UNIVERSITY OF MEDICINE AND PHARMACY
”GR. T. POPA” IASI
FACULTY OF PHARMACY

ABSTRACT OF DOCTORAL THESIS

THE ESTABLISHMENT OF NEW METHODS FOR
QUALITY CONTROL OF A FEW DRUGS OF THE
ANGIOTENSIN I CONVERTING ENZYME INHIBITOR
CLASS

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CONTENS

INTRODUCTION ................................................................................. 5

AIM AND OBJECTIVES ..................................................................... 6

GENERAL PART ............................................................................... 7

I. HYPERTENSION ........................................................................... 7
   I.1. Definition .............................................................................. 7
   I.6. Treatment ............................................................................. 7

II. HEART FAILURE ......................................................................... 8
   II.1. Definition ............................................................................. 8
   II.5. Treatment ............................................................................. 8

III. THE TREATMENT WITH ANGIOTENSIN I CONVERTING ENZYME INHIBITORS ............................................. 9
   III.1. The renin-angiotensine-aldosterone system ......................... 9
   III.3. Principles of treatment ...................................................... 9

IV. LISINOPRIL – ANALYTICAL PROFILE ..................................... 10
   IV.1. Nomenclature and chemical structure .................................... 10
   IV.4. Analysis methods .............................................................. 10

V. RAMIPRIL – ANALYTICAL PROFILE ......................................... 11
   V.1. Nomenclature and chemical structure .................................... 11
   V.4. Analysis methods .............................................................. 11

VI. THE VALIDATION OF THE ANALYTICAL METHODS .................... 12

EXPERIMENTAL PART .................................................................... 12

VII. QUANTITATIVE ASSAY OF LISINOPRIL THOUGHT HIGH PERFORMANCE LIQUID CHROMATOGRAPHY .............................. 12
   VII.1. Aim and objectives .......................................................... 12
   VII.3. The characteristics of the chromatographic method .......... 12
   VII.4. Validation of chromatographic method ............................. 14
   VII.5. Stability study of lisinopril solution ................................... 15
   VII.6. Evaluation through dissolution test of Lisinopril Atb® 20 mg and Zestril® 20 mg tablets ......................................................... 16
VIII. SPECTROPHOTOMETRIC ASSAY OF LISINOPRIL USING 2,4-DINITROPHENOL .................................................. 17
VIII.1. Aim and objectives .................................................. 17
VIII.3. Establishing optimum working conditions ................. 17
VIII.4. Procedure .................................................................. 17
VIII.5. Validation of analysis method ........................................ 18

IX. SPECTROPHOTOMETRIC ASSAY OF LISINOPRIL USING POTASSIUM PERMANGANATE IN ALKALINE MEDIUM ..................................................................................... 20
IX.1. Aim and objectives ....................................................... 20
IX.4. Procedure ...................................................................... 20
IX.5. Validation of analysis method .......................................... 21
IX.6. Spectrophotometric assay of lisinopril in Lisihexal® 20 mg tablets ................................................................. 22

X. TURBIDIMETRIC ASSAY OF LISINOPRIL USING FOSFOMOLIBDENIC ACID ........................................ 23
X.1. Aim and objectives ......................................................... 23
X.3. Establishing optimum working conditions ..................... 23
X.4. Procedure ........................................................................ 23
X.5. Validation of analysis method ........................................... 24
X.6. Spectrophotometric determination of lisinopril in Ranolip® 20mg tablets ............................................................ 25

XI. TURBIDIMETRIC ASSAY OF RAMIPRIL USING PHOSPHOMOLIBDENIC ACID ........................................ 26
XI.1. Aim and objectives ....................................................... 26
XI.3. Establishing optimum working conditions ..................... 26
XI.4. Procedure .................................................................... 26
XI.5. Validation of analysis method ........................................... 27
XI.6. Spectrophotometric determination of ramipril in Tritace® 5mg tablets ............................................................... 28

XII. TURBIDIMETRIC ASSAY OF LISINOPRIL USING PHOSPHOTUNGSTIC ACID ........................................ 28
XII.1. Aim and objectives ....................................................... 28
XII.3. Establishing optimum working conditions ..................... 28
The numbering of the chapters, figures, tables and references was maintained the same as in the doctoral thesis.
INTRODUCTION

Cardiovascular disease is the first cause of death in Romania, which ranks third in Europe, after Bulgaria and Moldova. Also, mortality from complications of hypertension places Romania among the countries with the most elevated values in Europe [1].

