, intermediary at 31-100 μM and severe above 100 μM [49]. Nowadays it is generally accepted that HHcy promotes thrombosis [50-52].

Homocysteine in pathology

Nowadays there are two theories regarding homocysteine role in pathology:

a. first hypothesis considers that homocysteine is a risk factor [53]
b. the second one considers Hcy as a marker of vascular chronic diseases [54].

Both opinions are based on scientific arguments, and although the debate continues, the is a general consensus that there is an unquestionable link between homocysteine and vascular endothelial dysfunction [55-57]. In the following are shortly presented clinical and preclinical studies in the literature and the proposed mechanisms.

a. Hypothesis that considers that homocysteine is a risk factor

Clinical studies found high blood homocysteine in a wide range of diseases: cardiovascular diseases [53, 58-62], neurological diseases [63-66], miscarriage [67-69], thrombophilia [70-75], bone fragility [76-81], diabetes [82-88], inflammation [89-91].

The proposed mechanisms through which hyperhomocysteinemia induces illnesses are the following: oxidative stress [92-98], inflammation [99-109], cellular signaling [110-127].

Cardiovascular diseases

Nowadays it is considered that even slightly higher than normal Hcy concentration is linked to the cardiovascular risk. A 5 μM increase in Hcy levels is equivalent to a 20 mg/dL increase in blood cholesterol [58,59], which in fact doubles the cardiovascular risk. Furthermore recent-data [53,62] show that a surprising 30% of cardiovascular mortality occurs in patients who do not present already known risk factors (dyslipidemia, hypertension, diabetes, smoking, obesity) [53, 60-62] but present high homocysteine concentration. These data indicate an escalation of risk when hyperhomocysteinemia is added to hypercholesterolemia.

Neurological diseases

Association of HHcys to Alzheimer's disease was reported by Seshadri [63 bis] who shows that high homocysteine levels may doubles the risk of developing the disease. The mechanism that links Hcy to Alzheimer's is supposed to be the chronic central nervous system ischemia [64-66] that cause neuronal damages. This observation led to the association of HHcy to the vascular disfunction.

Miscarriage and thrombophilia

Enzymes in homocysteine metabolism that deficiently work due to inherited/acquired specific mutations cause HHcy, condition encountered in miscarriage. It is the case of MTHFR deficiency. Literature indicates a 3,3-fold increase in the risk of miscarriage [67,68] due to this mutation. Moreover, in patients with MTHFR C667T mutations associated to factor V Leiden and/or prothrombin gene mutations were found in the recurrent miscarriages [69]. In addition to the prothrombotic effect, HHcy seems to decrease the fibrinolytic process. The mechanism of fibrinolysis depends on specific proteins, among which protein C plays a key role. Low protein C activity favors clot formation. Scientific studies show that HHcy blocks the activity of protein C by forming disulfide bridges with it and thus the thrombotic process is promoted [70]. As a consequence, both venous and arterial thrombosis [71-74] may occur. So, a high homocysteine level may lead to miscarriage, deep vein thrombosis and also acute events as pulmonary thromboembolism. These mechanisms are widely presented in our published paper [75].

Osteoporosis

In bone fragility, the increased risk of bone fractures in the elderly were associated with high levels of homocysteine [76-81]. It seems that HHcy disturbs the structure of collagen thus weakening the tissue resistance.

In the above presented pathologies HHcy seems to be a risk factor for many diseases but mainly in the cardiovascular disease.

b. Hypothesis that considers Hcy as a marker of vascular chronic diseases
Some scientists consider HHcy as a marker of an already altered vascular state rather than a risk factor [54,82]. These authors argued that in chronical hypertension or in diabetes the atherosclerosis may reach the stage where kidney function is severally affected. As a consequence, the HHcy removal decreases and its blood concentration increases. Atherosclerosis and diabetes are silent disease that develop years before being diagnosed and are accompanied by the unnoticed decline in renal function. Thus, chronical vascular disfunction contributes to the elevation of circulating Hcy as result of the progressive decline in renal function and in fact HHcy reflects the severity of atherosclerosis. As a conclusion HHcy signals the moment when the vascular disease reaches an irreversible stage [1]. The above considerations were supported by the fact that the decrease in homocysteine levels did not necessarily reduce the symptoms and didn't improve the recovery in cardiovascular diseases [82].
In this light hyperhomocysteinemia become a supplementary danger for patients with diabetes compared to non-diabetic [83-85]. The increase in Hcy levels noticed in diabetes is considered to be due to the degree of diabetes-induced nephropathy [86-88]. All this data suggests more for a marker role of homocysteine rather than a risk factor.

Homocysteine mechanism of action
Hyper homocysteinemia is generally accepted to be involved in endothelial dysfunction. Normal endothelial function consists in maintaining the vascular relaxation and the anticoagulant status. Disturbances of its normal function led to changes in vascular morphology, tonicity, coagulability, etc. Inflammation has the key role in endothelial response to injury and triggers a chain of events including defense, annihilation, or cellular signaling. HHcy interferes somewhere in this chain and disturb the endothelial normal response. Several hypotheses have been proposed for the HHcy mechanism of action: the reactive oxygen species generation [89], the inflammatory process [90], and the cellular signaling [91]. These mechanisms will be shortly presented below, along with scientific evidence.

Hyperhomocysteinemia involvement in oxidative stress.
Specific processes or stimuli trigger in the body the synthesis of reactive oxygen species (ROS). In their turn ROS trigger a cascade of events leading to: release of proinflammatory cytokines, activation of adhesion molecules, generation of intracellular messengers that lead to a cellular response that includes gene activation or repression [92-94]. Scientific studies, including ours, have shown that HHcy can generate reactive species both directly and by autooxidation [95,96]. In our published paper, we showed that HHcy promotes the generation of hydrogen peroxide and decrease the total antioxidant capacity in experimentally induced HHcy in rats [97, 98].

Hyperhomocysteinemia involvement in inflammation
The survival of the cell is based on its ability to remove any type of aggression/lesion and to restore the initial healthy structure. Thus, cells develop network of systems, named inflammasome, able to communicate in order to mobilize defending and healing structures and also to store information about the aggression. These complex actions require the mobilization of many components that are coordinated by chemical messenger or active peptides known also cytokine [99]. These

molecules trigger modification in ions fluxes, free radicals' production, specific genes transcription or repression, etc. Latest data demonstrates that the activation of inflammasomes (NLRP3 complex) represent a key step in HHcy-aggravated atherosclerosis [100,101]. Recent studies [102,103] had advanced the idea that HHcy triggers endothelial damage by promoting chronical inflammation. This type of inflammation is characterized by the fact that tissue lesion is not limited to the damaged tissue but also progresses to the surrounding tissues. In systemic inflammation, encountered in atherosclerosis or diabetes, high levels of chemical mediators such as interleukins (IL-6, IL-8 and TNFα) were found [104,105]. Recently, HHcy was positively associated to inflammatory markers IL-6, TNFα [106-109].

Hyperhomocysteinemia involvement in cellular signaling.
Inflammation generates ROS, ROS activates cellular signaling, cellular signaling generates active molecules including other ROS molecules. So, the generation of reactive species and inflammation are intertwined processes on which cell survival depends. Currently scientific evidences show that ROS serve in pathological as well as physiological processes [110]. Normal ROS concentration act to regulate biological and physiological processes on contrary higher ROS concentration is strongly associated with oxidative stress [111]. Among this hydrogen peroxide is considered the most likely secondary messenger [112]. Scientific data show that when H_2O_2 is added to a living system it could mimic growth factor activity and that the growth factors in turn could stimulate the endogenous production of H_2O_2 within cells [113-115]. Thus, a double direction is established between ROS and cellular signaling. A major route in signal transduction is activation of the mitogen-activated protein kinases or MAPK kinases where ROS play a key role according to the scientific data [116-121]. Considering the above data, it is possible that Hcy a H_2O_2 generator according to scientific data, may interfere in this signaling process promoting mitogenic activity [122-127].
All these data lead to the conclusion that endothelial normal functions (vascular relaxation and anticoagulation activity) are disturb in high homocysteine concentrations thus thrombogenic status being promoted [71-74].
Our team studied the influence of hyperhomocysteinemia on oxidative stress markers in various experimentally induced pathologies in rats.

II.2. Hyperhomocysteinemia effects on antioxidant capacity, in rats

Aim: To assess the possible correlation between the hyperhomocysteinemia and oxidative stress

This study investigated the influence of homocysteine concentrations on oxidative status in experimental induced hyperhomocysteinemia in rats. Hyperhomocysteinemia was experimentally induced by oral administration of a single daily dose of methionine, 2 mg/kg body weight, for 15 days [128]. All experiments were performed according to the standards of the European legislation concerning the care and use of experimental animals.

Material and Method
In order to determine Hcy plasma concentrations, a new HPLC analytical method was developed and thereafter submitted to validation procedure. The method uses derivatization followed by HPLC separation and UV detection.

Reagents

2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), trichloroacetic acid, tri-butylphosphine (TBP), perchloric acid, phosphate buffer, LiOH, acetonitrile, and methanol were purchased from Fluka and were for analysis or HPLC use.

Chromatographic system characteristic

The chromatographic system used for analysis consisted of Agilent 1200 HPLC 6520, Binary Pump, Zorbax SB-C 18 (4,6mm x 250mm) (5µm) and Detector UV-VIS (DAD) at 355 nm.

The mobile phase consisted of two solutions:

A. acetonitrile

B. mixture of 0.1 M trichloroacetic acid, 0.1 M LiON at pH = 1.65.

Elution occurred in the concentration gradient as follows:

- 4 minutes 11% A and 89% B
- 8 minutes 35% A and 65% B
- 12 minutes 11% A and 89% B

The flow rate of the mobile phase was 1.2 ml / min, sample volume was 20 µl and temperature was maintained at 25^0C.

Sample preparation procedure

There were used 0.3 ml of rat plasma to which 0.3 ml of 0.2M phosphate buffer pH 7.6, and 30 µl of 10% TBP in methanol were added. The mixture was heated for 30 minutes at 60^OC. After cooling at room temperature, 30µl of 0.1M CMQT and 0.3 ml of 3M perchloric acid (PCA) were added. After centrifugation at 12000 rpm for 10 minutes, an aliquot of 20 µl of supernatant was injected into the column.

Modificated procedure

We adapt a HPLC method for human plasma [129] by modifying the derivatisation and elution steps. We used tri-butylphosphine (TBP) instead of tri-n-butylphosphine (TnBP) as the reducing agent for oxidized and protein-bound homocysteine. The derivatisation agent was 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) [130]. We also use a gradient elution instead an isocratic elution.

Experimental procedures

The experiment was performed on two groups of 10 adult Wistar male rats, each weighing 150-200 g. Hyperhomocysteinemia was experimentally induced by methionine loading for 15 days as presented above. The animals from the two groups were fed differently, as follows:

- Group I - beside methionine, received food deprived of folic acid and vitamin B12, but containing apples 5g/100g animal, carrots 5g/100g animal, black bread 10g/100g animal and barley 10g/100g animal, for 15 days.
- Group II – beside methionine, received standard food containing 0.5 mg/ kg body weight folic acid and 10 mg/kg body weight vitamin B12 for 15 days.

Samples of blood were taken in order to determine homocysteine plasma concentration and oxidative stress parameters, initially and after 15 days of methionine exposure. Total homocysteine was determined in the rat plasma using the modified HPLC method.

In order to investigate the response of the antioxidant systems against high levels of homocysteine, the activities of intra-erythrocytes superoxide dismutase (SOD), glutathione peroxidase (GPx) and also plasma total antioxidant status (TAS) were measured using standardized Randox kit for research. The statistical analysis was realized by comparing the two groups using the variance

(ANOVA-one-way); a coefficient of $p<0.05$ was considered to indicate a statistically significant difference between groups.

Results and Discussions

For homocysteine determination in rats' plasma standard and calibration curves were performed. For standard curve, homocysteine stock solution of was prepared at a concentration of 10 mM. Working standard solutions were prepared by appropriate dilution of the stock one in the range of 1-32 μmol/ml and prepared as presented above in sample preparation. Aliquots of 20 μl of working standard solutions was injected into the HPLC column separated in gradient elution and detected in UV at 355 nm as presented above in modification procedure.

Calibration curve

Working similar standard solutions in the range of 1-32 μmol/ml were added to 0.3 ml of rat plasma and processed as described above in sample preparation procedure.

Chromatograms obtained for naïve rat plasma and for two concentration from calibration curve are presented in figures 31, 32, 33.

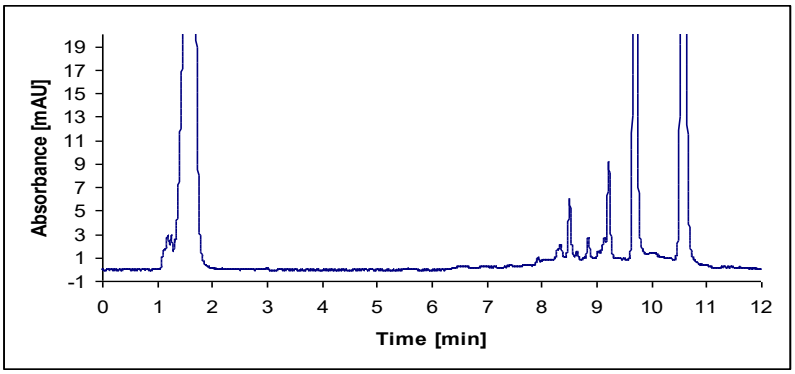


Fig. 31. Chromatogram obtained after elution of rat plasma sample, to which no homocysteine was added.

The retention time for homocysteine is tR = 8.6 significantly spaced from the elution time of the solvent which occurs at 1-2 minutes. Several peaks are observed in the chromatogram: at tR = 8.6 homocysteine is observed, in very small quantity, which comes from the endogenous plasma homocysteine, at tR = 9.1 cysteine, at tR = 9.7 cysteine-glycine and at tR = 10.5 CMQT used in excess.

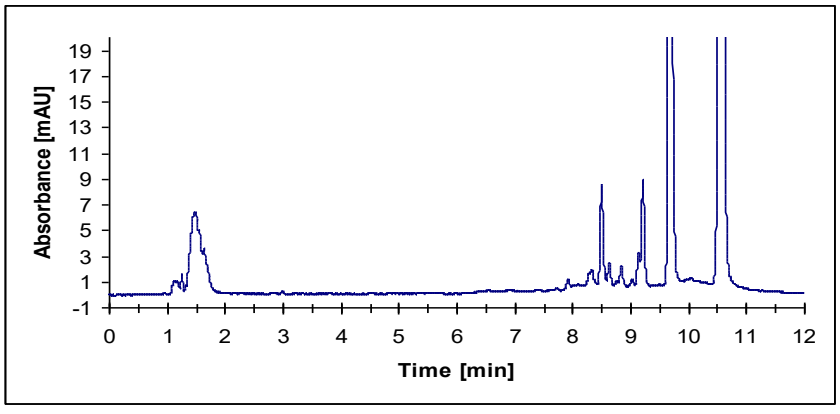


Fig. 32. Chromatogram obtained after eluting rat plasma sample, to which 4 μmoli of homocysteine were added.

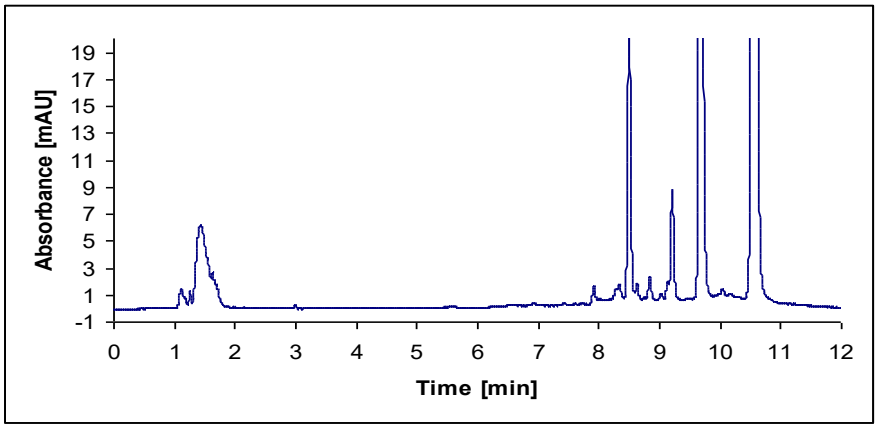
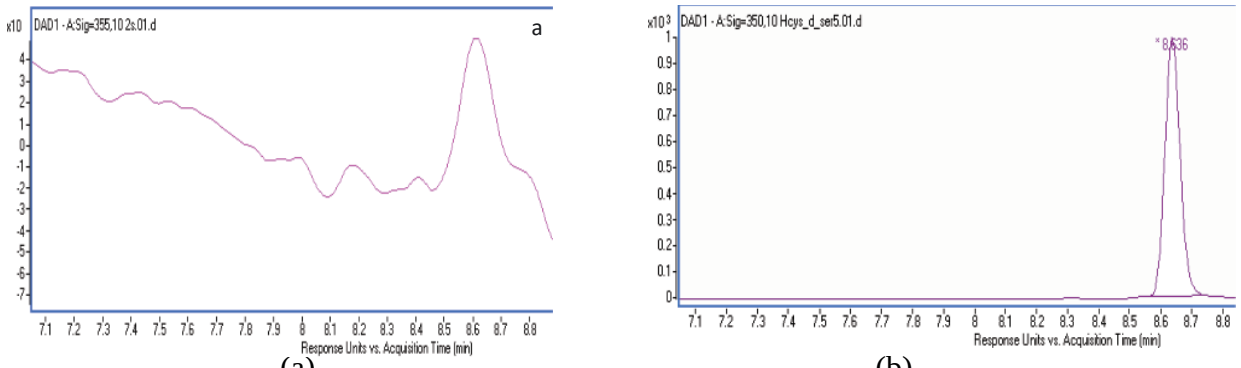


Fig. 33. Chromatogram obtained after eluting rat plasma sample, to which 24 μmoli of homocysteine were added.

By the chosen chromatographic method a good elution time for homocysteine is obtained, spaced from the solvent front and a good separation from other compounds with SH groups which are found in important concentrations in plasma. Thus homocysteine is eluted at a distance from glutathione, cysteine and cysteine-glycine. The chosen method is selective, preventing, through spaced retention times, the coelulation of compounds with similar structures.
The calibration curve for homocysteine was linear in the range of 1-32 μM. The linear regression equation for Hcys was Y= 30.97 X – 8.9676 (where Y represents the area of the peak and X represents the concentration), with a correlation coefficient r2=0.9975. The coefficient of variation in Hcys determination for 5 replicates was within 5% and recovery 86,6% (higher than the average retrieval recommendation of ± 15% of the value). Retention time for Hcys was 8,626 ±0,008 minutes.

Plasma homocysteine concentration in rats
Chromatograms obtained from rat plasma elution initially and after methionine loading are presented in Figure 34 a, b. Homocysteine was efficiently separated from other substances with similar structure. Starting from minute 7.1 to minute 8.8 (the interval during which homocysteine elutes) no interfering compounds were observed figure 34 b.



(a) (b)

Fig.34. Chromatograms of plasma samples obtained from the same rat, initially (a) and after methionine administration (b).

Homocysteine levels determined in rat plasma initially and after methionine administration are presented in table VIII.

Table VIII. Homocysteine concentration in rats after 15 days of methionine loading

	Homocysteine concentration (μM) Mean ±SD	
N = 10 animals/series	Initial	Final
Group I (animals receiving vitamin-deprived food exposed to methionine loading)	12,96±1.03	>32
Group II (animals receiving standard food and exposed to methionine loading)	11,33±0.97	>32

Our data show that in both groups' homocysteine concentrations are significantly increased after 15 days. Our data show that hyperhomocysteinemia was installed after 15 days of methionine administration thus confirming the experimental model in both groups. Despite vitamins supplementation, Hcy concentration did not decrease to a significant level and no difference was found between the group with and without vitamins administration. In cardiovascular diseases, the administration of vitamins is considered effective in reducing homocysteine levels [131-135], but in the hyperhomocysteine model made on rats, the administration of vitamins had no effect. We assume that the high homocysteine level found in both groups might have been generated as a result of disturbances in both the remethylation and the trans-sulfuration pathways or other unknown mechanisms [136-138]. On the other hand, our data are similar with other authors that consider that vitamin supplementation has only a slight effect in lowering homocysteine levels [139-140].

Under HHcy conditions, the antioxidant defense system show significant changes in both specific enzymes activities and plasma antioxidant compounds concentrations.

The total antioxidant status determined in rat plasma and the superoxide dismutase and glutathione peroxidase activities, determined in rat red blood cells, are presented in tables IX, X and XI respectively.

Table IX. Total antioxidant status determined initially and after methionine administration for a 15-day period.

	TAS	(mmol/l plasma) Mean ±SD
N = 10 animals/series	Initial	Final
Group I (animals receiving vitamin-deprived food and exposed to methionine loading)	0.908±0.022	0.760±0.024

Group II (animals receiving standard food and exposed to methionine loading)	0.897±0.036	0.756±0.041

Table X. Superoxide dismutase activity, in rat red blood cells, determined initially and after methionine administration for a 15-day period.

	SOD	(U/ml blood) whole Mean ±SD
N = 10 animals/series	Initial	Final
Group I (animals receiving vitamin-deprived food and exposed to methionine loading)	202.88±16.7	944.7±40.1
Group I (animals receiving standard food and exposed to methionine loading)	195.30±17.2	945.7±42.1

Table XI. Glutathione peroxidase activity, in rat red blood cells, determined initially and after methionine administration for a 15-day period.

	GPx	(U/L haemolysate) Mean ±SD
N = 10 animals/series	Initial	Final
Group I (animals receiving vitamin-deprived food and exposed to methionine loading)	60211±4926	34259±3897
Group II (animals receiving standard food and exposed to methionine loading)	64254±5067	39835±3524

TAS levels were significantly decreased no matter what the animal diet was. It is known that auto-oxidation of excess homocysteine produces free radicals [141-146] and as a consequence, decreased TAS levels are to be expected. The decay in TAS, as significant as it was, was not as severe as the changes observed in intracellular enzyme activity.

The activity of SOD suffered a huge increase of about four times the initial values explained by an increased presence of superoxide radicals. HHcy can generate reactive species by autoxidation [147,148] which could be an explanation to the increase recorded in SOD activity. On the other hand, the increase in reactive species concentration usually activates GPx and as a consequence we expected an increase in GPx activity. In fact, GPx activity decreased to almost half of its initial

activity. This result can be explained by a severe decrease in intracellular levels of antioxidant glutathione (the main cofactor of this enzyme) that leads to the observed decay in GPx activity.

Conclusions

As a conclusion HHcy, once installed, led to a severe imbalance in the intracellular antioxidant enzymes defense systems. Our findings are supported by the fact that homocysteine is mainly metabolized within the cell [149, 150].