Antihypertensive drug substances are very numerous and varied, both structurally and in terms of mechanism of action. They can be classified by the mechanism of action as:

1. peripheral vascular-acting drug substances;
2. drugs with inhibitory action on the adrenergic nervous system;
3. drugs acting on the renin-angiotensin-aldosterone system;
4. diuretic drugs [2].

In the Romanian drug market there already are 10 inhibitors of angiotensin I converting enzyme in the form of Romanian or imports generic drugs. Therefore, we consider that the development of new methods of quantitative evaluation of drugs inhibitors of angiotensin I converting enzyme class, the study of their stability in formulations used to treat hypertension and heart failure, diseases with a disturbingly widespread in population, is one of the top priorities of research teams from pharmaceutical companies, universities and research laboratories, and it is an important focus of research particularly in the context of national and international scientific research. The theme of the project therefore falls on the issue of concern to the European scientific community and the national control methodology employed in the harmonization of analytical drug research [6-7].
AIM AND OBJECTIVES

Aim:
The Doctoral Thesis “The Establishment Of New Methods For Quality Control Of A Few Drugs Of The Angiotensin I Converting Enzyme Inhibitor Class” proposes an analytical study of some drugs of this class (lisinopril and ramipril) in order to develop and validate new analytical methods for their accurate and efficient quantitative assay.

Objectives:
- information and documentation in the literature on already known qualitative and quantitative methods for drugs included in the study;
- achievement of a literature review on angiotensin I converting enzyme inhibitors, in general, and on lisinopril and ramipril in particular;
- development of new instrumental methods of quantitative evaluation of inhibitors of angiotensin I converting enzyme class included in the study;
- validation of the analytical methods for the two listed inhibitors of angiotensin converting enzyme I, according to European organizations and national regulations in effect, in order to be used in the control of these drugs in finite pharmaceutical forms and throughout various stages of manufacture;
- application of analytical methods developed and validated in the control of various pharmaceutical products (indigenous and foreign), by adapting the methodology of each method for the control of pharmaceutical products used in therapy.
GENERAL PART

I. HYPERTENSION

I.1. Definition: Hypertension is a syndrome characterized by increased systolic and diastolic pressure. The World Health Organization considers as normal maximum pressure values between 140-160 mm Hg, according to age, sex and weight, and normal minimum pressure values between 90-95 mm Hg [8].

I.6. Treatment

Depending on the location and mechanism of action, hypotensive agents can be classified as follows:

* vasodilators;
* hypotensive agents active on sympathetic receptors, blocking adrenergic transmission;
* hypotensive agents active on sympathetic or postganglionic nerve fibers, or on nerve endings: guanethidine, reserpine and alpha-methyldopa; the last two are active on the central nervous system;
* hypotensive agents active on sympathetic autonomous ganglia;
* hypotensive agents active on the central nervous system;
* hypotensive agents active on blood volume;
* antagonists of the renin-angiotensin system:
  - angiotensin II antagonists: saralazina;
  - angiotensin I converting enzyme inhibitors such as: captopril, enalapril, lisinopril and ramipril.
  - angiotensin II receptor antagonists: losartan, valsartan [2-8].
II. HEART FAILURE

II.1. Definition

Heart failure is a clinical syndrome resulting from the inability of the heart to expel the entire amount of blood received and to maintain appropriate blood flow for the needs of the organism, because of the unsatisfactory venous fills [10, 14].