In conclusion, hyperhomocysteinemia is linked to reactive species generation and the intracellular space seems to be more affected than the extracellular one.

Acknowledgements

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The results of this study have been published in two articles:
1.Central European Journal of Medicine, 2010, 5(5): 620-625
2.Medical Hypotheses, 2012, 78(4):554-555
(the title of the work, the authors and the complete data are presented at the end of the chapter, pg 66).

II.3. Investigating the influence of new synthetic compounds on homocysteine levels in experimental studies on laboratory animals

II.3.1. Introduction

Nowadays we can talk about two "epidemics" if we take into account the number of people affected, namely: diabetes and cardiovascular disease. The two conditions are interconnected, but obviously for each there is a specific medication. Whenever the two conditions are associated, there is a danger of over-medication. For this reason, an attempt was made to combine two active principles: one with a lipid-lowering action and another with improved glycemic control activity, through a chemical bond. The two principles chosen are:

- rutin, also named rutoside, quercetin-3-rutinoside and sophorin, is a citrus flavonoid glycoside with pharmacologic activity, found in buckwheat, the leaves and petioles of Rheum species and asparagus [151]. Rutin inhibits platelet aggregation [152,153] and prevents atherosclerotic lesions in mice [154], rats [155], hamsters [156] and rabbits [157]. Rutin has also an antioxidant activity [18]. Recent studies pointed out that rutin reduces lipid levels [158-162].

- a pyridine group (similar to niacin) in L103 and a pyrimidine (rosuvastatin like) in L3, both chemically bound to a Rutin molecule. It is considered that both molecules present lipid lowering effect in blood, as follows: the pyridine ring act mainly on triglycerides and the pyrimidine group on cholesterol. Niacin exerts antihypertensive effects at low doses, hypolipidemic effects at high doses [163] and is used in cardiovascular therapy [164].

The two principals were chemically bonded resulting two new derivatives coded L103 and L3 that contains bond a pyridine group respectively a pyrimidine group to the rutoside part.

The synthesis of the new compounds was performed at the at the department of Pharmaceutical Chemistry from the Faculty of Pharmacy of the UMF"Grigore T.Popa" Iasi. The two compounds were coded L3 and L103 and their structures are presented in figures 35 and 36.

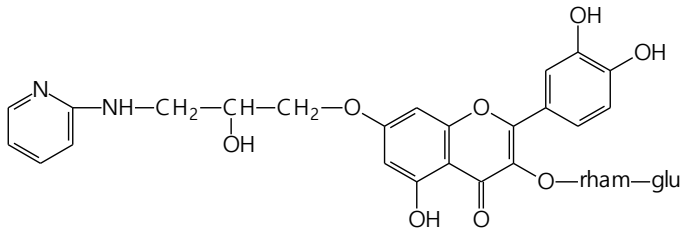

Fig.35. The structures of the compounds coded as L3

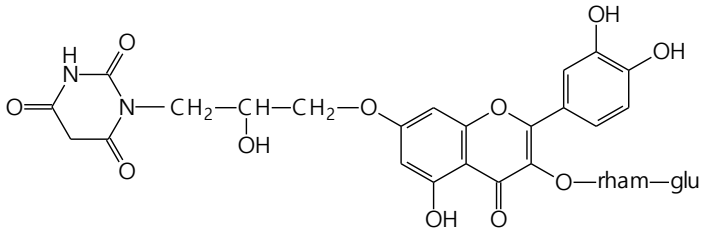

Fig.36. The structures of the compounds coded as L103

Elemental analysis of these two compounds was performed on "Exeter Analytical" CE-440 elemental analyzer.
The therapeutic dose for both derivatives L3 and L103 has been calculated as 1/20 from 50% lethal dose (DL50) at the department of Pharmacology at the Faculty of Medicine UMF "Grigore T.Popa" Iasi.
In the following will be presented the investigations regarding the influence of the 2 compounds on Hcy levels, atherosclerotic risk parameters and antioxidant status in two experimental induced models: hyperhomocysteinemia and hyperglycemia.

II.3.2. The influence of o new Rutin derivative (L103) in an experimental hyperhomocysteinemia model induced in rats

Aim: This study investigates the influence of the new synthesized rutin derivative coded as L103, on homocysteine, cholesterol and antioxidant status in rats with experimentally induced hyperhomocysteinemia by methionine loading.

Material and Method
The study was performed on three groups of 10 adult Wistar male rats, weighing 150-200g. The rats received standard food (containing folic acid and vitamin B12) and water ad libitum. All procedures were performed according to the European legislation concerning the care and use of animals for scientific purposes (Directive 86/609/EEC)[21].
Hyperhomocysteinemia was experimentally induced to all groups by oral administration of methionine 2g/kg body weight (b.w.) single dose daily, for 30 days [128].

- Group I was control and received only methionine as presented before;
- Group II received methionine similar to group I and niacin orally 50 mg /kg b.w. single dose daily, for 30 days;
- Group III received methionine similar to group I and orally L103 in 36,76 mg/kg b.w. single dose daily, for 30 days.

The blood samples were taken from retro-orbital plexus. Homocysteine, TAS, and cholesterol concentrations were determined before and after 30 days of methionine loading and administration of nicotinic acid and L103.
TAS and cholesterol concentrations were measured using a Randox kit for manual use.
Total plasma homocysteine was determined by previously presented HPLC validated method (presented in paragraph II.2). The statistical analysis used the variance ANOVA-one-way and Turkey-Kramer multiple comparisons; a coefficient $p< 0.05$ was considered to indicate a statistically significant difference within or between groups.

Results and Discussion
Homocysteine concentrations determined in rat plasma are presented in table XII.
The obtained data show significant increase in homocysteine level for all groups at final moment compared to initial moment, thus confirming HHcy status.

Table XII. Homocysteine concentrations determined in rat plasma, after methionine loading

	Hcys (μm/L); Mean ±SD	
N = 10 animals/series	Initial	Initial
Group I - only methionine	9.692 ± 0.692	31.056 ± 0.616*
Group II - methionine plus nicotinic acid	10.555± 0.668	28.06 ± 0.540*Ψ
Group III - methionine plus L103	10.167± 0.852	26.828 ± 0.505*Ψ

*Statistical difference within group. ΨStatistical difference between groups

Data are consistent with the literature [165] and our previous studies [166]. Hcy levels in groups II and III were significantly lower as comparing to control group at the final moment (< 0.001 for both groups), but Hcys concentration is still high when comparing to initial moment. Between group II and group III there is no statistical difference in Hcy levels in the final moment ($p< 0.055$). Hcy concentrations in human blood are classified as follows: normal range 5-15 μM; moderate 16-30 μM; intermediary 31-100 μM; severe above 100 μM [165]. Hcy levels appear to be influenced by the administration of nicotinic acid and L103 but to a small extent. Extrapolating to human classification the Hcys levels fit to moderate /intermediary HHcy. Thus, we can assume L103 fails to prevent the increase of homocysteine levels. Even so, lowering HHcy level from intermediary to moderate, might be a benefit for L103 administration.
The total antioxidant status determined in rat plasma is presented in table XIII.

Table XIII. TAS status determined in rat plasma, after methionine loading

	TAS (mmol/l plasma); Mean ±SD	
N = 10 animals/series	Initial	Final
Group I - only methionine	1.810±0.111	1.064±0.095*
Group II - methionine plus nicotinic acid	1.737± 0.134	1.237±0.203*
Group III - methionine plus L103	1.859± 0.049	1.469±0.116*Ψ

*Statistical difference within group. ΨStatistical difference between groups

Between groups II and control there is no statistical difference for the final moment ($p=0.192$). The fact that TAS levels are slightly higher in group II versus control suggests a minor antioxidant effect due to niacin presence. Literature shows that the oxidant/antioxidant activity of niacin

depends on the time of exposure as follows: the antioxidant activities of niacin in the later phases (3 weeks) of lipid peroxidation are much stronger than those in the earlier phases (1 week) [167]. Even if niacin has been administrated for 4 weeks, we assume that its antioxidant capacity was exceeded by the free radicals generated through the autooxidation of Hcy high concentrations [168,169]. Group III presents significantly higher TAS concentration as compare to control group in final moment (p= 0.0011). In fact, the drop in TAS concentrations was the smallest of all three groups, suggesting a protective antioxidant activity attributable most likely to the rutin moiety. The comparison between groups II and III shows no statistic difference at the final moment.
Cholesterol concentrations determined in rat plasma are presented in table XIV.

Table XIV. Cholesterol concentrations determined in rat plasma, after methionine loading

	Cholesterol (mg/dL); Mean ± SD	
N = 10 animals/series	Initial	Final
Group I - only methionine	85.94 ± 3.31	97.43 ± 4.16*
Group II - methionine plus nicotinic acid	89.27 ± 5.11	88.07 ± 3.07Ψ
Group III - methionine plus L103	86.47 ± 9.79	87.30 ± 2.72Ψ

* Statistical difference within group. Ψ Statistical difference between groups

Cholesterol concentrations were significantly increased at the final moment as compared to the initial one in control group (p=0.0013). Literature shows [170,171] that after methionine administration, cholesterol level increases because of the stimulation of its hepatic synthesis; our data being consistent to that. Within groups II and III no statistic difference in cholesterol concentrations was found when comparing initial to final moment (p = 0.702 respectively p=0.844). For group II, this result is justified because niacin a known hypolipidemiant drug has been administered. The similar behavior for group III suggests a nicotinic acid-like activity for L103. Both groups II and III exhibits significant decreased cholesterol levels as compare to control group at the final moment (p=0.0035 respectively p=0.0008) suggesting the lipid-lowering activity for niacin as well as for L103. Cardiovascular disease has multiple determinants: accumulation of lipids, the onset of inflammation, disruption of the redox balance [172,173]. Decreasing any of these factors favors recovery or at least prevents the evolution of the condition.

Conclusions

The new rutoside derivative L103, presents anti-oxidative properties and lowering cholesterol level in the experimental induced hyperhomocysteinemia in rats. We assume that in the case of the moderate/intermediary hyperhomocysteinemia the decrease of two aggravating risk factors (cholesterol and reactive species) justifies the use of L103. Since L103 exerts similar lipid-lowering effect to niacin, its administration might avoid the niacin side effects being a benefit in dyslipidemia. The fact that L103 pulls back homocysteine levels, even in a very small amount, may be useful in cardiovascular disease.

Acknowledgements

This study was supported from scientific grant from Romanian Education Ministry; program PN-II IDEI, cod 1225, Grant no. 223/2007

This study was published in Farmacia, 2017, 65(4):596-599 (the title of the work, the authors and the complete data are presented at the end of the chapter, pg 66).

II.3.3. The influence of a new rutin derivative (L3) on homocysteine, cholesterol and total antioxidative status in experimental diabetes in rat

Diabetes is a widely spread metabolic disease [174-178] that involves disturbances in the glucose, lipid and mineral levels, as well as in redox reactions. One of the major risk factors in diabetes is the cardiovascular disease and its complications [179]. Several biochemical parameters such as hypercholesterolemia [180], hyperhomocysteinemia [181,182] and the reactive species increse [183] are considered aggravating factors for the diabetes.

Aim: This study investigates the influence of a new rutin derivative coded L3, on homocysteine, cholesterol and antioxidant status in rats with experimentally alloxan-induced diabetes.

Materials and Methods

The study was performed on three groups of 10 adult Wistar male rats, weighing 150-200g, standard food and free access to water. All procedures were carried out in accordance with the Directive 86/609/EEC (24th November 1986), regarding the protection of animals used for experimental and other scientific purposes.

Experimental diabetes was induced by intraperitoneal (i.p.) administration of a single dose of 110 mg/kg b.w. of alloxan single dose [184-186] to all of animals.

The animals were divided into 3 groups as follows:

- control group that received no substance;
- group that received only alloxan;
- group that received alloxan and L3 in 10.96 mg/ kg b.w. as single daily dose by oral route, for a 30 days period.

Samples of blood were taken initially and after 30 days and glucose, cholesterol, homocysteine concentration and total antioxidant status (TAS) were determined.

For glucose, cholesterol and TAS determination, Randox kit for manual were used.

For plasma homocysteine determination the immunoassay technique using an Axis Homocysteine EIA kit was used.

The statistical analysis for all determined parameters was realized by comparing groups using the analysis of variance (One-Way ANOVA) followed by the Bonferroni post hoc test; a coefficient $p< 0.05$ was considered to indicate a statistically significant difference between groups.

Results and Discussions

Glucose, cholesterol, homocysteine and TAS levels, determined in rat plasma, initially and after 30 days are presented in tables XV, XVI, XVII and XVIII respectively:

Table XV. Concentration of glucose in rat plasma determined initially and after 30 days.

Group N = 10 animals/group	Glucose concentration (mg/dL) Mean ±SD	
	Initial	Final
Control	78.31 ± 4.32	80.3±3.25
Alloxan i.p. 110mg/kg b.w. single dose	72.12±2.29	99.67±3.34*
Alloxan i.p. 110mg/kg b.w. single dose; L3 orally 10.96 mg/kg b.w. single daily dose, 30 days	76.85±3.98	80.028 ±1.79
* The mean difference is significant at the $p = 0.05$ level		

For the control group glycemia remains constant while for group that receive alloxan glucose levels were, as expected, significantly high, thus confirming the hyperglycemic status.
A significant decrease in glucose concentration is found when comparing animals treated and not treated with L3, at final moment (p=0.0001 compared to the final measurement). Even so, we cannot assume that L3 certainly has a hypoglycemic activity. Rather, we believe that early administration of L3 to some extent prevents hyperglycemia.

Homocysteine concentrations determined in rat plasma initially and after alloxan administration are presented in table XVI:

Table XVI. Homocysteine concentrations in rat plasma determined initially and after 30 days.

Group N = 10 animals/group	Hcys (μm/L) Mean ±SD	
	Initial	Final
Control	7.43 ± 1.04	7.28 ± 1.21
Alloxan i.p. 110mg/kg b.w. single dose	8.62 ± 1.47	8.97 ± 1.37
Alloxan i.p. 110mg/kg b.w. single dose; L3 orally 10.96 mg/kg b.w. single daily dose, 30 days	7.62 ± 0.61	6.67 ± 1.04
* The mean difference is significant at the $p = 0.015$ level		

Homocysteine levels in the control group and alloxan receiving group remains constant, suggesting that high glucose levels for a 30 days period do not affect homocysteine levels.
A significant decrease in the homocysteine levels was observed when comparing the group receiving derivative L3 to the group that received only alloxan, at the final moment (p=0.0015). However, we cannot interpret the data obtained as a direct effect of L3 on the Hcys level. Early administration of L3 may attenuate the effects of hyperglycemia on protein metabolism, somehow lowering homocysteine levels (p = 0.015).
The total antioxidant condition determined in rat plasma initially and after alloxan administration is presented in table XVII:

Table XVII. Total antioxidant status in rat plasma determined initially and after 30 days.

Group N = 10 animals/group	Hcys (μm/L) Mean ±SD	
	Initial	Final
Control	1.61 ± 0.295	1.55 ± 0.296
Alloxan i.p. 110mg/kg b.w. single dose	1.48 ± 0.328	1.02 ± 0.074*
Alloxan i.p. 110mg/kg b.w. single dose; L3 orally 10.96 mg/kg b.w. single daily dose, 30 days	1.55 ± 0.443	1.45± 0.086
* The mean difference is significant at the $p = 0.05$ level		

TAS levels present similar decreasing trends or all groups at the end of the study, except the group receiving only alloxan where the decrease is significantly statistic (p=0.0251. This suggests the influence of hyperglycemia on the antioxidant status disruption.
In contrast, when comparing the two groups that received alloxan, a significantly higher value of the TAS concentration was observed in the group that also received the L3 derivative (p = 0.0484) at the

final time. These data suggest that in the group that received alloxan and the L3 derivative, the oxidative-reducing balance was less affected by either lower glucose levels or by the antioxidative activity of L3 derivative.

The cholesterol concentrations determined in rat plasma initially and after the alloxan administration are presented in table XVIII:

Table XVIII. Cholesterol concentrations in rat plasma determined initially and after 30 days.

Group N = 10 animals/group	Cholesterol (mg/dL) Mean ±SD Initial	Final
Control	48.29 ± 6.95	50.91 ± 5.38
Alloxan i.p. 110mg/kg b.w. single dose	56.03 ± 6.16	58.07 ± 8.82
Alloxan i.p. 110mg/kg b.w. single dose; L3 orally 10.96 mg/kg b.w. single daily dose, 30 days	58.26 ± 8.43	46.6925 ± 5.09* Ψ
* The mean difference is significant at the $p = 0.05$ level		

*Statistical difference within group. ΨStatistical difference between groups

For the first two groups there is no significantly changes cholesterol concentrations at the final moment. These data show that high glucose levels do not necessarily trigger the rise of cholesterol levels.
For the group that received alloxan and the L3 derivative, cholesterol levels present a significant decrease (p=0.0103) after a month of L3 exposure when compared with the initial measurements
Lower concentration of cholesterol was also observed when comparing the group that received alloxan and the L3 derivative to the group that received only alloxan at the final moment (p=0.012).
We assume that the pyridine part in the compound L3 (rosuvastatin like) is responsible for the cholesterol-lowering effect.

Our data show that hyperglycemia does not increase substantially the homocysteine and the cholesterol levels. In a diabetic-like condition we experimentally induced, TAS concentration decreases significantly but the cholesterol and the homocysteine show an almost normal level. These data suggest that after a month of exposure to hyperglycemia, the redox balance is the most affected parameter.
In diabetes, cholesterol as well as homocysteine represent aggravating factors and, as a consequence, keeping their concentrations closer to the normal levels is a benefit from the L3 derivative.
Recent studies show that the excess of homocysteine conduces to an auto-oxidative reaction and generates free radicals [187,147]. When homocysteine levels are low, the free radical's generation is scarce and, as a consequence, TAS levels are less affected. In L3 administration TAS levels decrease only slightly and correlate with the measured low homocysteine levels.
Therefore, our research suggests that L3 administration may exert a protective activity for diabetics' condition, reducing risk factors such as cholesterol, homocysteine and free-radicals.

Conclusions

The new rutin derivative taken into study, noted L3, presents antioxidative properties and a slightly lowering-cholesterol and homocysteine levels in a similar diabetes state.

It seems also effective in decreasing glucose levels when administered in the early stages of disease.

It may be assumed that this effect is obtained through a decrease of the known risk factors mentioned above.

Acknowledgements

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This work was published in the article, Farmacia, 2013, 61(6), 1167-1177 (the title of the work, the authors and the complete data are presented at the end of the chapter, pg 66).

General conclusions on the chapter

The chapter has presented a reliable model of hyperhomocysteinemia performed on rats.

Using this model, it was possible to study the influence of HHcy on antioxidative defense system and to conclude there is a certain positive correlation between them.

We also be able to study the activity of two new synthesized compounds that proves benefic effect on some parameters such as glycemia, cholesterol, total antioxidant status but with low effects on HHcy.

The results of the studies were published in 4 scientific papers and 2 book chapters in international publication presented below:

The results of the studies presented in Chapter II were published in the following publications*:*

Full articles in ISI journals

1. **Cristiana Filip**, Elena Albu, Dan Lupascu, Nina Filip. The influence of o new Rutin derivative in an experimental model of induced hyperhomocysteinemia in rats, Farmacia, 2017, 65 (4), 596-599
2. Elena Albu, Dan Lupascu, **Cristiana Filip**, Irina M Jaba, Nina Zamosteanu, The influence of a new rutin derivative on homocysteine, cholesterol and total antioxidative status in experimental diabetes in rat, Farmacia, 2013, 61(6), 1167-1177
3. Elena Albu, **Cristiana Filip**, Nina Zamosteanu, Irina Maria Jaba, Ines Strenja Linic, Ivan Sosa, Hyperhomocysteinemia is an indicator of oxidant stress, Medical Hypotheses, 78(4),554-555, 2012
4. **Filip C**, Albu E, Zamosteanu N, Jaba I.M, Silion M, Gheorghita N, Ostin.C.Mungiu - Hyperhomocysteinemia effect's on antioxidant capacity, in rats, Central European Journal of Medicine, 2010, 5(5), 620-625, ISSN 1895-1058,

Book chapters published in international publishing houses

1. **Filip Cristiana**, Zamosteanu Nina, Albu Elena - Blood Cell -An overview of studies in hematology, Chapter 3. Homocysteine in red blood cells methabolism- Pharmacological Approaches, Edited by Terry E. Moschandreou, 2012, pg 33-73, ISBN 978-953-51-0753-8 InTechopen Croatia,
2. **Cristiana Filip,** Elena Albu, Hurjui Ion, Catalina Filip, Cuciureanu Magda, Radu Florin Popa, Demetra Gabriela Socolov, Ovidiu Alexa and Alexandru Filip. Chapter 3. Is Homocysteine a Marker or a Risk Factor: A Question Still Waits for an Answer, in Non-Proteinogenic Acids, edited by Nina Filip and Cristina Elena Iancu, 2018, pg. 33-52, Print ISBN 978-1-78984-728-4, Online ISBN 978-1-78984-729-1, InTechopen Croatia **http://dx.doi.org/10.5772/intechopen.81799**

Chapter III. Investigation of antioxidant status in acute and chronic inflammation

III.1. Introduction.

A radical is a chemical species that possesses a single unpaired electron in outer orbitals and is able to independently exist, known also as free radical. Radicals are highly reactive in extracting an electron from any neighbor molecule in order to complete their own orbitals. There are two main groups of free radicals: ROS or reactive species of oxygen and RNS or reactive species of nitrogen. ROS and RNS can act individually or together in physiological processes or damaging cells causing the so-called nitro-oxidative stress. Therefore, these two species are often collectively referred to as ROS/RNS.

Reactive oxygen species

Reactive species of oxygen refers to a group of highly reactive O_2 metabolites, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($1O_2$), and hydroxyl radical (OH·), that can be formed within cells. Reactive oxygen species are constantly formed as necessary products:

- in order to kill invading pathogens (in neutrophils)
- signaling molecules
- byproducts in normal enzymatic reaction in all human cells through normal aerobic processes as mitochondrial oxidative phosphorylation

The ground state of oxygen is triplet oxygen meaning that the molecule has two unpaired electrons occupying two different molecular orbitals (Figure 37).

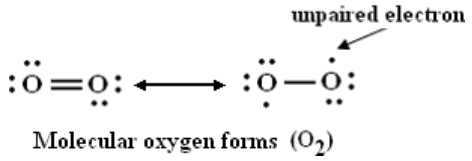


Figure 37. The structure of oxygen molecule

Oxygen O_2 is both kinetically stable and very reactive, depending the surrounding conditions.
It is stable as it cannot react rapidly with already paired electrons in the covalent organic bonds. It is very reactive in the presence of transition metals (eg Fe, Cu, Mn) where it rapidly captures an electron [188,189].
In living cells oxidative attack of O_2 generates superoxide radical (O_2^-) which tend to be slow (as transitional metals are bounded to proteins). Once an electron acquired, additional electrons are easier added, further reactions quickly occur [190] and reactive species of oxygen are generated.
Additional electrons convert superoxide to hydrogen peroxide (not a radical) than to hydroxyl radical (OH·) considered the most reactive free radical and finally hydroxyl ion (Figure 38) [191].

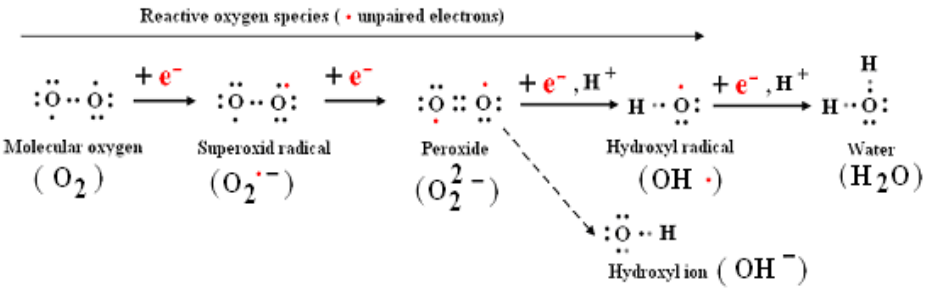


Figure 38. Reactive oxygen species generation

From biological point of view superoxide radical (O_2^-) acts quickly with any suitable structure encountered, so that it cannot diffuse at a distance. Even so, its harmful potential comes from its ability to generate two additional free radicals: strong hydroxyl radical (through Haber-Weiss reaction) and peroxynitrite (by collision with NO•) [192].

Hydrogen peroxide (H_2O_2) is not a free radical but it generates (Haber-Weiss reaction) [193-195] the most powerful hydroxyl radical OH•. Hydrogen peroxide (H_2O_2) is lipid soluble and as a consequence it can diffuse through and across lipid membranes. Its limited reactivity allows it to disperse on a large scale and any metal structure encountered will favor the formation of the OH• radical thus damaging local protein structure.

The hydroxyl radical (OH•) is the highly reactive structure because of its indiscriminate reactivity. It reacts with any biostructure encountered and as a consequence it diffuses only over a short distance. Even so it makes big damages because it can initiate autocatalytic radical chain reaction [196].

Reactive nitrogen species

Nitric oxide (NO•) is a signal molecule, synthesized by many cells, it plays different roles depending on the physiological conditions as follows: vasorelaxation and anti- inflammatory effect under normal physiological conditions [197,198] or pro-inflammatory mediator under pathological conditions. Its effect depends on the type and proximity of the target cell, as well as its chemical concentration [198]. The enzyme that synthesizes NO is nitric oxide synthase and it presents three isoforms: NOS-1 and NOS-3 are constitutive isoenzymes and NOS-2 which is the inducive isoform [199, 200]. The generation and role of NO• is subjected to complex mechanisms, because blood oxygenation dictates superoxide radical versus NO• radical formation. [201, 202].

Systems that neutralize reactive species.

Antioxidant defense systems of living organisms can be classified into enzymatic and non-enzymatic. Intracellular acts the enzymatic systems of superoxide dismutase and peroxidase. In the blood acts the so-called "total antioxidant capacity" which includes all compounds with reducing functions such as: glutathione, vitamin C, uric acid, bilirubin etc. The consequences of the intensity of the oxidative attack can be measured by malondialdehyde (MDA) which levels directly correlate to the extent of oxidative damages. Malondialdehyde (MDA) is the most frequently used biomarker of oxidative stress in many health problems such as cancer, psychiatry, chronic obstructive pulmonary disease, asthma, or cardiovascular diseases.

III.2. The Effects of Two Nitric Oxide Donors in Acute Inflammation in Rats

It is generally accepted that atherosclerosis has an inflammatory base. Its treatment includes vasorelaxant drugs such as NO donors. Moreover, NO is a signal molecule that plays a key role in the pathogenesis of inflammation [203, 204] and also participates in the generation of reactive species [205]. Literature mentions that NO as well as NO donors show ambivalent anti-inflammatory or pro-inflammatory activity depending on the physiological and pathological conditions [206, 207]. Thus, the present study investigated the effects of NO donors on oxidative and inflammatory status in rats with experimentally induced acute inflammation. The study was carried out as part of a doctoral thesis that investigated the pharmacodynamic effects of some NO donors used in the treatment of cardiovascular disease.

Aim: The study investigates the effects of two nitric oxide donors, named Nebivolol and S-Nitroso-glutathione (GSNO) on the antioxidant status, in experimental-induced acute paw inflammation in rats.

Material and Method

Reagents

Carrageenan, nebivolol and S-nitroso-glutathione were purchased from Sigma Chemical Aldrich Co Germany. Nebivolol, indomethacin and the saline solution were obtained in the form of standardized preparations available on the pharmaceutical market. The saline solution was ready to use, and the other solutions were prepared extemporaneously.

Materials and Methods

The experiment was carried out on Wistar rats (200-250 g) receiving standard pellets food and water at libitum. The inflammation was experimental induced by locally subcutaneous injecting a solution of 0.2 mL 1% carrageenan into the hind paws of all animals.

Experimental animals were randomly assigned to 4 groups (5 animals each) which received intraperitoneally (i.p.) the following substances:

- Group 1 (SS) - saline solution 0.1mL/100 g body weight (b.w.)
- Group 2 (IND): indometacin 150 mg/kg b.w.
- Group 3 (NEB): nebivolol 1 mg/kg b.w.
- Group 4 (GSNO): S-nitroso-glutathione 1 mg/kg b.w.

The extent of local inflammation was assessed by measured the posterior paw volume, using a plethysmograph (PanLab Apparatus) initially (before carrageenan injection), at 1, 3, 5, 24 h and 3 days after the inflammation was induced [208, 209].

Blood samples were taken from the retroorbital plexus under general anesthesia and analyzed for: hematological, biochemical as well as immunological determinations initially, at 24 hours and 72 h after irritant injection. Histopathological examination was also performed on fragments of hepatic tissue taken at the end of the experiment from euthanized animals (under general anesthesia with 2% enflurane).

Hematological assessment was performed by Cells blood counting on HEMAVET 950 automatic analyzer, with fluorescence flow cytometry.

Biochemical assessment was performed by measuring the activities of alanine aminotransferase (ALT), aspartate amino- transferase (AST) and lactate dehydrogenase (LDH) using an ACCENT 200 analyzer.
Oxidative stress was assessed by measuring the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) using standardized Randox kit for manual use.
Inflammatory response was assessed by measuring the levels of interleukin IL-6 and tumor necrosis factor alpha (TNF-α) using standardized Bio.kit for manual use. Complement fractions C3 and C4 activity was determined using Hartmann-Bracy technique.
Histological examination was performed after preparation (formaldehyde fixation, paraffin inclusion, Masson 's trichrome staining) by optical microscopy, using a Nikon E600 Ti Eclipse apparatus.
The statistical analysis for all determined parameters was realized by comparing groups using the analysis of variance (One-Way ANOVA) implemented in the SPSS 17.0 software for Windows. The values of the coefficient p below 0.05 were considered to be statistically significant compared to control group.
The experiment was performed under international ethical regulations and the EU Directive 2010/63/EU regarding the investigations performed on laboratory animals [21] and according to the recommendations of the Committee for Research and Ethical Issues of "Grigore T. Popa" University.

Results and Discussions

Anti-inflammatory effect

Following the injection with 1% carrageenan solution, the inflammatory process developed and reached its maximum intensity 3-5 h after the irritant injection and was maintained for about 24 hours. Subsequently, the inflammatory process gradually decreased, but retained its specific characteristics for up to 72 hours (Figure 1) [210, 211].
The inflammation increase is determined by comparing the paw volume obtained for each group with its own initial volume and expressed as a percentage of paw volume inflammation (% PVI), using the following formula: %PVI = (determined paw volume - initial volume) x 100 / initial volume.
The anti-inflammatory activity was evaluated as the percent of paw inhibition of edema (%PIE) according to the equation: %PIE = (%PVI control -%CVL treated) x 100 / %CVL control where:

-% PVI control represent the increase percentage for the saline solution receiving group
-% CVL treated represent the increase percentage for the NEB/GSNO receiving group
- % CVL control represent the increase percentage for indomethacin receiving group

The variation of the paw volume following the administration of IND, NEB and GSNO are presented in figure 39.

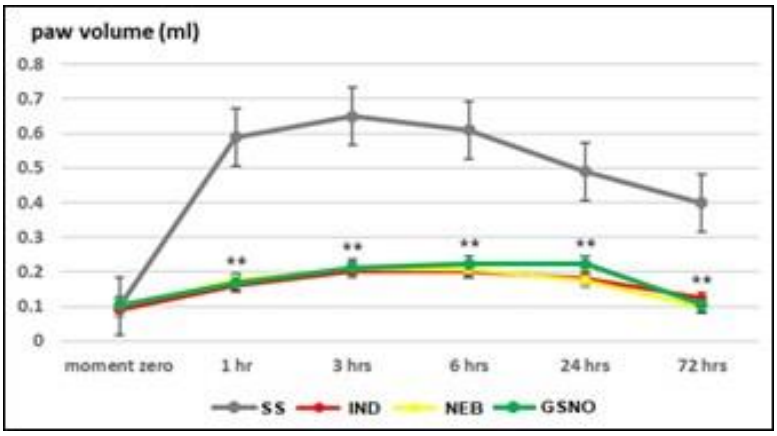


Fig. 39. Effects of NEB and GSNO on the carrageenan-induced paw volume in rats. Each point represents the mean ± standard deviation (SD) of the average paw volume, for 5 animals in a group. **p<0.01 statistically significant compared to control group.

Figure 39 shows that in the IND group a rapid and progressive decrease in paw volume was observed even after one hour (**p<0.01), much more pronounced in the range of 3 to 6 h (**p<0.01), but statistically significant also at 24 and 72 h (**p<0.01). The administration of NO donors NEB and GSNO produced a significant decrease in the paw volume, statistically significant (**p < 0.01) compared to control in the experiment (Figure 39) and similar with the IND group.

The anti-inflammatory effect of IND, NEB and GSNO is presented in figure 40.

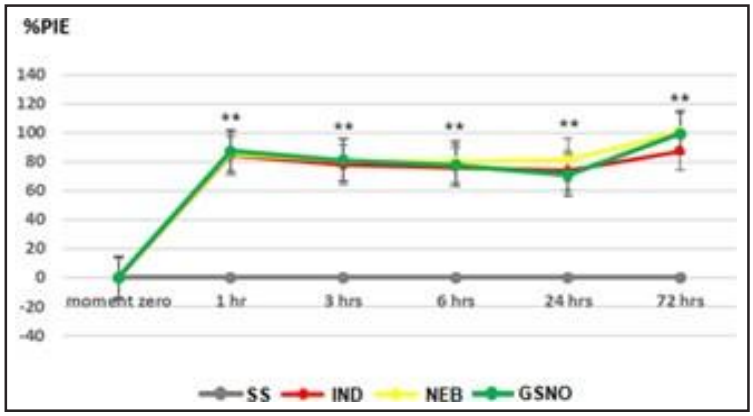


Fig. 40. %PIE determined by NEB and GSNO in the carrageenan- induced paw inflammation in rats. **p<0.01 statistically significant compared to control group.

IND manifested the most pronounced %PIE after one hour (84.13 ± 3.25) and at 72 h (87.41 ± 4.55). NEB and GSNO produced the highest %PIE at 1 h (85.19 ± 3.10 and 87.44 ± 5.28) respectively and at 72 h (100.65 ± 7.62 and 99.36 ± 6.23), respectively.

Obtained data show that the use of NEB and GSNO induced substantial anti-inflammatory effects especially at 24 h in this standard experimental test [212]. Nebivolol exhibited more pronounced

anti-inflammatory effects than GSNO, but less intense than indomethacin at different times during the experiment.

The reactive species assessment

The use of IND, NEB and GSNO produced a marked elevation ($p<0.01$) in SOD and GPx values after 24 h, compared to the initial moment (or moment zero-MO) (Figures 41,42).

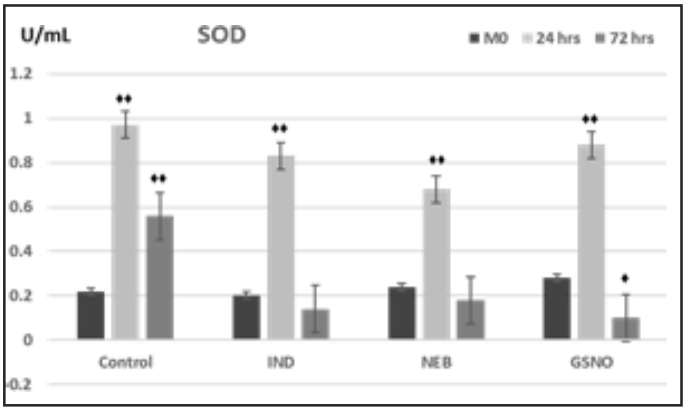


Fig. 41. The effects of NEB and GSNO on the activity of SOD (U/mL). Data were expressed as arithmetic mean ± S.D. of mean for 5 animals in a group. •$p<0.05$, ••$p<0.01$ significant vs M0.

Induction of paw edema triggers the activity of the antioxidant defense system at different intensities depending on the administration of anti-inflammatory agents or its lack. Being in the first line of antioxidant defense, increased SOD activity indicates the presence of oxidative aggression directly correlated to inflammation.

SOD activity significantly increases for all groups, the highest activity for SOD being recorded to the control group (receiving no anti-inflammatory agent).

SOD activity to groups receiving IND, NEB and GSNO induced a smaller increase in SOD activity. Thus a lower level of inflammation indicates a lower reactive species generation in the presence of these compounds. The lowest level of SOD activity found in the group receiving NEB indicates that this compound has the strongest limitation of inflammation and its subsequent effects.

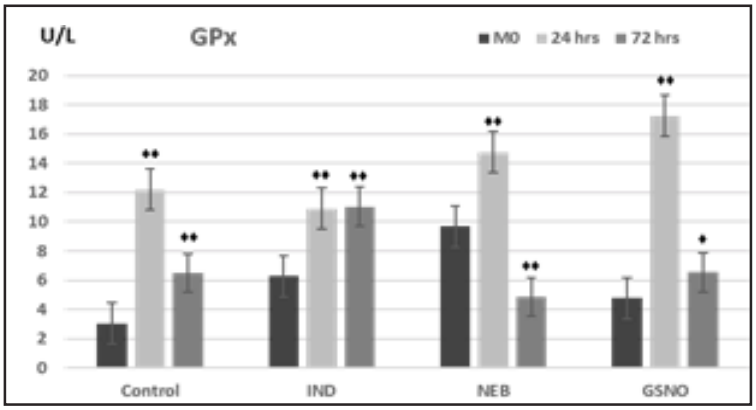


Fig. 42. The effects of NEB and GSNO on the activity of GPx (U/L). Data were expressed as arithmetic mean ± S.D. of mean for 5 animals in a group. •$p<0.05$, ••$p<0.01$ significant vs M0

The second in line of antioxidant defense GPx activity was found increased in all group as compared to initial moment but to different extent.
Following IND administration, the GPx activity increases (•• p <0.01) to similar values at both 24 and 72 hours (•• p <0.01) after injection of the irritant agent. Obtained data suggests the IND limitation of inflammation and subsequent oxidative aggression as expected for the anti-inflammatory consecrated drug.
The administration of NEB and GSNO shows a similar increasing pattern, that indicates an over-induction of enzyme activity followed by a severe decrease at 72 hours. The recorded decay in GPx activities can be justified by depletion of the enzyme co- substrate the antioxidant molecule of glutathione.
The huge activity recorded for GSNO recorded at 24 hours can be attributed to the glutathione component present in the GSNO structure, which acting as a reserve can be released as needed. Thus, the GSNO structure can prove to be a benefit for the detoxification capacity of the entire anti-oxidant system.

Following the carrageenan-induced paw inflammation, the percent of leucocyte formula elements significantly changed at 24 and 72 h, presented data in table XIX.
In the control group polymorphonuclear neutrophils (PMN) gradual increases, lymphocytes (Ly) and eosinophils (E) progressive decreases.
In IND and NEB receiving groups a similar path was registered, thus PMN, eosinophils (E) and monocytes (M) increase and Ly significantly decreases and eosinophils (E) progressive decreases at 24 h (*p<0.05). 72 h after administration the path changes as the percentage of PMN decreased and the percentage of Ly increased, statistically significant compared to SS group after 72 h.
For the group receiving GSNO PMN percent significantly decreases (*p<0.05) while Ly and M percent, statistically increase (*p<0.05) compared to control at 24 and 72 h in the experiment

Tab. XIX. The effects of NEB and GSNO on the leucocyte formula elements in the carrageenan-induced paw inflammation in rats. Values are presented as Mean ± SD of the leucocyte's formula for 5 animals per group. *p<0.05 statistic significant compared to control.

		Leukocyte formula (%)				
		PMN	**Ly**	**E**	**M**	**B**
SS	M0	17.62±1.70	75.12±1.47	2.40±0.27	4.84±1.02	0.10±0.07
	24 h.	32.64±2.36	63.76±3.40	1.32±0.27	2.42±0.41	0.00±0.00
	72 h.	33.70±1.85	61.92±1.87	1.20±0.16	3.20±0.54	0.00±0.00
IND	M0	13.40±1.30*	78.20±0.20*	1.80±0.80	6.55±0.65	0.05±0.05
	24 h.	28.40±3.10*	61.20±4.50*	4.70±0.60*	5.30±0.80*	0.00±0.00
	72 h.	33.55±6.25	61.05±5.15*	2.20±0.50	3.10±1.60	0.10±0.00
NEB	M0	17.20±2.50	75.30±2.10	1.45±0.65	6.05±1.05*	0.00±0.00
	24 h.	28.60±5.20*	61.70±3.00*	5.00±2.30*	4.60±0.10*	0.10±0.00
	72 h.	28.70±0.10*	64.25±0.05*	2.05±0.55	4.95±0.75*	0.05±0.05
GSNO	M0	17.10±1.90	75.60±3.00	1.00±0.40*	6.25±0.65*	0.05±0.05
	24 h.	23.40±0.90*	68.05±0.25*	1.75±0.05	6.75±1.15*	0.05±0.05
	72 h.	29.70±1.60*	63.90±1.40*	1.75±0.45	4.55±0.65*	0.10±0.00

For the reactive C3 protein levels, no significant variation between control and IND, NEB, GSNO at 24 hours and 72 h was found. The administration of IND and GSNO causes the increase in the serum C3 fraction level, statistically significant (*p<0.05) compared to the control group at 24 and 72 h. The administration of NEB induced an important increase in C3 fraction activity only at 24 h (Figure 43).

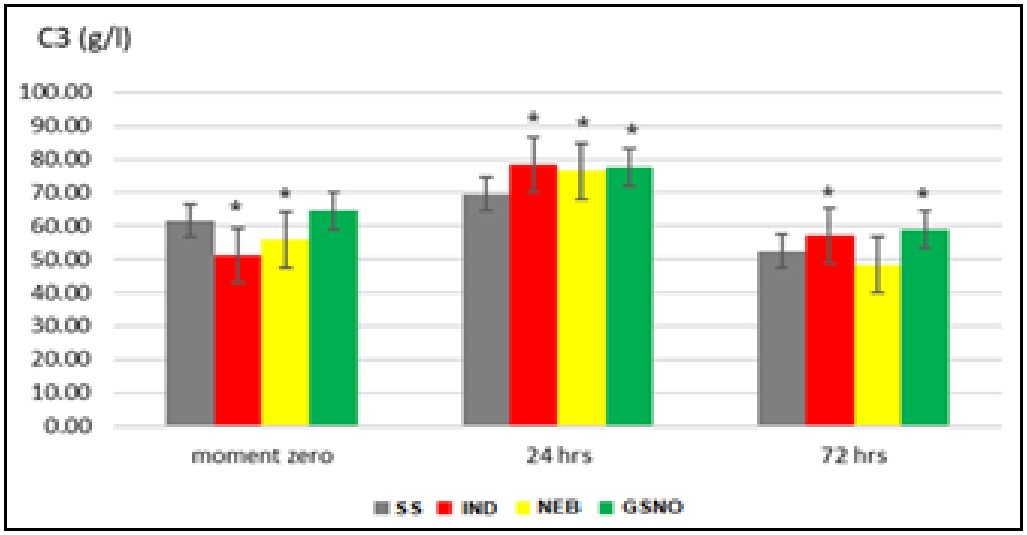


Fig. 43. Effects of NEB and GSNO on the C3 serum complement fraction values in the carrageenan-induced paw inflammation in rats. Each point represents the mean ± SD of the C3 fraction values for 5 animals in a group. *p<0.05 statistically significant compared.

No statistically significant variations in C4 activity between the groups receiving IND, NEB, GSNO and the control were observed during the experiment (Figure 44).

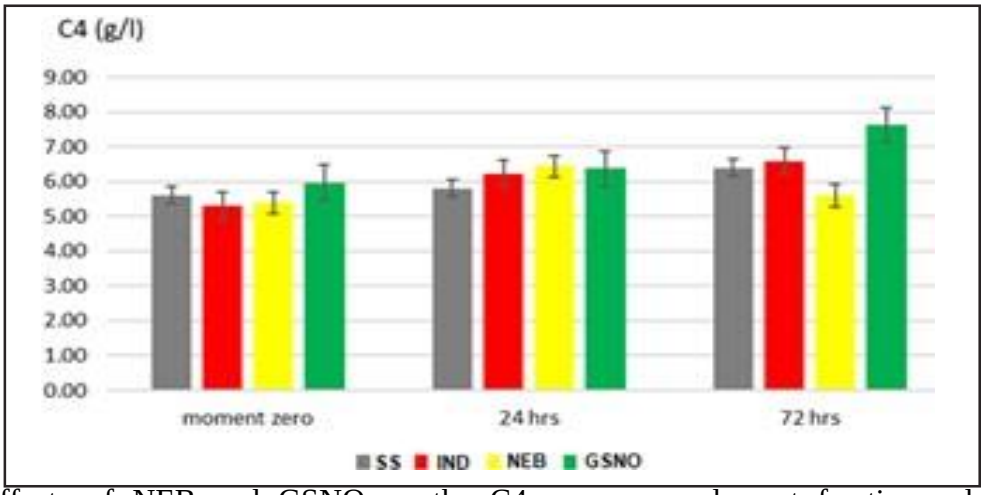


Fig.44. Effects of NEB and GSNO on the C4 serum complement fraction values in the carrageenan-induced paw inflammation in rats. Each point represents the mean ± SD of the C4 fraction values for 5 animals in a group

The serum IL-6 levels significantly increase for all groups at 24 h (**p<0.01), respectively at 72 h (*p<0.05) after carrageenan-induced paw inflammation compared to control group (Figure 45).

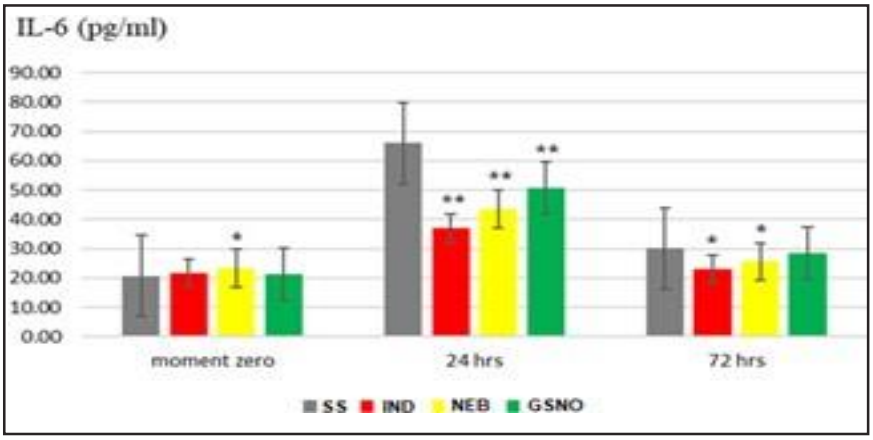


Fig. 45. Effects of NEB and GSNO on the serum IL-6 values in the carrageenan-induced paw inflammation in rats. Each point represents the mean ± SD of the IL-6 values for 5 animals in a group.

Serum TNF-α levels decrease in all groups at 24h and 72h, after induction of inflammation, but to a different extent.
At 24 h the decrease in the group receiving IND being the most pronounced compared to control (**p<0.01).
At 72 h after pow edema induction TNF-α tend to restore the normal value but without reach them. The decreased path keeps at 72 hour but with a slightly lower statistical significance (*p<0.05) (Figure 46).

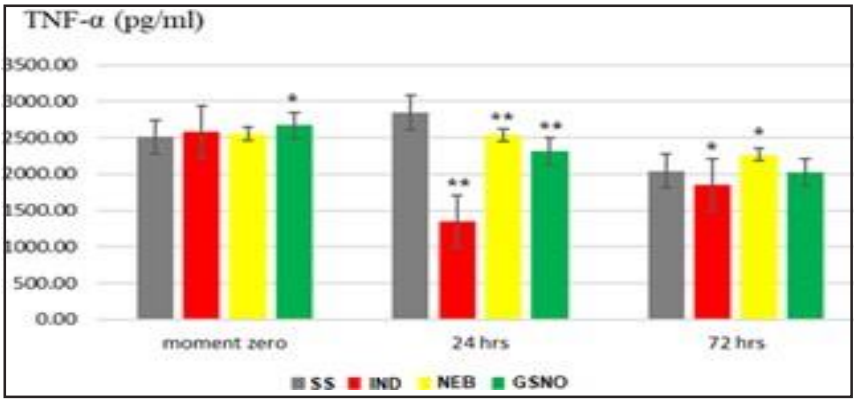


Fig. 46. Effects of NEB and GSNO on the serum TNF-α values in the carrageenan-induced paw inflammation in rats. Each point represents the mean ± SD of the TNF-α values for 5 animals in a group.

Nitric oxide is soluble structure, thus diffusing easily through biological membranes. Within cell is a signal molecule influencing immune system and contributing to the generation of the reactive species with both pro-inflammatory and anti-inflammatory effects depending the physiological conditions [213-216].

Literature shows that the activity of inducive iNOS enzyme under inflammatory conditions leads to a larger amount of NO over a longer period of time allowing NO to participate in all three phases of this process: inflammatory, proliferative and remodeling [217]. Thus, initially NO neutralize pathogenic agents via reactive oxygen species [218] and then mediates the activity of inflammatory and immune cells, including: macrophages, mast cells, neutrophils, T lymphocytes, natural killer and antigen presenting cells thus modulating the immune response [219].
In this study following the carrageenan-induced paw inflammation a biphasic response was developed: an early inflammatory reaction and neurogenic pain (up to 6 h), and a second late phase, inflammatory hyperalgesia (peak intensity around 72 h) which will progressively diminished after approximately 96 h [220]. The positive control drug, IND determined a significant reduction in local inflammatory process after intraperitoneal administration, similar to literature [221-223].
The presence of the NO molecule generated either as a result of the iNOS induction performed by IND or GSNO or by the NEB structure indicates a decrease in the intensity of all inflammatory stages subsequent to irritant administration.

Biochemical assessment of hepatic functions was performed by determining the activity of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) initially, 24 h and 72 h after injection of irritant agent.
A significant increase in ALT activity was observed only in group receiving IND at 24 h from the initial moment (♦♦$p<0.01$) (Figure 47).
In group receiving NEB and GSNO the variation of the ALT activity at 24 and 72 hours are very small and quite similar to the control group. Instead, enzyme activity is well lower for both groups compared to IND receiving group.

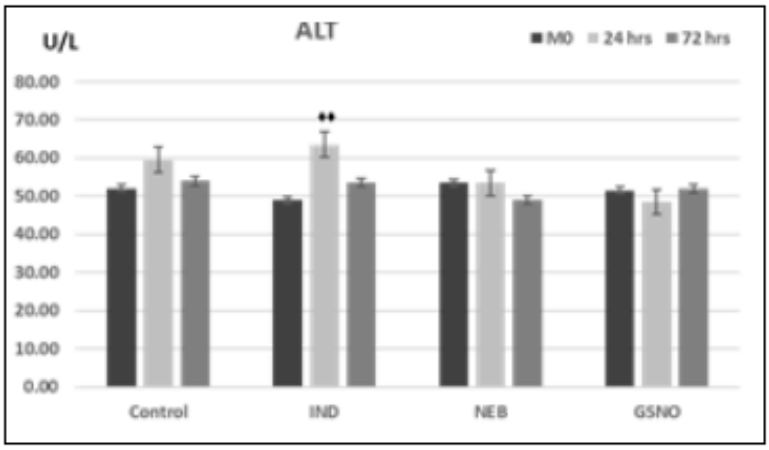


Fig. 47. The effects of NEB and GSNO on the ALT levels (U/L). Data were expressed as arithmetic mean ± S.D. of mean for 5 animals in a group. ♦♦$p<0.01$ significant vs M0.

For the all groups except for IND the pattern of variation is quite similar, after an initial decrease at 24 h the activity of enzymes recovers at 72 h suggesting a relatively low toxicity for the both substances.
For AST activity in the IND group there was a statistically significant increase in enzyme activity from baseline at 24 hours (♦$p<0.05$), only for IND as expected suggesting IND toxicity. The toxic effect of IND, although not very large, is confirmed by ALT activity. At 72 h, AST activity is restored as the administered drugs have been eliminated (Figure 48). The path of variation of ALT

and AST are similar to ALT suggesting a moderate toxicity for both administered compound NEB respectively GNSO.

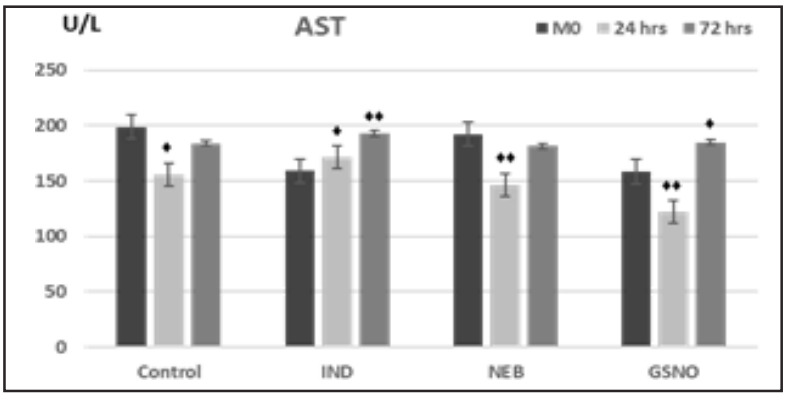


Fig. 48. The effects of NEB and GSNO on the AST levels (U/L). Data were expressed as arithmetic mean ± S.D. of mean for 5 animals in a group.♦p<0.05, ♦♦p<0.01 significant vs M0

The variation profile of the two enzymes ALT and AST suggests a moderate toxicity for both compounds administered NEB and respectively GNSO and significantly lower than IND [224, 225].

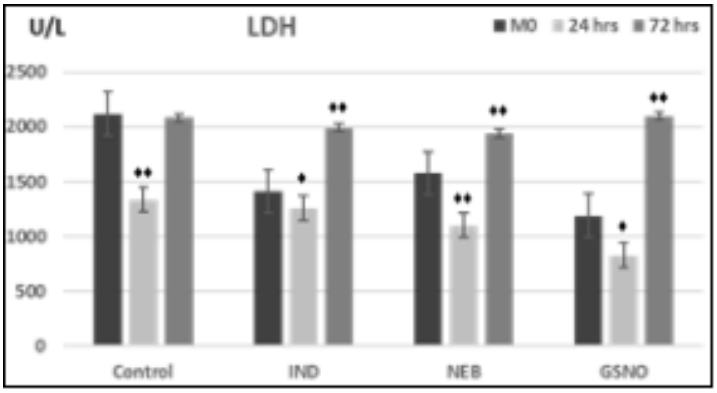


Fig. 49. The effects of NEB and GSNO on the LDH levels (U/L). Data were expressed as arithmetic mean ± S.D. of mean for 5 animals in a group. ♦p<0.05, ♦♦p<0.01 significant vs M0

At 24 hours the pattern of decrease in LDH activity is similar for all groups including control. We assume that this behavior indicates a common adaptation reaction probably triggered by the administration of the studied compounds. At 72 hours, a significant increase is observed for the groups that received IND, NEB and GSNO (Figure 49). We can only assume that this pattern is linked to the anaerobic mechanism of enzyme function: in normal oxygenation LDH activity is low to normal, in hypo-oxygenation LDH activity is higher. We can only speculate that the administration of these compounds somehow promotes anaerobic metabolism thus suggesting a late injury.

Experimental data reveals that NO has different effects [226] according to the source from which it originated: protective if it is generate by the eNOS but if derived from iNOS-derived NO has damaging action, because it acts primarily as a pro-inflammatory mediator [227-232]. This

intriguing identical behavior manifested by all groups indicates a pattern that certainly deserves further investigation.

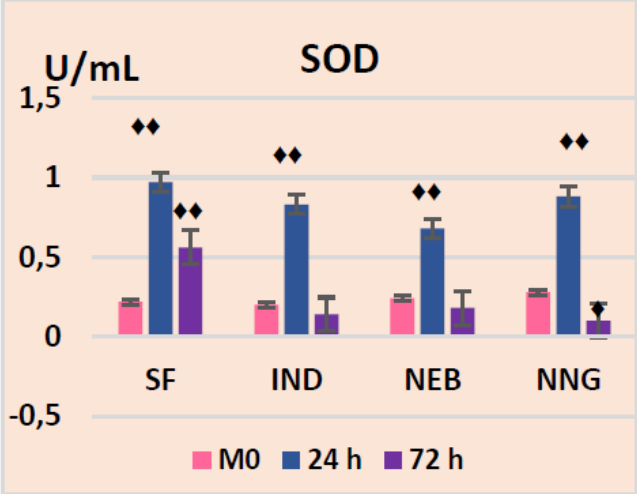


Fig. 50. The effects of NEB and GSNO on the SOD levels (U/mL). Data were expressed as arithmetic mean ± S.D. of mean for 5 animals in a group. ♦p<0.05, ♦♦p<0.01 significant vs M0

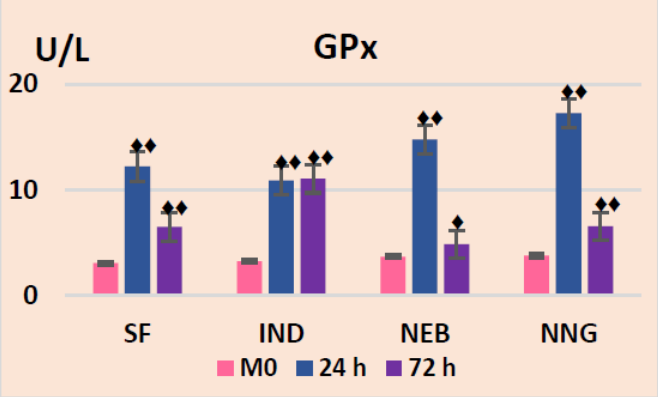


Fig.51. The effects of NEB and GSNO on the GPx levels (U/L). Data were expressed as arithmetic mean ± S.D. of mean for 5 animals in a group. ♦p<0.05, ♦♦p<0.01 significant vs M0

The activity of SOD and GPx after IND, NEB and GSNO administration of recorded a marked elevation (p<0.01) after 24 h, compared to the moment zero of the experiment (Figures 50, 51). At 72 hours after initial moment SOD activity return to initial values. On the contrary, GPx activity remain elevated in group that received IND (♦♦p<0.01) as well as for GSNO group (♦p<0.05) (Figure 50). The higher activity was noticed in the GSNO administered group for both enzymes SOD and GPx suggesting the lowest anti-inflammatory potential.

The intensity of the oxidative attack can be confirmed by the increased levels of malondialdehyde, the final product of lipid peroxidation. The more intense the oxidative aggression, the greater the lipid destruction, especially in the phospholipid bilayer of the cell membrane. Malondialdehyde is

the final product of lipid degradation and therefore its concentration will increase directly proportional to the destruction.
The data obtained were positively correlated with the values of malondialdehyde (Figures 52) thus confirming the extent of the oxidative aggression.

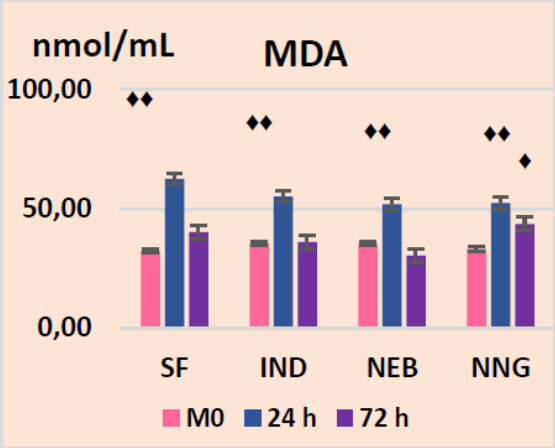


Fig.52. The effects of NEB and GSNO on the MDA levels (nmol/mL). Data were expressed as arithmetic mean ± S.D. of mean for 5 animals in a group. ♦p<0.05, ♦♦p<0.01 significant vs M0

The data obtained were positively correlated with the values of malondialdehyde and also to the histopathological liver alterations (performed but not presented in this paper). Antioxidants system is an important operator in the control of nitro-oxidative stress involved in hepatic sinusoidal endothelial cell physiology [233, 234].
The results of this study have been published in two articles:
1. Revista de Chimie (Bucharest) 2018, 69(10): 2899-2903
2. Revista de Chimie (Bucharest) 2019, 70(4):1360-1363
(the titles of the works, the authors and the complete data are presented at the end of the chapter, pg 95).

Conclusions
The positive control drug IND shows anti-inflammatory activity in this experimental model in rats as it was expected. The administration of nitric oxide donors NEB and GSNO on experimentally induced paw inflammation cause significant decreases in local edema, by influencing as well blood elements, and specific serum inflammatory and immune markers.
The use of NEB and GSNO induced anti-inflammatory-like effects, especially at 24 hours in this experimentally induced inflammation model. Nebivolol exhibited more pronounced anti-inflammatory effects than GSNO, but less intense than indomethacin at certain times during the experiment.
We can appreciate that nebivolol as well as S-nitroso-glutathione show anti-inflammatory effects on local acute inflammation experimental induced in rats.
The administration of NEB and GSNO present a degree of hepatic toxicity not as higher as indomethacin.

LDH levels following the administration of NEB and GSNO show significant changes in its activity which is directly related to the level of intracellular anaerobic metabolism. This observation leads us to speculate an association with high oxidative stress.
Even though it is a preclinical study, the data obtained suggest the anti-inflammatory-like potential of nitric oxide donors that are used in the therapy of cardiovascular diseases.

III.3. Assessment of the oxidative stress and inflammatory parameters in patients with middle chronic suppurative otitis media ear disorders

Chronic suppurative otitis media (CSOM) is a local inflammatory process, involving hearing loss and purulent discharge [235, 236]. Bacterial otitis media is caused by the migration of pathogens from the nasopharynx to the middle ear. The endotoxin in the bacterial cell wall triggers the immune response. General factors (inflammatory mediators, proteins, cytokines, oxygen free radicals), and local factors (Eustachian tube dysfunction, malformations, tympanic membrane perforation) maintain inflammation and disease progression [237]. During the inflammatory process of the middle ear and mastoid, superoxide radicals are generated to increase bactericidal activity leading to free radical growth followed by the alteration of oxidative balance [238] and proximal structures. The implications of oxidative stress have not been explored in CSOM pathogenesis. In the literature there are only a few articles that explore this topic [239-242].
There also are few data in literature [243] regarding the possible link between cytokine levels and the processes that occur in the middle ear during otitis. Both Interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNFα) are cytokines required for activating the innate immune response. At the injury site keratinocytes produce mediators of inflammation such as IL-1 α and IL-1β, IL-6 and IL-8 [244-246]. Initially IL-1α promotes the recruitment of inflammatory cells at the site of inflammation and activates tumor necrosis factor-alpha. Tumor necrosis factor has a pyrogenic function and increases the vascular permeability. Secondly interleukin-6 increases the antibody production and the acute-phase reactants, dictates the transition from acute to chronic inflammation, has proinflammatory and pyrogenic function. Interleukin-8 promotes the recruitment of neutrophils. Thus, a possible correlation between inflammation and reactive species in chronic suppurative otitis media must be investigated.

Aim: The aim of this study was to evaluate the antioxidative and the inflammatory status in patients with chronic suppurative otitis media (CSOM).

Materials and Methods
The study was performed on patients diagnosed with chronic suppurative otitis media with and without cholesteatoma admitted to the Clinical Rehabilitation Hospital, Iasi, Romania, for surgery. The inclusion criteria for the study were: clinical and imagistic diagnosis of CSOM with or without cholesteatoma. Exclusion criteria were represented by other chronic or acute pathology other than ear disease, history of smoking, under medication, including vitamins.
The study included 90 patients divided into 4 groups:

- group M is the control group including 30 healthy patients (with no chronic or acute diseases, medication administration or history of smoking.
- group O including patients with CSOM without cholesteatoma, composed of 28 patients
- group C including patients with CSOM with cholesteatoma composed of 21 patients

- group R including patients with CSOM with cholesteatoma recidivism composed of 11 patients.

Blood sample were taken a jeun before surgery from all patient and parameters of the oxidative stress (CAT and SOD, TAC and MDA) as well as the marker of inflammation (IL-1α, IL-6, IL-8) were assessed.

Blood sample were portioned in two aliquots differently prepared:

- in one aliquot erythrocyte were separate and subsequently lysed getting the so-called erythrocyte lysate
- the other aliquot was used to obtaining serum.

In the erythrocyte lysate the catalase and SOD activity were performed. In the serum TAC, malondialdehyde and IL-1α, IL-6, IL-8 were performed.

The CAT and SOD activities were determined by using standardized test kit for research use as follows: CAT Assay Kit (Abnova- KA0884) respectively SOD Assay Kit-WST, Sigma Aldrich.

TAC and malondialdehyde ware determined by using standardized test kit for research use as follows: TAC Assay Kit from Sigma Aldrich, and Lipid peroxidation (MDA) Assay Kit from Sigma Aldrich.

Interleukins IL-1α, IL-6, IL-8 were determined using Human ELISA standardized test kit for manual use produced by Bio Scientific Corporation.

Statistical analysis was performed using SPSS database version 18.0 (SPSS, Inc.) and processed with specific statistical functions. Obtained data were calculated as mean ± standard deviation. The Skewness and Kurtosis test (-2<P<2), were used to evaluate the distribution of continuous variables. The student's t-test, a parametric test that compares the average values recorded in two groups with normal distributions was used. The F test (ANOVA) was also used to compare three or more groups with a normal distribution. Post hoc analysis was made with Bonferroni test.

Results and Discussion

The reactive species assessment

SOD activity was evaluated indirectly by measuring the degree of inhibition of a reaction in which the superoxide anion is a substrate. Thus, a large amount of SOD in situ will determine the consumption of superoxide and thus will cause a decrease/ inhibition in the reaction rate in which it participates.

The activity of SOD for all the groups are presented in figure 53.

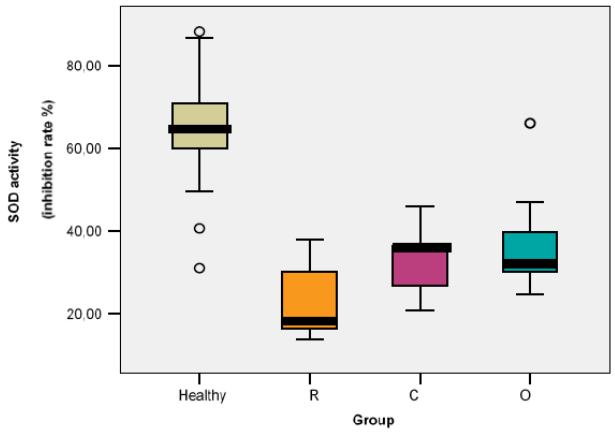


Fig. 53. Mean levels variations of SOD activity in healthy subjects' group (M), patients with cholesteatoma recidivism (R), CSOM with cholesteatoma (C), CSOM without cholesteatoma (O)

In the healthy subject's group, enzymes activities values ranged between 31.006 and 88.408, suggesting that the activity of antioxidant defense is relatively close to its full capacity.
The mean level of SOD activity in patients with cholesteatoma recidivism, followed by patients with CSOM with cholesteatoma, was found to be significantly lower compared to the control group (22.929; 32.541 ($p = 0.004$) vs 64.475; $p = 0.001$). For patient with CSOM without cholesteatoma mean SOD values are 36.13 still lower than control.
A high concentration of reactive species results in a proportionally high activity of detoxifying enzymes, up to a point represented by the maximum capacity of the system. If the generation of reactive species continues, the enzymatic activity may either remain constant for a limited period or it may decrease. The decrease is attributed either to the inhibition by substrate excess or to the exhaustion of the defense system. In our case, we consider that the decrease in the activity of the defense system is caused by its exhaustion due to the chronicity of the process, especially in the recidivism group.
Low activity of SOD was found in literature [242,247] in children with otitis media with effusion and showed that ventilation tubes insertion and adenoidectomy decreased the oxidative stress [247]. The lowest values were recorded in patients with cholesteatoma recidivism. As a conclusion the highest generation of reactive species and therefore the higher alteration was identified in the relapse group. A chronic inflammatory process can diminish the ability to recover and thus trigger the cholesteatoma recidivism. In the case of chronic ear inflammation, the production of reactive oxygen species may increase the dysregulation processes and development of preneoplastic condition [248,249].
The Skewness/Kurtosis tests ($-2 < p < 2$) suggest that the SOD and CAT series values were homogeneous (table XX).

Table XX. Statistical description of SOD values in studied groups

Parameter	Group M	Group R	Group C	Group O
Number of subjects/patients	30	11	21	28
Mean Median	64.475	22.929 [a)]	32.541 [a) b)]	36.316[a)] a) ns)
Standard deviation	64.723	18.276	36.019	32.123
Variance	12.818	9.290	7.639	10.129
Skewness	19.882	18.312	28.349	12.589
Test	-0.351	0.769	-0.032	1.938
Std. Error of Skewness	0.427	0.661	0.501	0.441
Kurtosis	0.756	-1.081	-0.726	1.916
Std. Error of Kurtosis	0.833	1.279	0.972	0.858
Minim	31.006	13.744	20.853	24.562
Maxim	88.408	37.915	45.972	66.173
Percentiles 25	59.027	16.256	26.094	30.191
50	64.723	18.276	36.019	32.123
75	71.549	30.028	36.967	39.922

a) $p < 0.001$, b) $p < 0.05$, ns) $p > 0.05$

CAT activity was expressed as mU/mL (one unit of CAT is the quantity of catalase that decomposes 1,0 µmol H_2O_2 per minute when the pH is 4.5 at 25ºC) and presented in fig 54.

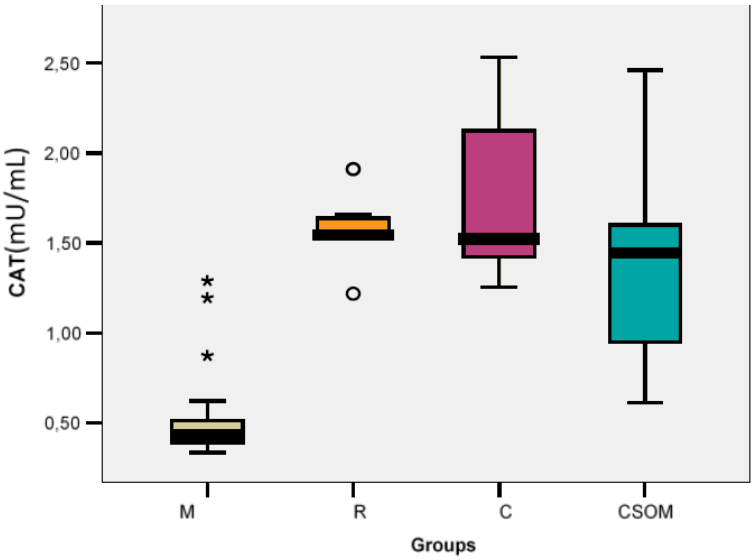


Figure 54. Mean levels variation of CAT activity in the healthy subject's group (M), patients with cholesteatoma recidivism (R), CSOM with cholesteatoma (C), CSOM without cholesteatoma (O)

In healthy group the activity of CAT ranged between 0.334 mU/mL and 1.289 mU/mL.
For the patients tested with CSOM without cholesteatoma (group O) enzymes activities values is 0.611 mU/mL and 2.461 mU/mL.

For the patients tested with CSOM with cholesteatoma (group C) enzymes activities values is 1.253 mU/mL and 2.531 mU/ mL.
For the patients tested with cholesteatoma recidivism (group R) enzymes activities values is 1.216 mU/mL and 1.910 mU/mL.
Obtained data show that the CAT activity increase in all group as comparing to the healthy group. This suggests an increased enzymatic activity proportional to the concentration of H_2O_2 and the generation of reactive species. The pattern is similar for all groups of patients, thus indicating an inflammatory process. The higher activity was registered for the cholesteatoma recidivism. The increased activity of CAT may also be attributed to a hydrogen peroxide molecules action on mRNA expression [250-252] as literature noticed. The intensity of oxidative stress was similar regardless the patient age or gender [253].
Literature data are contradictory as some authors found lower CAT values in patients with CSOM compared to the healthy subject's group [242, 247] and others found was increased [252] CAT activity in patients with chronic otitis media.
The TAC mean values are presented in figure 55.

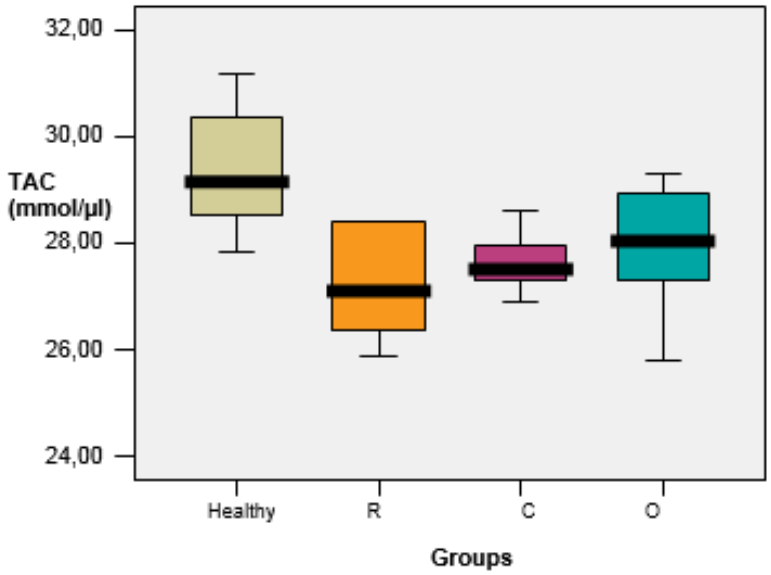


Fig. 55. TAC mean values in study groups: healthy control group, R- patients with cholesteatoma recidivism, C- represents patients with CSOM with cholesteatoma, O- patients with CSOM without cholesteatoma

Statistical description of TAC values in studied groups are presented in table XXI.

Table XXI. Statistical description of TAC values in studied groups.

Parameter		Group M	Group R	Group C	Group O
Number		30	11	21	28
Mean		29.401	27.210 a)	27.657 a) ns)	27.834 a) ns) ns)
Median		29.162	27.111	27.519	28.045
Standard Deviation		1.036	1.054	0.514	1.094
Variance		1.073	1.111	0.264	1.197
Skewness Test		-0.037	0.029	0.341	-.0577
Std. Error of Skewness		0.427	0.661	0.501	0.441
Kurtosis		-1.243	-1.657	-0.773	-0.586
Std. Error of Kurtosis		0.833	1.279	0.972	0.858
Minimum		27.834	25.902	26.908	25.815
Maximum		31.185	28.411	28.608	29.313
Percentiles	25	28.518	25.932	27.207	27.323
	50	29.162	27.111	27.519	28.045
	75	30.379	28.411	27.958	28.960

The measurement of TAC is used as an indicator of the entire system's capacity to counteract oxidative stress-induced injury and it is measured in the serum sample. Our study showed statistically significant higher levels of TAC in the healthy group compared to groups with CSOM with or without cholesteatoma, thus demonstrating the existence of the imbalance in the oxidant-antioxidant systems produced by the inflammatory process. Leukocyte infiltration, an inflammation signal, was linked to the overproduction of oxidants and the tissue damage in the middle ear thus highlight the link between inflammation and oxidative stress. In literature, up to now, there are only 2 studies in literature that measure TAC values in the patients' serum in this type of illness.

The extent of the damages produced by the oxidative species attack is quantified by MDA levels and presented in figure 56.

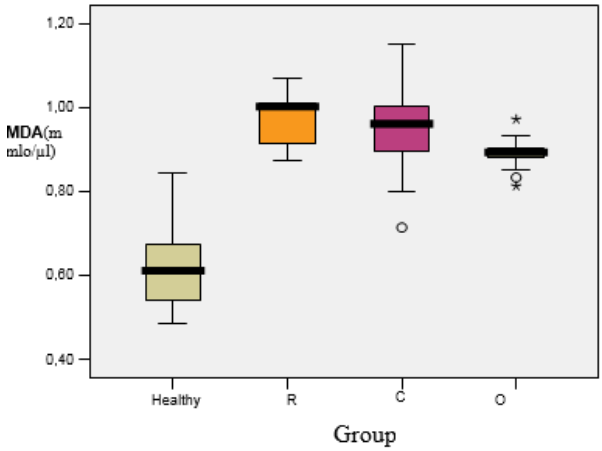


Fig. 56. MDA mean values in study groups: healthy control group, R- patients with cholesteatoma recidivism, C- represents patients with CSOM with cholesteatoma, O- patients

Statistical description of MDA values in studied groups are presented in table XXII.
Table XXII. Statistical description of MDA values in studied groups.

Parameter		Lot M	Lot R	Lot C	Lot O
Number		30	11	21	28
Mean		0.625	0.979 [a)]	0.948 [a) ns)]	0.891 [a) ns) b)]
Median		0.612	1.003	0.962	0.894
Standard Deviation		0.096	0.069	0.107	0.030
Variance		0.009	0.005	0.011	0.001
Skewness Test		0.668	-0.277	-0.157	-0.120
Std. Error of Skewness		0.427	0.661	0.501	0.441
Kurtosis		-0.189	-1.203	0.363	1.557
Std. Error of Kurtosis		0.833	1.279	0.972	0.858
Minimum		0.488	0.875	0.715	0.814
Maximum		0.843	1.071	1.150	0.973
Percentiles	25	0.540	0.897	0.892	0.879
	50	0.612	1.003	0.962	0.8894
	75	0.680	1.011	1.005	0.903

Lipid peroxidation represents the degradation of lipids that occurred as a consequence of oxidative attack and is a very reliable marker for the extent of this damage. All cells and cell orgonites contain polyunsaturated acids membrane that suffer lipid peroxidation under free radicals' actions. At the end of the interaction, polyunsaturated acids are subsequently hydrolyzed into biological compounds among them MDA is the most significant as reflects the lipid peroxidation magnitude. In this study, the MDA levels, as expected, were higher in all patient's group with CSOM with and without cholesteatoma and cholesteatoma recidivism compared to the healthy group. In middle year diseases may be a very good marker of disease severity. Literature show that administration of antioxidant (antioxidant vitamins for example) added to the treatment decrease the MDA levels. This confirms the involvement of oxidative stress in inflammation as well as the antioxidant drugs potential in healing /preventing these types of illnesses.
As a conclusion the data obtained indicated a definite association of inflammation with the generation of reactive species and the activation of the defense system. The extent of the antioxidant system depends on how long the injury lasts.
Obtained data show statistically significant lower SOD activity in all patients similar to literature. Instead for CAT activity obtained data show statistically increased, compared to the healthy group similar to only some data in literature [254-256].

The inflammatory maker assessment
The inflammatory mediators investigated in the pathogenesis of chronic suppurative otitis media (CSOM) and cholesteatoma were IL-1α, IL-6 and IL-8.
The serum concentrations of IL-1α recorded for the studied groups are presented in figure 57.

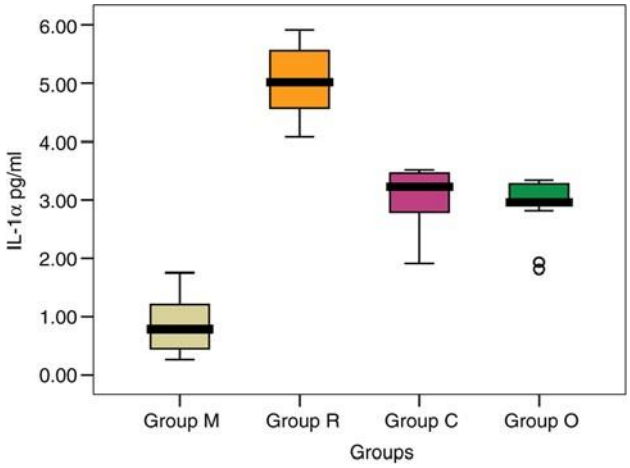


Figure 57. IL-1α mean values (pg/ml) for study groups. Group M included healthy individuals, group C included patients with CSOM with cholesteatoma, group R comprised patients with cholesteatoma recidivism, and group O included patients with simple CSOM

For the IL-1α values the mean level recorded in group M was 0.815 pg/ml represent the normal range values which are, as expected, significantly lower (P=0.001) (Figure 54) compared to all the other patient's groups.
In group O, IL-1α mean values were 2.911 pg/ml close to that obtained for group C which mean values were 3.011 pg/ml. For the group R the mean level of IL-1α were 5.021pg/ml significantly higher (P=0.001) as comparing to both normal and pathological other two groups.

In both males and females, the highest mean level of IL-1α was found in patients with recurrence, and the lowest in controls. Within the group, no significant differences between sexes were recorded (P>0.05) (Table XXIII).

Table XXIII. Mean IL-1α values (pg/ml) compared by sex and study group.

Sex	Group M	Group R	Group C	Group O	FANOVA test[a]
Male	0.86±0 .42	5.07±0.66	2.88±0.69	2.81±0.50	0.001
Female	0.79±0.42	4.97±0.63	3.13±0.49	3.03±0.37	0.001
Student's t-test	0.653	0.811	0.365	0.192	-

[a]Bonferroni post hoc test was used. Group M included healthy individuals, group C included patients with CSOM with cholesteatoma, group R comprised patients with cholesteatoma recidivism, and group O included patients with simple CSOM. IL, interleukin; CSOM, chronic suppurative otitis media.

The IL-1α levels in patients with CSOM with and without cholesteatoma are significantly lower compared to patients with cholesteatoma recidivism but higher as comparing to the healthy group. As expected, the lowest value for IL-1α were found in the healthy group (both males and females) [257] and the highest in patients with cholesteatoma recidivism. Obtained data suggest the degree of bone destruction [258] and the significant lesions in the middle ear is correlated to the highest values of for IL-1α [259-261]. Furthermore, obtained data indicate that the inflammatory status is more acute than chronic as can noticed for cholesteatoma recurrence.
The serum concentration of IL-6 recorded for the studied groups are presented in presented in figure 58.

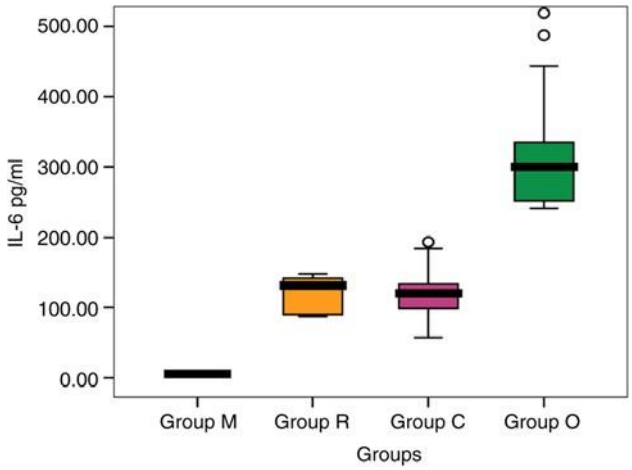


Figure 58. IL-6 mean values (pg/ml) for study groups. Group M included healthy individuals, group C included patients with CSOM with cholesteatoma, group R comprised patients with cholesteatoma recidivism, and group O included patients with simple CSOM.

The IL-6 serum mean values recorded in group M are 5.81 pg/ml. It is the lower mean value (P=0.001) from all of the groups as seen in figure 55. For groups C and R, recorded IL-6 serum values are almost similar 121.23 pg/ml respectively 117.77 pg/ml and both are significantly higher

than control. In group O, the mean values of IL-6 are 316.59 pg/ml, which is significantly higher than the values of the healthy group, as well as those of groups C and R.
In both males and females, the highest mean level of IL-6 was found in patients from group O and the lowest in group M. Within the group, only in patients from group O was there a significantly higher mean value in males than females (P=0.001) (Table XXIV).

Table XXIV. Mean IL-6 values (pg/ml) compared by sex and study group.

Sex	Group M	Group R	Group C	Group O	FANOVA test[a]
Male	5.89±1.72	123.86±27.91	107.43±43.19	356.38±80.94	0.001
Female	5.75±1.61	110.47±24.40	133.78±34.61	270.69±32.16	0.001
Student's t-test	0.817	0.424	0.138	0.001	-

[a]Bonferroni post hoc test was used. Group M included healthy individuals, group C included patients with CSOM with cholesteatoma, group R comprised patients with cholesteatoma recidivism, and group O included patients with simple CSOM.

The IL-6 lowest level was found in the healthy group and it was expected as it known that IL-6 is considered a marker of the bacterial infection [262-264].
The highest level of IL-6 was found in patients with CSOM without cholesteatoma (O), which suggests the presence of an acute phase of the lesion and the beginning of the chronicity process. Instead in groups with cholesteatoma recidivism (R) and CSOM cholesteatoma (C) lower level of IL-6 was found; the obtained data suggest the beginning of the transition from the acute to the chronic phase of inflammation. Thus IL-6 levels can be a first signal for chronicity processes installing. Literature notice that elevated levels of serum IL-6 are related to the degree of middle ear destruction and to the severity of the disease [265,266]. High level of IL-6 and CRP may be predicting factors in streptococcal otitis media [264] and may lead to cholesteatoma epithelial hyperplasia [267].
The serum concentration of IL-8 recorded for the studied groups are presented in figure 59.

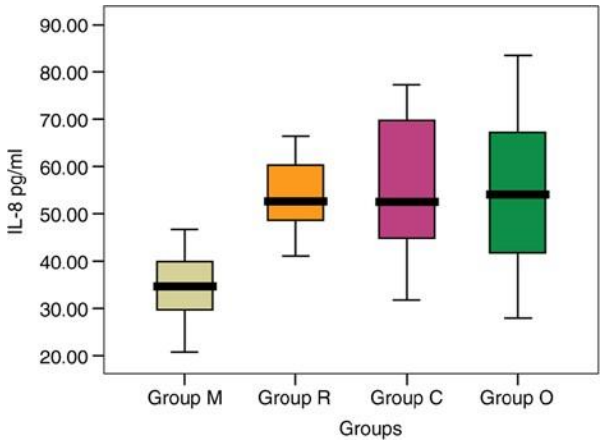


Figure 59. IL-8 mean values (pg/ml) for study groups. Group M included healthy individuals, group C included patients with CSOM with cholesteatoma, group R comprised patients with cholesteatoma recidivism, and group O included patients with simple CSOM.

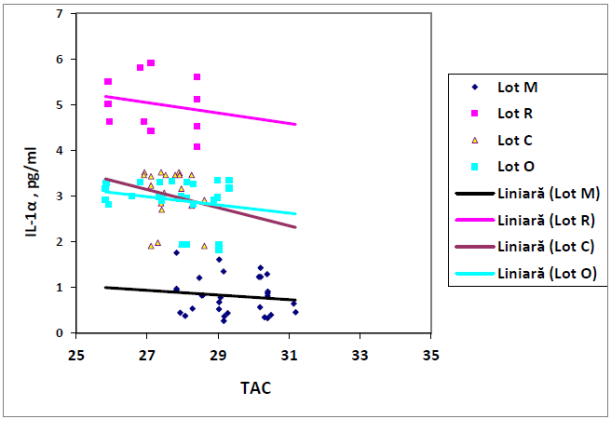
The serum mean value for IL-8 are 34.31pg/ the lower (P=0.001) in all the studied groups. The IL-8 mean values for all patient's groups recorded almost similar values as follows: 54.20pg/ml in group O, 53.9 pg/ml in group C and 53.74pg/ml in group R. In males, the highest mean level of IL-8 was found in patients from group O, while in females, the highest mean level of IL-8 was found in patients from group C, and in both sexes the lowest level average IL-8 was observed in group M (Figure 56). Within the group, no significant differences between sexes were registered (P>0.05) (Table XXV).

Table XXV. Mean IL-8 values (pg/ml) compared by sex and study group.

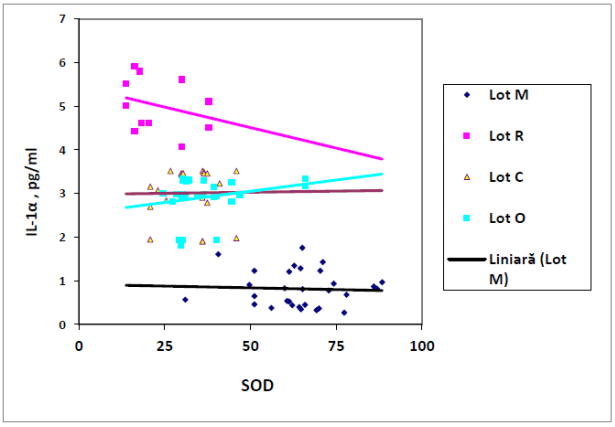
Sex	Group M	Group R	Group C	Group O	FANOVA test[a]
Male	35.34±7.80	56.15±11.33	50.18±13.45	58.33±19.45	0.002
Female	33.62±7.06	50.85±2.32	57.31±15.46	49.44±12.76	0.001
Student's t-test	0.537	0.335	0.275	0.172	-

[a]Bonferroni post hoc test used. Group M included healthy individuals, group C included patients with CSOM with cholesteatoma, group R comprised patients with cholesteatoma recidivism, and group O included patients with simple CSOM.

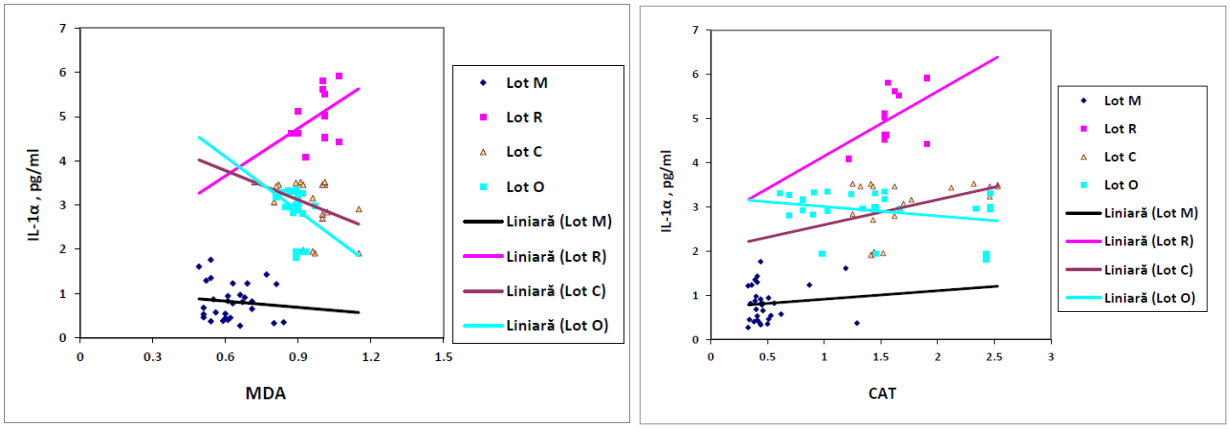
The correlation between the individual values of IL-1α and those of the antioxidative defense components is presented comparatively by group in figure 60 (a) (b) (c) (d).



a



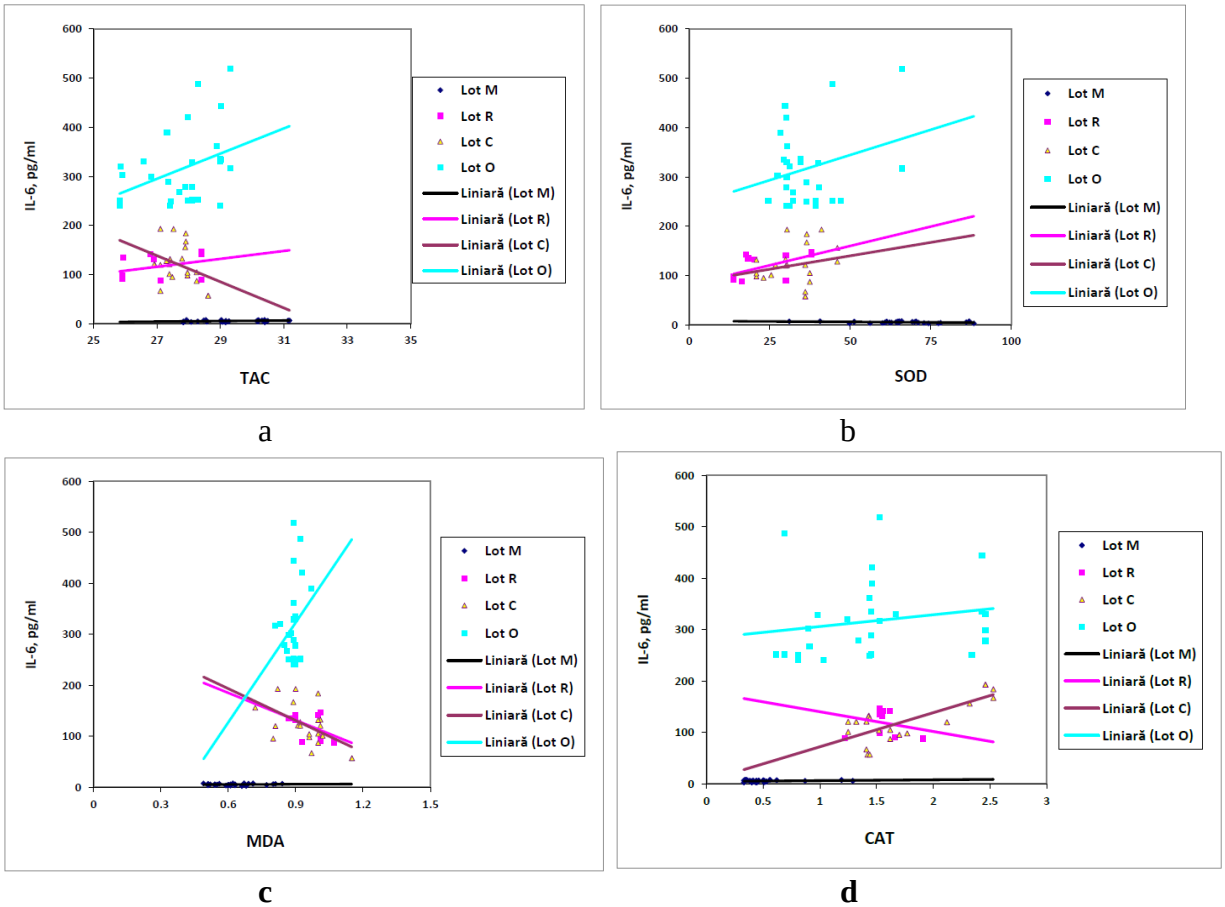
b



c d

Figure 60: Correlation between the values of IL-1α and TAC (a), SOD (b), MDA (c) and CAT (d)

The correlation between the individual values of IL-6 and those of the antioxidative defense components is presented comparatively by group in figure 61 (a) (b) (c) (d).



a b

c **d**

Figure 61: Correlation between the values of IL-6 and TAC (a), SOD (b), MDA (c) and CAT (d)

Literature specifies that IL-8 promotes the collagenases involved in the process of bone lysis [260], the production of adhesion molecules and neutrophil migration during the acute inflammatory response [268]. Literature [269-272] notice that IL-8 can initiate the tissue damage leading to chronic inflammation by releasing the lysosomal enzymes. The obtained data for IL-8 are found in similar concentrations that are significantly higher in all groups compared to the control group in agreement with literature. We consider that obtained data suggest that IL-8 can be associated with bone lysis and tissue that continues ultimately lead to irreversible ear damage [260, 268-270]. To summarize, high levels of Il-1alpha, Il-6, IL-8 are associated with the degree of bone destruction [259,260,161, 258] in individuals diagnosed with CSOM diseases.
The correlation between the individual values of IL-8 and those of the antioxidative defense components is presented comparatively by group in figure 62 (a) (b) (c) (d).

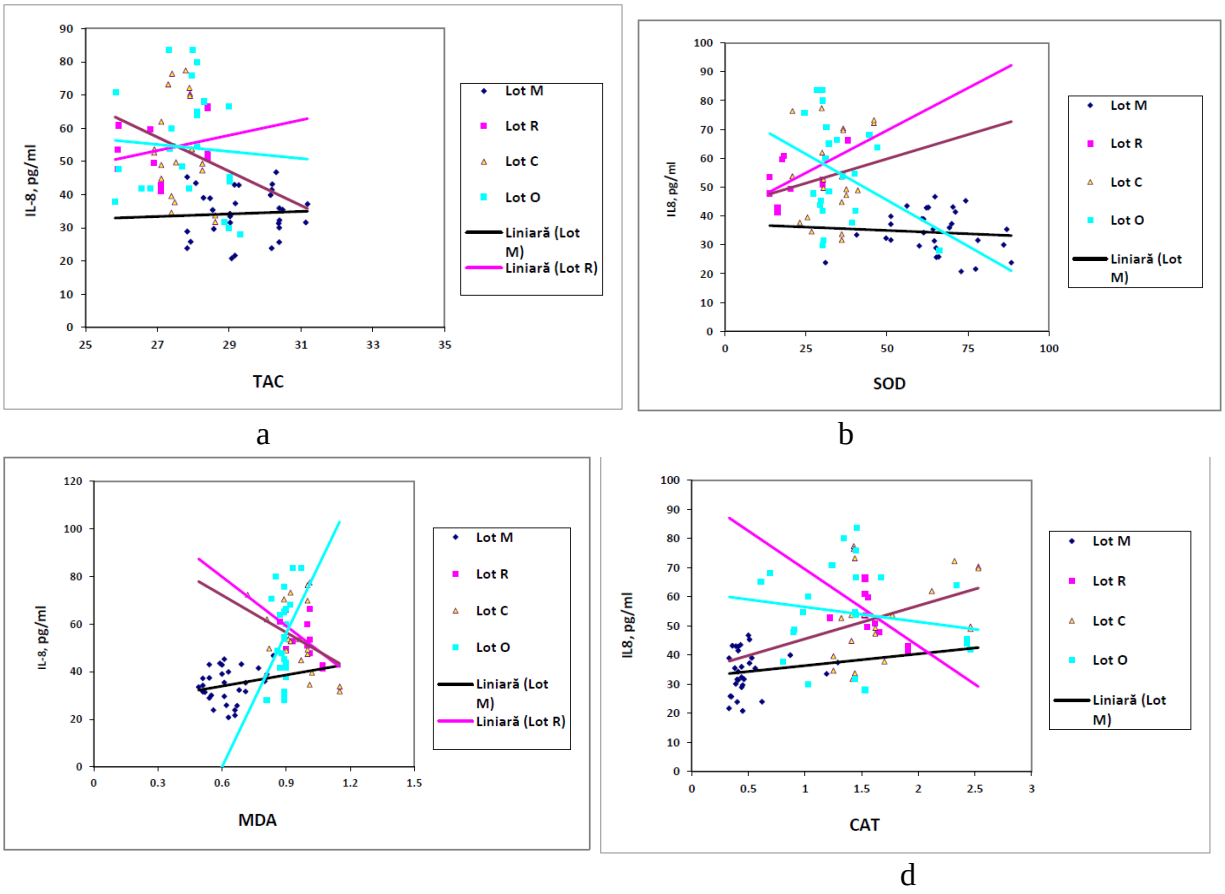


Figure 62: Correlation between the values of IL-6 and TAC (a), SOD (b), MDA (c) and CAT (d)

Conclusions

In conclusion, high serum levels of Il-1α, Il-6 and IL-8 were recorded in all otitis media groups compared to the healthy group. IL-1α had the highest value in patients with cholesteatoma recidivism suggesting the massive bone in destruction. IL-6 and IL-8 had the highest value in patients with CSOM.

The current study has some limitations. Firstly, the study enrolled a relatively small number of patients, making it difficult to generalize the results. Secondly, children and adults were included together in the

analysis and we consider that in the future differentiated studies on pediatric and adult populations are needed, which would allow a more accurate quantification of the inflammation parameters.
The results of this study have been published in two articles:
1. Farmacia, 2018, 66 (6): 984-988
2. Experimental and Therapeutic Medicine, 2021, 22: 1226
(the titles of the works, the authors and the complete data are presented at the end of the chapter, pg 95).

III.4. Antioxidant status after biogenic amines administration in rats

Aim: In this paper, the total antioxidant capacity of rats in which biogenic amines (histamine, tyramine, cadaverine) were administered was evaluated in order to identify a possible correlation with their serum levels.

Materials and Methods
The study was an application for the validation of the method developed to simultaneous separation of histamine, tyramine and cadaverine in biological fluids in this case plasma taken from rats to which these biogenic amines were administered.
The study was performed on three groups of rats, of 10 animals each. Each batch was its own witness and they received:

- group I - 10 mg / kg histamine, i.p., single administration
- group II - 10 mg / kg tyramine, i.p., single administration
- group III - 5 mg / kg histamine and 5 mg / kg tyramine, i.p., single administration

For the chromatographic determination, the method previously presented and which was subject to validation procedure in the chapter I.1.2. “Determination of histamine and tyramine levels following their administration to rats”.
The experiment complied with the specific legislation on working with experimental animals and obtained the agreement of the Ethics Commission of the University of Medicine and Pharmacy "Gr.T.Popa" Iasi.
The animals were anesthetized with ketamine (75 mg / kg i.p.) and blood samples were collected from the initial retroorbital plexus, 24 and 72 hours after amine administration under anesthesia.
The concentrations of the following parameters were determined in the collected blood samples:

- histamine and tyramine concentration
- the activity of SOD and GPx enzymes as well as the total concentration of antioxidant species in serum (TAS)

To determine histamine and tyramine levels, plasma samples collected from the experimental animals were processed according to the previously validated HPLC-MS / MS determinations working protocol.
To determine the activity of SOD, GPx and TAS, standardized Kits for manual use purchased from RANDOX were used.
The ANOVA one way test and the Tukey test for post-hoc analysis was used for the statistical interpretation of the results. Results were expressed as mean ± standard deviation. The value of $p < 0.05$ was considered significant.

Results and Discussion

The chromatogram obtained from the analysis of rat plasma to which each of the three amines in a concentration of 5 mg / kg body weight was added is shown in figure 63.

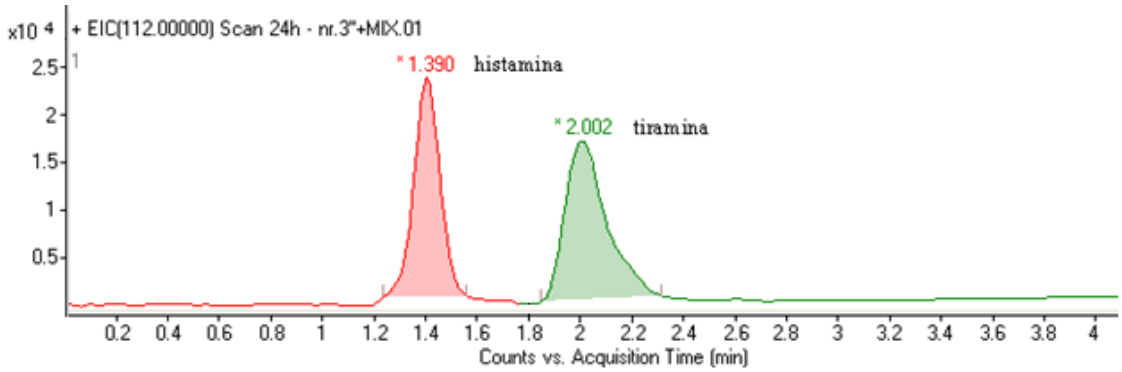


Fig. 63. Chromatogram obtained for a plasma sample collected 1 hour from Lot III, in which the mixture of histamine, tyramine and cadaverine was administered at a dose of 5 mg / kg each.

Plasma concentration of biogenic amines is presented in Tables III, IV, V (cap I.1.3.).
Folowing biogenic amine administration both histamine and tyramine levels reach a significant increse, but still in the normal range. 24 h after their administration they almost disappear from the blood. The changes in total antioxidant status initial, 24 and 72 hours after amines administration is presented in table XXVI.

Table XXVI. Total antioxidant capacity after histamine, tyramine and a mixture of, histamine, tyramine and cadaverine, administration in rats

		TAS mmol/L (mean± SD)	
	Initial	24 hours after administration	72 hours after administration
Series I (n=10 animals) (Histamine 10mg/kg body)	1.423±0.25	1.160±0.14	0.885±0.18
Series II (n=10 animals) (Tyramine 10mg/kg body)	1.428±0.25	1.224±0.13	0.927±0.18
Series III (n=10 animals) (Histamine, Tyramine, Cadaverine 5mg/kg body)	1.424±0.19	1.285±0.09	0.935±0.17

Obtained data show a similar pattern after all biogenic amines administration. The concentration of TAS progressively decreases from initial to 24 and finally to 72 hours. Regarding the simultaneously administration of both amines there was not noticed a cumulative decreasing effect on the TAS concentration.

Obtained data show that at 24 hours the concentrations of biogenic amines return to normal. Even so the toxic efect on antioxidative defence system stil maintains and and disturbance prologes to 72 hours. We can assume that there is a pozitive correlation between biogenic amine generation and oxidative stress.

Stress alters the balance that normally exists in the body between oxidizing and antioxidant systems in favor of the former. Stremoukhov et al. demonstrated that histamine has a pro-oxidant effect favoring the generation of hydrogen peroxide [273].

Our results show that the total antioxidant status shows a significant decrease 72 hours after the administration of the studied amines in all three groups. Although biogenic amines are known to be eliminated within a maximum of 24 hours, the literature indicates that tyramine may accumulate [274,275].

The data obtained can be interpreted in the sense that although after 24 there are no significant amines concentrations in blood, the imbalance produced is not yet overcome 72 after their administration. Therefore, the antioxidant balance is not restored at this time. Thus 72 hours after the administration of biogenic amines, the level of total plasma antioxidant capacity is still low. The administration of the amine mixture indicates the highest toxicity probably justified by their cumulative effects [276].

The obtained data indicate that elevated plasma histamine and tyramine concentrations affect the total plasma antioxidant capacity [277]. Therefore, it was possible to disturb the balance of antioxidant defense processes both in the case of administration of individual amines and their mixture thus trigerring toxic effects [278,279].

Conclusions

Significant decrease in total antioxidant status at 72 h thus suggesting a severe impairment of antioxidant defense capacity since the effects of their administration still persist.
The data obtained suggest a cumulation of oxidative effects in case of their associated administration.
Increased concentrations of biogenic amines ingested with food in which they may be present, generate an increase in the concentration of reactive oxygen species, which disrupts the balance of antioxidant processes, most likely through hydrogen peroxide resulting in oxidative deamination processes of biogenic amines .

The results of this study have been published in Rev Med Chir Soc Med Nat Iasi, 2009; 113(2): 502-504 (the titles of the works, the authors and the complete data are presented at the end of the chapter, pg 95).

The results of the studies presented in Chapter III were published in the following publication:

Full articles in ISI journals

1. Buca BR, Tartau Mititelu L, Rezus C**, Filip C**, Pinzariu AC, Rezus E, Popa GE, Panaite A, Lupusoru CE, Bogdan M, Pavel L, Lupusoru RV, The Effects of Two Nitric Oide Donors in Acute Inflammation in Rats Experimental data, Revista de Chimie (Bucharest) 2018, 69(10): 2899-2903
2. Beatrice Rozalinda Buca, Liliana Mititelu-Tartau, **Cristiana Filip**, Ciprian Rezus, Cristina Iancu, Elena Rezus, Alin Constantin Pinzariu, Sorin Aurelian Pasca, Catalina Elena Lupusoru, Raoul Vasile Lupusoru. The Influence of Nitric Oxid Donors Nebivolol and S-Nitrosoglutathion of the Oxidatives Stress and Liver Function in Rats. Revista de Chimie (Bucharest) 2019, 70(4):1360-1363
3. Șerban R, Badescu C, Filip A, Cobzeanu MD, Badescu M, Butnaru C, Huzum B, **Filip C.** The cat and sod activities in patients with chronic suppurative otitis media, Farmacia, 2018, 66 (6): 984-988
4. Roxana Șerban, **Cristiana Filip**, Luminita Mihaela Radilescu, Minerva Codruta Badescu, Magda Mariana Badescu, Bogdan Mihail Diaconescu, Filip A, Mihail Dan Cobzeanu, Bogdan Mihail Cobzeanu MD. IL-1α, IL-6 and IL-8 serum values in patients with chronic suppurative otitis media, Experimental and Therapeutic Medicine, 2021, 22: 1226

Full articles in BDI journals

1. Zamosteanu N, **Filip C**, Albu E, Ungureanu D, Cuciureanu R, Total antioxidant status in the blood serum of rats after biogenic amines administration. Rev Med Chir Soc Med Nat Iasi, 2009; 113(2): 502-504.
2. **Filip Cristiana**, Albu Elena, Zamosteanu Nina. Superoxide Dismutase: Therapeutic Targets in SOD Related Pathology, Health, 2014, 6, 975-988, ISSN: 1949-4998

Editor / Book chapters published in international publishing houses

1. Reactive Oxygen Species (ROS) in Living cells. **Edited by Cristiana Filip** and Elena Albu, 2018, Print ISBN 978-1-78923-134-2, Online ISBN 978-1-78923-135-9, Printed in Croatia, InTechopen
2. **Filip Cristiana** - Reactive Oxygen Species (ROS) in Living cells - Introductory Chapter: The Biology of Reactive Species, 2018, pg.3-6, **Edited by Cristiana Filip** and Elena Albu, 2018, Print ISBN 978-1-78923-134-2, Online ISBN 978-1-78923-135-9, Printed in Croatia, InTechopen

Chapter IV. Determination of specific biochemical and biomechanical parameters in fragility fracture.

IV.1. Introduction

Nowadays osteoporosis is one of the most common bone diseases encountered in older population. The major consequence of osteoporosis is the risk of fractures called fragility fractures (FF). Fragility fractures are serious medical problems, both due to the high costs of treatment and the severe decrease in the patient's quality of life. Fragility fracture occurs when structural changes suffered by the bone affect its biomechanical characteristics such as resistance to compression and torsional forces. The World Health Organization (WHO) defines a fragility fracture as one caused by a trauma that would be insufficient to injure bone if the bone substance were normal [280]. The most common fragility fractures are located in the vertebrae, femur, proximal humerus, distal radius. A particular case is the fragility fracture of the pelvis (FFP) whose incidence has increased lately. This type of fracture raises supplementary problems compared to the other fractures due to the difficulties of diagnosis, surgical approach and to the longer recovery period. Fragility fractures are treated as literature recommends either conservative or by surgery [281,282]. Alternatively, to surgery, pharmacological therapy can reduce the risk of fracture by 30% to 40% by using anti-osteoporotic medication associated with vitamin D and eventually Ca supplementation.

Regarding the conservative treatment, it is known that the healing process of a fracture involves the participation of endogenous factors as well as exogenous factors such as vitamin D and calcium. Osteoporosis is characterized by increased calcium level but it is generated by the process of the systemic bone loss. More than 50% of the hypercalcemia cases, found in women, are associated with osteoporosis. In osteoporosis, detected calcium levels were greater than 2.56 mmol/L, the reference range being 2.15–2.55 mmol/L [283] In the case of osteoporotic bone, the healing process is much slower [284] and the calcium and vitamin D deficiency has more severe consequences on systemic bone mineralization. The risk is even higher in the case of fractured osteoporotic bone when the increased calcium requirement for fracture-callus mineralization is provided by systemic bone resorption. In other words, post-traumatic bone loss leads to exacerbation of osteoporosis, increasing the risk of secondary fracture, 3 times [285]. The literature indicates that the subsequent bone loss after a fracture is estimated between 2-15% of the bone mass [286] Therefore, the need for the use of bone anti-resorption agents associated with calcium and vitamin D supplementation appears obvious. [285].

Regarding the surgical treatment, the main approach was the use of prostheses made of different materials including synthetic polymers. From the beginning of their discovery, polymers have been used for medical purposes. Polymers are generally classified in natural (biopolymers) and synthetic polymers, both has useful mechanical properties. The very first reported synthetic polymer for medical use is poly (methyl methacrylate) (PMMA) by a British ophthalmologist, Sir Nicholas Harold Ridley, in 1949 for making intraocular lens [287]. Polymers are macromolecules with high versatility in structure and properties that allows their use in various fields such as mechanics, medicine, electronics, and other branches of science and technology. Biomedical polymers [288-290] are used in medical prosthetic, heart valves, stents, cartilage scaffolds, joints, making of artificial skin, blood vessels, urinary catheters, ureteral stents, artificial kidney/hemodialysis membranes, stemmatological use and drug delivery systems. Regardless of their medical application, polymers must meet two conditions namely biocompatibility and biodegradability [291].

In these researches polyurethanes were used in a biomechanical study to assess their possible use in the fracture fixation, particularly in the fragility fractures of the pelvis. Bone fractures are usually repaired by using different types of devices such as: wires, pins and screws, plates and nails most of them made of stainless steel. These devices meet all biological and mechanical requirements but their major disadvantage is the need for a second intervention to remove them after healing. For healthy bone, surgical intervention does not involve major risks. In contrast, for osteoporotic bone, the fixation of the fracture presents the major risk of failure or weakening of the implant due to the altered bone structure. To reduce the risk of failure, prosthetic materials have been improved with various cements [292-294]. Different types of cements can be obtained by chemical manipulation in order to provide a more efficient transition between the metal surface and the real bone structure to ensure a perfect cohesion between the implant material and the natural bone [295-297]. Moreover, currently, the synthetic materials used in prosthesis are improved by associating with natural components of the bone, such as hydroxyapatite [298-300]. For osteoporosis the association of the implanted material with hydroxyapatite is expected to improve the bone mineralization after implantation.

The studies presented below is part of a larger study conducted in a doctoral thesis in which the fragility of the pelvis was studied.
From this larger study, two aspects will be presented:

IV.2. the involvement of vitamin D and calcium in the fragility fracture healing
IV.3. the testing of the use of some polymers in order to improve the surgical fixation of the fragility fracture

IV.2. The involvement of vitamin D and calcium in the fragility fracture healing

Aim: The aim of our study was to evaluate the role of calcium and vitamin D in the healing process of patients with pelvic fragility fracture in osteoporotic patients.

Materials and methods

The study was realized on patients who experienced pelvic fracture, admitted at the "St. Spiridon" Emergency Hospital, Iasi, Romania. The inclusion criteria for the patients in the study were: osteoporotic patients aged 60 years and older. The fractures had been confirmed by X-ray or computer tomography. Informed consent from all patients included in this study was obtained. The current research has been conducted in accordance to the ethical principles set out by the Helsinki Declaration and by the Ethical Committee from our university.

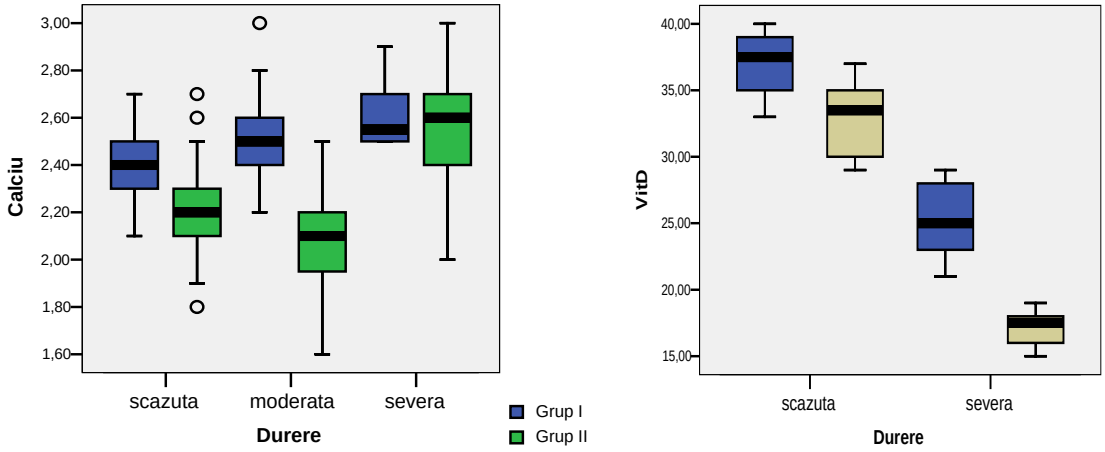
Patients were divided in 2 groups: Patients were divided in 2 groups: Group I-patients receiving osteoporotic treatment (bisphosphonates) before fracture and supplemented with calcium and vitamin D, Group II- patients receiving osteoporotic treatment (bisphosphonates) before fracture but not supplemented with calcium and vitamin D by voluntarily not complying with the prescription or ceasing osteoporotic therapy, due to negligence, or other reasons. Group I contains 22 patients aged 63-85 years (16 women, 6 males). Group II contains 31 patients aged between 60-81years (23 women, 8 males).
Patients in both groups was conservatively treated and received pain medication. Patients were monitored for a short-term follow-up during hospitalization, in the admission clinic or in specialized recovery department, for 14 days and 1 month after fracture. During hospitalization

patients were monitored for the time required to reduce pain by using the visual analog scale or VAS. At admission, routine analyses including serum calcium levels were determined for all patients in hospital laboratory. Vitamin D was determined in the biochemistry department by using a 25-OH vitamin D DRG Elisa kit 5396 for manual use. Determination was performed at hospitalization for all patients and at one month after discharge only for patients who presented for re-evaluation.

Results and discussions

In group I, the appearance of FFP, in patients undergoing osteoporotic therapy and calcium and vitamin D supplementation, may be explained by the older age in this group. Literature indicates [301,302] that bisphosphonates administration prevents fractures in 40% of osteoporotic patients over 70 years (femur data reported), but lower improvements over 80 years of age. In group II, the fracture may be attributed is to non-complying to the therapy. An incidence of 30% - 50% including all types of fragility fractures, according to literature, were found in patients who do not follow the prescribed treatment [303]. In any type of fractures, including FFP, pain reduction is an indication of the healing process. Pain has been assessed with a VAS score whose interpretation after Jensen [304,305] is realized on a scale of 10 cm (100 mm) as follows: no pain (0–4 mm), mild pain (5–44 mm), moderate pain (45–74 mm) and severe pain (75– 100 mm).

The profile for serum calcium levels (mM/l) initially at 14 and 30day after fracture correlated to pain is presented in figure 64 (a).
The profile for vitamin D (ng/ml) initially and 30 days after fracture correlated to is presented in figure 64 (b).



a. b.

Fig. 64 Correlation of calcium (a) and Vitamin D (b) levels with pain intensity in the healing process of fragility fracture.

Calcium is essential for the callus mineralization. The calcium needed for this process is obtained on account of systemic bone resorption in favor of fracture healing. Therefore, the loss of systemic bone mass in the case of osteoporosis is much accentuated in the context of a calcium deficiency. As shown in Figure 64 (a), the low levels of calcium are associated with low to moderate pain

while high concentration to severe pain. Therefore, calcium supplementation in FFP becomes necessary not only to support the fracture healing but also to avoid additional fractures.
Vitamin D optimal level according to the literature is between 25-80 ng/ml, with vitamin insufficiency below 30 ng/mL and vitamin deficiency below 20 ng/ml [306].
Vitamin D concentration at the moment of fracture were for group I at the minimum level of the normal range, while in group II well below this value. Thus, in group I there is hypovitaminosis while in group II vitamin deficiency is installed.
One month after fracture the follow-up on patients (who returned to control) show that during the recovery, they continued/resumed the treatment with bisphosphonates and supplemented calcium and vitamin D. Thus, one month after discharge vitamin D levels are in the normal range (37±3.57 ng/ml) for group I, and near the near the lower limit range for group II. Obtained data indicates that the recovery evolved differently, being slower for the group II. Different authors suggest different vitamin D levels for optimal bone metabolism: greater than 30 ng/ml [307] or better between 40-60 ng/ml [308]. Literature mentions the importance of vitamin D in callus mineralization [309,310] and indicate the intervenes in its formation, as a consequence a vitamin deficiency may slow down the fracture recovery.
To summarize obtained data show that osteoporotic patients with calcium and vitamin D supplementation show a better recovery. The recommended amounts of calcium and vitamin D for osteoporotic patients over 50 years of age are 1200 mg [311] respectively 32 ng/ml (75 nmol/L) [312]. In osteoporotic patients, post-fracture vitamin D administration is recommended for 5 to 8 weeks with calcium levels checking [313]. Supplementation of osteoporotic treatment with calcium and vitamin D should become mandatory especially for osteoporotic patients who have suffered a fracture, as they have a 2 to 4-fold increased risk of future fracture. [314-316].

Conclusions
Calcium and vitamin D exert a positive influence on the fracture-healing outcome as they are the major compounds in normal as well as osteoporotic bone remodeling.
Obtained data emphasize the necessity of calcium and vitamin D supplementation in the healing process particularly after the fragility fracture in patients with insufficient calcium and vitamin D status.
Calcium and vitamin D levels should be constantly evaluated, especially in osteoporotic patients, in order to avoid recurrence.

This work was published in REV.CHIM.(Bucharest), 70, No. 10, 2019 (the title of the work, the authors and the complete data are presented at the end of the chapter, pg 107)

IV.3. Evaluation of the use of some polymers in order to improve the surgical fixation of the fragility fracture

Aim: The aim of this study was to evaluate the mechanical properties of orthopedic screws after coating with both a polyurethane polymer and its hydroxyapatite-containing derivatives in order to increase the stability of the fragility fracture fixation (FF) on an artificial bone.
The study pursued two objectives:

IV.3.1. The polymer synthesis that aimed two objectives:

- first, to identify a biocompatible non-brittle polymer with a certain degree of elasticity

- second, to improve it, by adding the components already existing in the bone structure, namely hydroxyapatite.

IV.3.2. The biomechanical assessment

IV.3.1. Polymers synthesis

Material and Method

In the study it was used a new type of polyurethane, named polyurethane acrylate (PUA) and its derivative obtained by the addition of different concentrations of hydroxyapatite (HA) labeled as PUA-10 %HA and PUA-30 %HA.

In order to simulate the osteoporotic bone, homologated synthetic bones that has similar density to the pathological bone were used.

For the fixation devices three types of homologated orthopedics screws such as cortical, malleolar and spongy screws were used. The selected screws were coated with both polyurethane and polyurethane containing HA. After coating, the screws was subjected to mechanical testing and the strength to fracture was assessed by comparing with identical uncoated screw.

The polymeric material used in this study, synthesized at the Institute of Macromolecular Chemistry "Petru Poni", Iasi, Department of Polyaddition and Photochemistry is a new type of polyurethane named polyurethane acrylate (PUA).

Results and discussion

The urethane group was obtained by the analogous polymer reaction between cycle polycarbonate (figure 65.a) and several types of diamines (figure 65.b) [317-321].

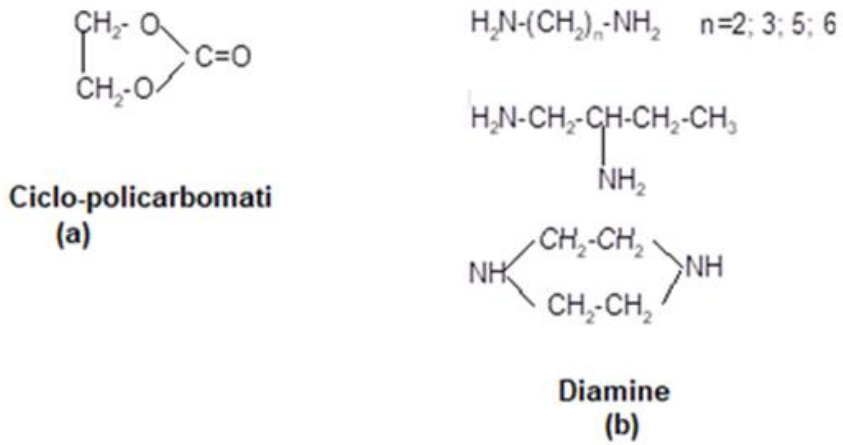


Fig. 65: The chemical structures of the starting compounds used to generate the urethane group.

After the reaction the resulted compounds were subjected to the condensation reaction with acrylic and methacrylic acid, resulting a series of vinyl monomers containing urethanes groups. The structures of the vinyl monomers are shown in figure 66.

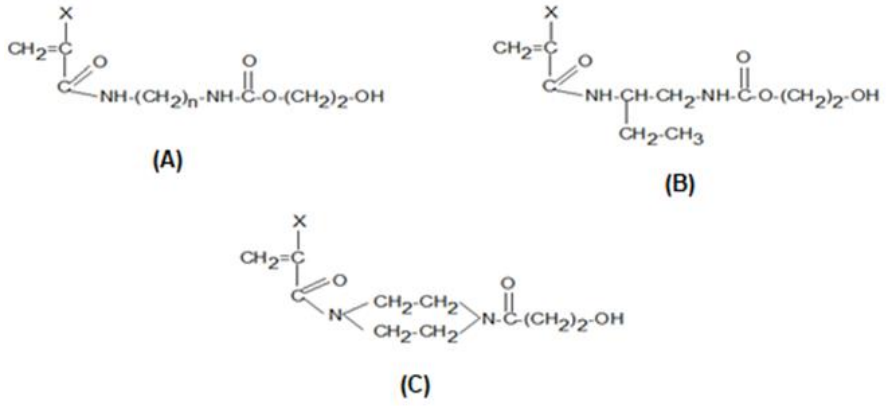


Fig.66. Vinyl monomers structures containing urethane groups

The vinyl monomers obtained, A, B, C were subjected to polymerization in the presence of $K_2S_2O_8$, at low temperatures between 20-40°C, resulting polymers with concentrations in dry matter 30-50% and viscosities ranging between 40000- 355000cP at 20°C. The structure selected for polymerization was the vinyl monomer A, where X represents H, presented in figure 67. The structure of the polymer resulted, named polyurethane acrylate polymer, is presented (figure 68).

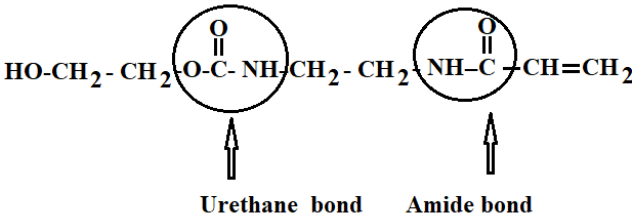


Fig.67. The structure of vinyl monomers A structures used for polymerization.

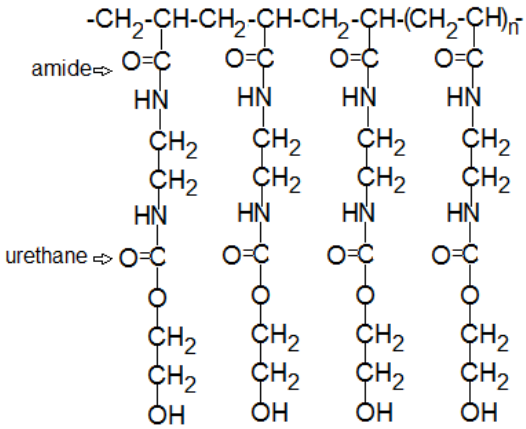


Fig. 68. The structure of the resulted polymer, named polyurethane acrylate (PUA).

The PUA polymer presents some additional advantages over other similar polyurethanes as follows: hydrophily, bio-compatibility, lack of toxicity, mechanical resistance and a certain degree of elasticity. The major PUA advantage is the lack of toxicity of the raw materials used for

synthesis: water as a solvent, amines, ethylene carbonate, acrylic acids [317]. The non-toxic synthesis process is possible due to the structure of the macromolecular chain that allows the fixation of OH terminal groups. Subsequently these groups facilitate the use of water as a solvent thus polymerization may occur in a non-toxic solvent [317]. The urethane groups generate hydrogen bonds with different other chemical groups, thus favoring the physical adhesion on certain surfaces. Another advantage of PUA is the low economic cost due to the mechanism of the polymerization that can take place at ambient temperature.

After synthesis, an aqueous solution of polyurethane acrylate polymer was mixed with hydroxyapatite in two concentrations representing 10% and 30% from the polymer mass, respectively. The mixture was stirred vigorously thus resulting a stable suspension of hydroxyapatite in the PUA. The hydroxyapatite suspension was fixed by a considerable number of hydrogen bonds formed with different active groups belonging to the polyurethane such as the amide, urethane and hydroxyl groups (Figure 69).

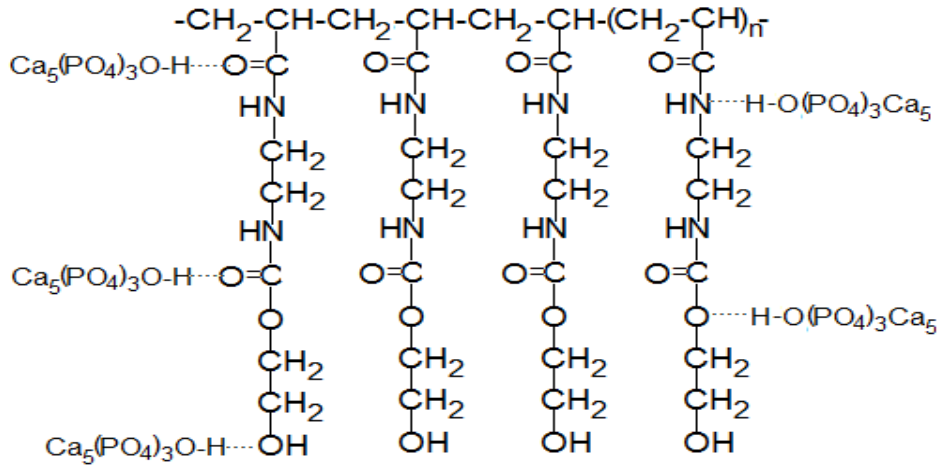


Fig.69. Hydrogen bonds formed between hydroxyapatite and PUA

The structure of the polymer was confirmed by the spectra IR and 1H RMN. In the IR spectrometry the polymer shows the following characteristic bands: 3300cm-1 (NH; OH hydrogen bridges); 2968cm-1(CH2 δ as); 1695cm-1(>C=O; amide band I); 1540cm-1 (NH and C-N amide band II; 1448cm-1 and 1395cm-1 (>CH2); 1325cm-1 and 1255cm-1 (>C – N and NH amide band III. In the 1H NMR spectrometry the polymer shows the following characteristic bands: 1,7 and 2 ppm protons from -CH2- CH<; 3,2; 3,3; 3,4; 3,5 ppm protons from OCONH-CH2- CH2-N<; 3,8; 4 p pm protons from OCO- CH2-CH2-OH; 7,8 ppm protons from urethane NH group.
The obtained polymer achieves an adhesion force of 0.2-0.3 kgf / cm2, thus proving to be a good adhesive for many types of materials.

In addition, in the case of a real bone, both acrylate polyurethane and hydroxyapatite have the potential to form a significant number of strong physical bonds (hydrogen bonds, van der Waals, etc.) with the active groups from collagen belonging to the bone matrix, thus promoting an intimate connection to the implant (Figure 70).

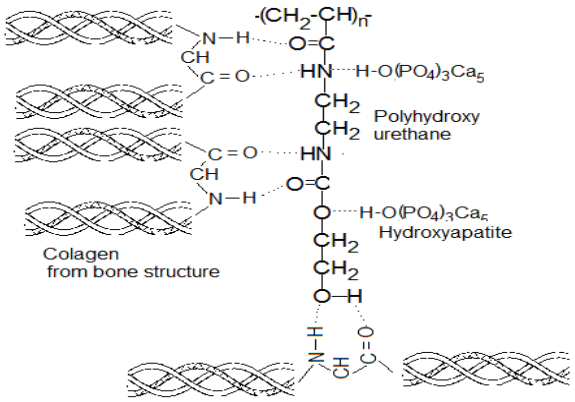


Fig.70. Hydrogen bonds that can be achieved within the matrix of polyhydroxy urethane, collagen fibers from bone structure and hydroxyapatite

The obtained polymer containing HA mimics some of the natural bond's present in the normal bone. The bone matrix is composed of organic components, such as elastic collagen fibers, as well as inorganic mineral components such as hydroxyapatite and calcium phosphate [322]. Collagen provides resistance to fracture due to its elasticity [323,324] and inorganic mineralization ensures bone rigidity [325]. Thus, the combination of the biocompatible, strongly hydrophilic and elastic PUA with the rigid hydroxyapatite, component belonging to the natural bone, brings together the elements required for an appropriate orthopedic cementation material.
As a conclusion for the biomechanical assessment, a polymer called polyurethane acrylate and its derivatives containing 10% and 30% hydroxyapatite was chosen.

IV.3.2. Biomechanical assessment

To evaluate the mechanical properties of a structure means to assess its strength in terms of displacement (deformation) produced by the application of an external force (stress). External forces are different such as compression, torsion, bending, traction, loading, shearing actions.
In the particular case of pelvic fracture, the main stress is the loading stress, nevertheless the literature [326, 327] considers more relevant for biomechanical evaluation the tensile test. The tensile force applied cause the sample deforms until the its fractures. The process representing the deformations that appeared under the applied force until sample brakes are shown in figure 71.

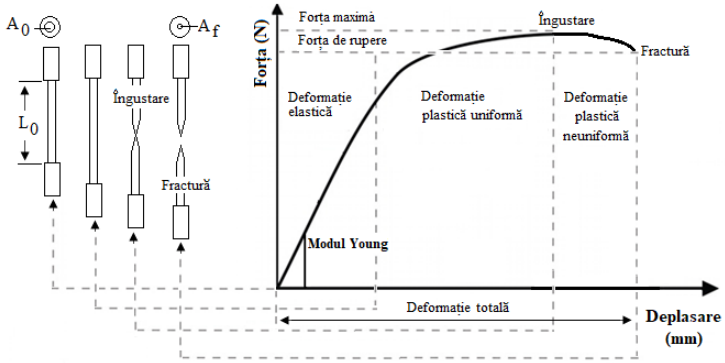


Fig. 71. Stages of deformation until the sample breaks: deformation up to the elastic limit, narrowing and rupture of the sample [modified after 328].

Material and method

In this study mechanical tests had evaluated the behavior of the three different type of orthopedic titanium screws covered with a polymeric film (with and without HA) that were fixed on synthetic bone samples.

The titanium screws (purchased from Biomatrix) used in the study have different diameters 4.5 cortical screw, 6.5 malleolar screw and 6.5 cancellous screw

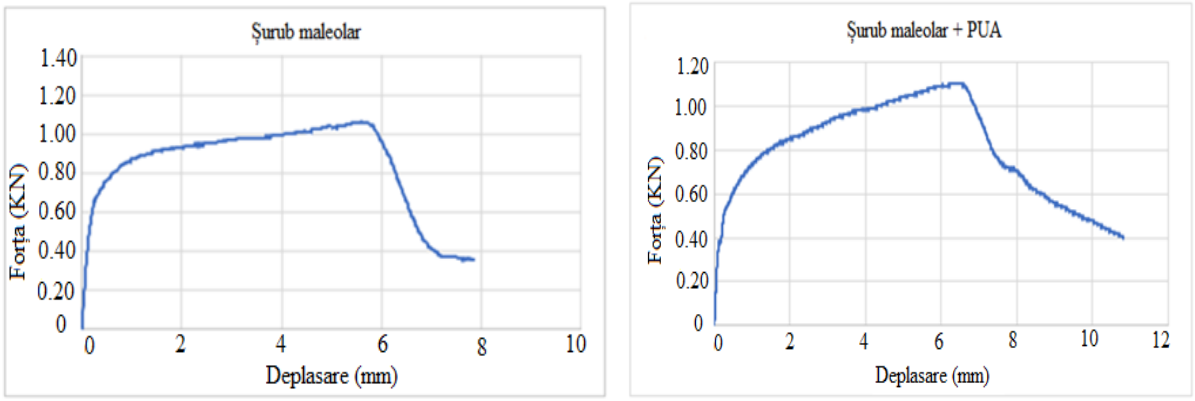
The synthetic bone samples (purchased from Sawbones-SKU:1522-09) which simulate the osteoporotic bone were 7.5 PCF cell polyurethane foam blocks (size 40 mm × 130 mm × 180 mm and density 0.16 g / cm^3 similar to osteoporotic density).

The polymer applied on the surface of the screws was left to stand up to 24 h to complete the polymerization reaction. Uncoated screws as well as those coated with PUA or PUA-HA were subjected to a tensile force. The applied tensile force was generated by a static test machine (WDW-50E) using a speed of 1 mm / min. The displacements to fracture caused by the tensile applied forces were recorded by a specialized WDW Universal Testing Machine Measure & Control System program.

Results and discussions

The mechanical testes of the joint composed of artificial bone and the polyurethane coated screws compared to uncoated screw fixation, was evaluated in terms of maximum tensile forces and displacements [329].

The diagrams of the displacement under the tensile stress for the joints performed using malleolar screws not covered and covered with acrylate polyurethane polymer are shown in figure 72. a. and 72. b. respectively.



a. b.

Fig. 72. Diagram obtained when applying the tensile force on the joint of the malleolar bone-screw not covered with polymer (a) and the malleolar bone-screw covered with PUA (b)

The obtained data show that application of the PUA improves the mechanical characteristics of the joint between the coated screw and the bone compared to the joint using non-coated screw.

The increased tensile forces and displacements to fracture, noticed in the coated screws, may be attributed to the elastic properties of the polymer. A greater deformation for a long time under a higher loading force indicate the join fails/breaks up much later.

This behavior can be attributed to the elastic character of the polymer which takes over part of the applied force. We can also assume that polymer coating provides a more efficient transition zone

between the metal surface and the synthetic bone structure [330-331], improving the cohesion of the entire prosthetic device. In the particular case of fragility fracture of the pelvis (FFP), an increased cohesion of the fixation system could be decisive for the success of the fracture surgical therapy. The best results after PUA coating were obtained for malleolar screw.

Similar mechanical test was performed for the polyurethane acrylate containing HA. Identical types of screws were coated with polyurethane acrylate containing 10% respectively 30% HA. The mechanical joints behavior was evaluated in terms of maximum tensile forces and displacements and compared to screw coated with polymer without HA.

Under condition of coating with PUA-10% HA and PUA-30% HA the improved mechanical properties were recorded only for lower HA concentration obtained for the same malleolar type of screw. The result suggests that the high percentage of hydroxyapatite of 30% HA added to the polymer decreases the adhesion between the implant and the synthetic bone. It is possible that under stress conditions hydroxyapatite precipitates and generates crystals that disrupt the homogeneous structure of the HA polymer, by introducing points/areas of instability thus disrupting the cohesion of the entire joint. The improved mechanicals properties were obtained for the malleolar screw with PUA-10% HA (Figure 73).

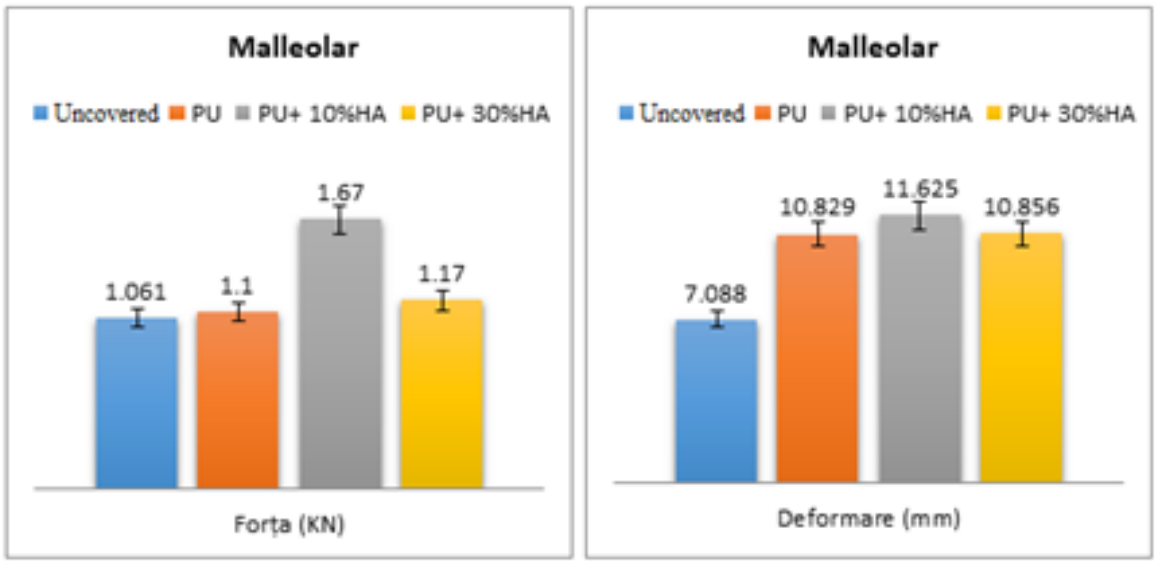


Fig 73. The comparative mechanical behavior of the malleolar screws uncoated and coated with PUA as well as PUA-10% HA and PUA-30%

Bonferoni's analysis for multiple comparisons, of forces and of displacements at break, is presented in the table XXV(a) respectively Table XXVII(b).

Table XXVII. Bonferoni analysis for multiple comparisons, of the average breaking forces (a), and of displacements at break (b) suffered by the three types of screws coated with polyurethane and polyurethane improved with hydroxyapatite compared to similar uncoated screws.

Bonferroni

Dependent Variable	(I) Surub	(J) Surub	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Simplu	cortical	maleolar	-,020100*	,004451	,000	-,03146	-,00874
		cancellous	-,109600*	,004451	,000	-,12096	-,09824
	maleolar	cortical	,020100*	,004451	,000	,00874	,03146
		cancellous	-,089500*	,004451	,000	-,10086	-,07814
	cancellous	cortical	,109600*	,004451	,000	,09824	,12096
		maleolar	,089500*	,004451	,000	,07814	,10086
PU	cortical	maleolar	-,015200	,006127	,059	-,03084	,00044
		cancellous	-,195400*	,006127	,000	-,21104	-,17976
	maleolar	cortical	,015200	,006127	,059	-,00044	,03084
		cancellous	-,180200*	,006127	,000	-,19584	-,16456
	cancellous	cortical	,195400*	,006127	,000	,17976	,21104
		maleolar	,180200*	,006127	,000	,16456	,19584
PU10HA	cortical	maleolar	-,487000*	,027034	,000	-,55600	-,41800
		cancellous	-,104000*	,027034	,002	-,17300	-,03500
	maleolar	cortical	,487000*	,027034	,000	,41800	,55600
		cancellous	,383000*	,027034	,000	,31400	,45200
	cancellous	cortical	,104000*	,027034	,002	,03500	,17300
		maleolar	-,383000*	,027034	,000	-,45200	-,31400
PU30HA	cortical	maleolar	-,065100*	,017809	,003	-,11056	-,01964
		cancellous	-,115400*	,017809	,000	-,16086	-,06994
	maleolar	cortical	,065100*	,017809	,003	,01964	,11056
		cancellous	-,050300*	,017809	,026	-,09576	-,00484
	cancellous	cortical	,115400*	,017809	,000	,06994	,16086
		maleolar	,050300*	,017809	,026	,00484	,09576

*. The mean difference is significant at the .05 level.

(a)

Bonferroni

Dependent Variable	(I) Surub	(J) Surub	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Simplu	cortical	maleolar	-2,092900*	,034789	,000	-2,18170	-2,00410
		cancellous	-1,977800*	,034789	,000	-2,06660	-1,88900
	maleolar	cortical	2,092900*	,034789	,000	2,00410	2,18170
		cancellous	,115100*	,034789	,008	,02630	,20390
	cancellous	cortical	1,977800*	,034789	,000	1,88900	2,06660
		maleolar	-,115100*	,034789	,008	-,20390	-,02630
PU	cortical	maleolar	,187000*	,046976	,001	,06710	,30690
		cancellous	-1,433000*	,046976	,000	-1,55290	-1,31310
	maleolar	cortical	-,187000*	,046976	,001	-,30690	-,06710
		cancellous	-1,620000*	,046976	,000	-1,73990	-1,50010
	cancellous	cortical	1,433000*	,046976	,000	1,31310	1,55290
		maleolar	1,620000*	,046976	,000	1,50010	1,73990
PU10HA	cortical	maleolar	-,309000	,166905	,225	-,73502	,11702
		cancellous	-,500000*	,166905	,017	-,92602	-,07398
	maleolar	cortical	,309000	,166905	,225	-,11702	,73502
		cancellous	-,191000	,166905	,788	-,61702	,23502
	cancellous	cortical	,500000*	,166905	,017	,07398	,92602
		maleolar	,191000	,166905	,788	-,23502	,61702
PU30HA	cortical	maleolar	-,827000*	,226647	,003	-1,40551	-,24849
		cancellous	,020000	,226647	1,000	-,55851	,59851
	maleolar	cortical	,827000*	,226647	,003	,24849	1,40551
		cancellous	,847000*	,226647	,003	,26849	1,42551
	cancellous	cortical	-,020000	,226647	1,000	-,59851	,55851
		maleolar	-,847000*	,226647	,003	-1,42551	-,26849

*. The mean difference is significant at the .05 level.

(b)

Conclusion

Obtained data shows that tested PUA improves the stability between the screw and the synthetic bone. We estimate that the effect obtained is caused by the partial damping of the loading force due to the elastic component of the polymer.

For the polymer containing HA obtained data show that the augmented screw can be obtained by incorporating the lower hydroxyapatite concentrations.

One limitation of the study is that the simulation was performed on artificial bone. In vivo hydroxyapatite is actively assimilated by the bone matrix, participating in the process of bone synthesis and thus promoting bone regeneration. Under real, physiological conditions, it is possible that the presence of HA, even at high concentrations (30%), generates centers / points of bone mineralization beneficial to the process of bone fixation and regeneration. It is paradoxical, but this limitation of the study opens up prospects for further research on animal models.

The results of this study have been published in three articles:
1. REVISTA DE CHIMIE 2019, 70(3): 1094-1097
2. MATERIALE PLASTICE, 2019, 56(3): 559-562
3. MATERIALE PLASTICE, 2019, 56, (4), 1028
(the titles of the works, the authors and the complete data are presented at the end of the chapter, pg 107).

The results of the studies presented in Chapter IV were published in the following publication:

Full articles in ISI journals

1. Alexandru Filip, Ovidiu Alexa, Paul Dan Sirbu, **Cristiana Filip**, Elena Cojocaru, Gabriela Puha, Mioara Florentina Trandafirescu, Oana Viola Badulescu, Calcium and Vitamin D Involvement in the Fragility Fracture of the Pelvis, REV.CHIM.(Bucharest), 70, No. 10, 2019
2. Alexandru Filip,, Ovidiu Alexa, Paul Dan Sirbu, **Cristiana Filip**, Liviu Andrusca, Ionut Alin Pascal, Stefan Oprea, Oana Viola Badulescu, Assessment of the Mechanical Properties of Orthopedic Screws Coated with Polyurethane Acrylate Containing Hydroxyapatite, Intended to Fix the Fragility Fractures, MATERIALE PLASTICE, 56, no. 4, 2019, 1028
3. Filip, A; Badulescu, OV; Sirbu, PD; Veliceasa, B; Puha, B; Pascal, IA; Andrusca, L ; Oprea, S; **Filip, C**; Alexa, O, Preliminary Investigation on Mechanical Properties of Polymer Coating Screws for the Future Fragility Fracture Fixation, MATERIALE PLASTICE, 2019, 56(3): 559-562
4. Alexandru Filip, Bogdan Veliceasa, Bogdan Puha, **Cristiana Filip**, Dragos Popescu, with Fragility Fracture of the Pelvis, REVISTA DE CHIMIE 2019, 70(3): 1094-1097

Full articles in BDI journals

1. A Filip, N Filip, B Veliceasa, **C Filip**, O Alexa The Relationship between Homocysteine and Fragility Fractures-A Systematic Review. Annual Research & Review in Biology, 2017, 16(5):1-8

Section II. The evolution and development of the professional, scientific and academic career

Professional achievements

I started my professional activity in 1986, when I was employed as a chemist researcher at the Department of Pharmacology within the Faculty of Medicine, University of Medicine and Pharmacy "Grigore T. Popa", Iași. Here for a period of 13 years I have participated in the research activity in the discipline, that was carried out on the basis of grants. During this period, I was a member of 33 grants and the research activity at the Department was materialized in the publication of 14 scientific papers. In this period, I participated in conferences and congresses both national and international in the field of pharmacological research, where I had 2 oral presentations on the research carried out.

In order to improve professional expertise, I attended training courses/workshops as follows:

- 1988 - Postgraduate course entitled "News in clinical biochemistry" organized by the Ministry of Health, Center for the improvement of senior health personnel, period 15.03.1988-30.04.1988
- 1995 - Postgraduate course entitled "News in biochemistry" organized by the Biochemistry Discipline, Faculty of Medicine, by UMF "Grigore T. Popa", Iasi, period 1.03.1995-1.04.1995
- 2001 - Confirmation of the title Doctor of Chemistry, based on the order of the M.E.N. no. 3951/ 05.06.2001;
- 2001 - Psycho-pedagogical course organized by the Teaching Staff Training Department of the University "Al. I. Cuza" Iasi, academic year 2000-2001, Certificate Series A No. 0014640 of April 23, 2001
- 2004 – title of Specialist chemist - clinical laboratory, confirmed by M.S.F Order no. 482/2002
- 2004 - Course entitled "Molecular Biology and Pathology. Biotechnologies" Organized under the patronage of the Romanian Academy and the Paris Academy of Sciences in collaboration with the University "Al. I. Cuza" Iasi, 5-17.07.2004
- 2006 – teaching Erasmus Internship (for three weeks) at the Faculty of Pharmacy, University of Renne 2, France
- 2010 – title of Principal medical chemist – clinical laboratory, confirmed by the Order of M.S. no. 990/2010
- 2012 – Molecular diagnostic course and workshop: "Amplifying (PCR) and non-amplifying techniques (in situ hybridization) for the analysis of nucleic acids in molecular diagnosis". Training system for medical personnel in the field of new technologies in the healthcare system (molecular diagnostics) Project co-financed from the European social fund through the program POSDRU/51/3.2/S/58819 S

Academic achievements

In 1999 I was admitted through examination, as Assistant in the Department of Biochemistry at the Faculty of Medicine, “Grigore T. Popa” University of Medicine and Pharmacy, Iași. As assistant I carried out hours of practical works with first- and second-year students from Medicine. I performed practical works with first year students in Medical Assistance in Iasi and Botosani and also with first year students at the Faculty of Nursing Iasi. At the same time, I guided graduation

theses for the graduates of the Faculty of Medicine. In 2002, I published a course book, as a co-author, for the students of the Faculty of Nursing and that of Medical Assistance (found in the complete list of publications).

In 2004 I have been promoted, through examination, to lecturer to the same department. As a lecturer, I was assigned the first-year biochemistry course for medical students. I continued the laboratory activities/practical work with the first-year students from Medicine and those from the General Medicine Assistants and Nursing Specialties. In 2005, I published a completed and revised course book, as a co-author, addressed to the students of the Faculty of Nursing and that of Medical Assistance. In 2007 I also published, as sole author, a book addressed to students at the Faculty of Medicine.

In 2008 I have been promoted, through examination, to Associate Professor of the same discipline. As an Associate Professor, I was assigned the same didactic attributions, in addition, I held practical classes for first-year English students.

For the all-academic activity:

- I guide the scientific research activities of the students that have contributed to scientific papers to local and national scientific meetings or congresses.
- I guide the elaboration of the graduation papers of more than 100 students from both the Romanian Direction of Study
- I held courses and practical works in Master's stages
- I held post-university courses for specialists in the medical field (biochemists, chemists, biologists) working in the National Health Network
- I have participated as course expert and workshop expert specific to biochemistry in educational projects as follows:

  "What would it be like to be a Dentist?BeStomatis" Project ROSE AG 130/ GU/PV/II 01(2019-2022)

  "Reducing university dropout by increasing the academic and socio-emotional performance of students from the first year of the undergraduate cycle of the Faculty of General Medicine" - University of Medicine and Pharmacy "Grigore T. Popa" Iasi - ProMedicis, ROSEAG228/SGU/NC/II (2021,2022)

Over the years, I have been involved in different activities in the university community, of which I mention here:

- president or member of the promotion commissions in the didactic contests at the Biochemistry, and Biophysics departments (2008-2021).
- president or member of the promotion contests for obtaining the titles of specialist chemist respectively main chemist (2008-2022) working in the National Health Network.
- head of the department of Biochemistry (2015-2022)
- member of the professional commissions for the admission exam at the of UMF "Grigore T Popa" Iași (2000-2015, 2017-2022)
- member of the UMF Residency Exam committee "Grigore T Popa" - Iași (2012, 2014, 2016-2022)
- member of commissions for the employment as chemist or biochemist in the National Health Network
- member of the doctoral thesis's guidance committee
- president or member of committees for administrative staff promotion or employment
- member of the evaluation committee from the dean's office of the teaching staff UMF

"Grigore T Popa" - Iasi, (2015, 2016)

To summarize in the entire academic activity, I have published: 11 books with didactic content addressed to students or to the graduates for the preparation of the entrance in Medicine faculty exam; one as sole author the others as coauthor (found in the complete list of publications).

Scientific achievements

In 1992 I was admitted as a candidate for a doctor's degree in the field of Analytical Chemistry with the research theme entitled" Analytical methods for the bioavailability estimation of some drugs bound on a polymeric support" and in 2001 I have been confirmed as Doctor in Chemistry (Diploma Seria C, no. 0001685).

The main themes that I have approached in my scientific research activity until now refer to:

- the elaboration and validation of new methods for the quantification of compounds that are either found in living bodies or are introduced in living bodies for medical purposes
- the study of hyperhomocysteinemia, a pathological situation encountered in the most widespread diseases today; atherosclerosis, diabetes and osteoporosis, diseases that can be aggravated by the presence of increased homocysteine concentrations. This fundamental study also contains an application part that aims to identify new compounds with therapeutic potential of these diseases.
- the study of reactive oxygen species in different diseases in order to identify some pathological mechanisms that can be interfered by medication.

The research carried out during my PhD provided me with the knowledge regarding the requirements that a correct determination must meet, namely the validation conditions. The doctoral experience later helped me in the selection of methods and techniques for the detection of compounds of interest, depending on the purpose, quantity or matrix in which they are found.

The research themes pursued throughout the scientific activity included two main aspects: the study of homocysteine and oxidative stress. In fact, these themes overlap because the current literature considers homocysteine a disturbing factor of the redox balance in the body, a phenomenon also confirmed by our researches. In these studies, the doctoral expertise was used because standardized chromatographic methods for the determination of homocysteine from biological fluids in experimental animals, were required.

In our studies the methods used were selected according to the type of investigated compound and the quantity in which it is present in living bodies. Thus, I used chromatography for homocysteine quantification, enzymatic methods coupled with colorimetric determinations for the components of oxidative stress and immunological methods as ELISA in the case of interleukins that are present in pg/µl quantities.

In the context of studying the variation of these parameters, which dictate the shift from physiological to pathological, we investigated the possibility of using new synthetic compounds as therapeutic agents, and the results obtained were satisfactory.

The entire scientific work had produced:

- 5 chapter in international publishers and 1 book as editor (found in the complete list of publications).
- 39 scientific grants as follows: 7 grants funded by CNCSIS and 32 research contracts

grants funded by Chemical-Pharmaceutical Research Institute (ICCF) and Institute of Macromolecular Chemistry Petru Poni Iasi, where I had participated as a member (found in the complete list of publications).

- 12 ISI papers (first author), 7 ISI (coauthor), 24 BDI papers (author or coauthor).

Future directions in scientific activity

Doctoral training is part of the acquisition stage of the scientific research process. The PhD student is considered a researcher at the beginning of a scientific career, which will later be able to:

- carry out independent research activities;
- critically evaluate the obtained scientific results;
- develop experimental strategies for obtaining scientific information
- bring original contribution to the own field of expertise (the main goal of scientific research);

As PhD supervisor, it is natural to continue the previous themes on both the fundamental aspects of elucidating the mechanisms of action and the applicative aspects of evaluating the therapeutic potential of the different new compounds.

Both types of research are based on interdisciplinary/multidisciplinary collaboration with medical (physiopathology, pharmacology) or pharmaceutical disciplines (drug synthesis). It is understandable that this approach can maximize both the efficiency of research and the chances of financing future scientific partnerships.

As I mentioned before, it is currently considered that hyperhomocyteinemia disrupts endothelial functions by promoting thrombosis. The normal physiological function of the vascular endothelium is anticoagulation and vasorelaxation. Thus, the vascular endothelium functions are at the bottom of the physiological process of tissue irrigation and supply as well as their detoxification. Consequently, any disturbance will lead to a pathological or injury process.

The injury process can be blocked by interfering with the pathological mechanisms. This goal can be achieved by studying on experimental models some new compounds, either natural or synthetic, which target either the path of initiation, transmission of the pathological phenomenon or the lesions caused by it. This field is not only a promising research direction, but also a broad one.

I intend to expand the previous themes by investigating the immunological parameters as well as some new aspects belonging to the field of molecular biology.

The two domains overlap because inflammation parameters intervene in the expression of genes involved in the immune response. In addition, the coordination of the whole process is achieved through the intervention of reactive species ROS and RNS (reactive nitrogen species). These species activate or, on the contrary, block the action of specific protein groups involved in the mobilization of the destruction of pathogens, the removal of the lesion and the morpho-functional restoration of the initial structure. The application of current molecular biology techniques allows the approach of the subcellular level of injury process.

It is already known that the most common genetic cause of elevated levels of homocysteine in the plasma (hyperhomocysteinemia) is Methylenetetrahydrofolate Reductase (MTHFR) deficiency. MTHFR mutation can lead, among other things, to recurrent pregnancy loss. As a perspective, we consider that the application of molecular biology techniques can be a new stage in the study of hyperhomocysteinemia.

Professional activity development

Being able to transmit knowledge is the ultimate goal of teaching. The improvement of the teaching skills can be obtained by continuously learning throughout the academic life and not only. Since the teaching is addressed to the students, they must be involved and trained through the methods and means of the present time that they understand best. Thus, the student is the fundament of the academic activities.

Even if the students are the ones being taught, the purpose of the relationship must have the mentor aspect in the first place and the examiner only in the second place.

Mentoring mainly involves uncovering the difficult and less common aspects of a problem as the student will become a future colleague.

Examining assumes fairness as the sole and absolute criterion; thus, the student will perceive the exam as a measure of his own level and not as a sanction.

In the teaching activity I aim to:

- adapt to the current digital methods and materials that make the theoretical issues very suggestive and accessible
- continuous update the didactic materials to current international trends
- always emphasize the particularities
- active involving of all students in working hours and practical applications
- challenge the learning through discovery and team study.
- challenge the ask of questions
- additionally explaining through consultations or Team’s meetings in order to clarify the misunderstandings
- continuation of the ProMedicis program

I hope that these intentions will maintain, or even better stimulate, students' interest in the field of biochemistry and help them acquire the knowledge and skills necessary for the future medical profession, whether it will be in a hospital, in a polyclinic office or in medical research.

Section III. References

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