II.5. Treatment

The most widely used and effective classes of drugs are drugs used for the treatment of pump failure (systolic heart failure). These include:

* angiotensine I converting enzyme inhibitors;
* angiotensin II receptor blockers: they block the action of certain chemicals in the body responsible for the constriction of blood vessels, leading to improved blood flow and reducing blood pressure; they are used as substitutes for angiotensine I converting enzyme inhibitors when side effects renders them inadvisable; recent studies have shown that the combination of an angiotensin II receptor blocker, candesartan and an angiotensin-converting enzyme inhibitor reduces the number of hospitalizations and deaths in patients with heart failure;
* diuretics;
* aldosterone receptor antagonists (spironolactone and eplerenona are diuretics but they have additional properties of preventing the worsening of heart failure and improvement of symptoms);
* digoxin;
* beta blockers;
* vasodilators (they lower the blood pressure and reduce heart strain) [4, 5].
III. THE TREATMENT WITH ANGIOTENSINE I CONVERTING ENZYME INHIBITORS

III.1. The renin-angiotensine-aldosterone system

The renin-angiotensine-aldosterone system is recognized as a key element in regulating blood pressure and fluid and electrolyte homeostasis. It is a proteolytic cascade in which angiotensinogen (a serum protein) is cleaved by renin (an aspartyl-protease) in order to form decapeptide angiotensin I, biologically inactive; it is activated when it is cleaved by a metallo-protease (angiotensin converting enzyme) resulting octapeptide angiotensin II [2].

III.3. Principles of treatment

Low blood achieved by angiotensin converting enzyme inhibitors is due to:

1) inhibition of the conversion of angiotensin I to circulating angiotensin II;
2) increased natriuresis and renal vasodilatation;
3) reduction of aldosterone secretion;
4) moderate inactivation of bradikinins;
5) inhibition of local formation of angiotensin II in myocard and other tissues;
6) increase of insulin sensitivity.

From a pharmacokinetic point of view there are three classes of angiotensin I converting enzyme inhibitors:

♦ Ist class includes captopril, active by itself and its metabolites;
♦ IInd class contains pro-drugs as enalapril, which becomes active after liver metabolism to its diacid form;
♦ IIIrd Class included lisinopril, soluble in water; it is excreted unchanged by the liver [4].
IV. LISINGPRL – ANALYTICAL PROFILE

Lisinopril, an analogue of enalaprilat, is a long-acting angiotensin I converting enzyme inhibitor which differs from captopril by lacking the sulfhydryl group. Lisinopril, discovered and developed by the Merck Sharp & Dohme Research Laboratories, is indicated for the treatment of hypertension and congestive heart failure [19-25].

IV.1. Nomenclature and chemical structure

From a chemical point of view, lisinoprilul (Figure 4) is L-proline, 1-[N-(1-carboxy-3-phenylpropyl)-L-lysyl]-dihydrate and its molecular formula is C$_{21}$H$_{31}$N$_{3}$O$_{5}$·2H$_{2}$O having a molecular weight of Mr = 441.52 [24].

![Figure 4. The chemical structure of lisinopril](image)

IV.4. Analysis methods

The literature cites numerous methods for the quantitative evaluation of lisinopril. In the literature there were cited 14 spectrophotometric methods, 5 spectrofluorimetric methods, four methods of thin layer chromatography and 23 high-performance liquid chromatography methods.
V. RAMIPRIL – ANALYTICAL PROFILE

Ramipril is an inhibitor of angiotensin I converting enzyme with long lasting action, indicated for the treatment of hypertension and congestive heart failure. It is a prodrug, it is activated by liver dezesterification to its active metabolite ramiprilat [82].

V.1. Nomenclature and chemical structure

According to IUPAC regulations ramipril (Figure 20) is (2S,3aS,6aS) -1-[(2S)-2-[(2S) -1-ethoxy-1-oxo-4-phenylbutan-2-yl] amino]propanoyl]-octahydrocyclopenta[b]pyrrole-2-carboxylic acid [83].

![Figure 20. The chemical structure of ramipril](image)

V.4. Analysis methods

There are many spectrophotometric and chromatographic methods of analysis, but conventional methods such as titration or voltammetry are an important group of analysis methods for ramipril. These methods can be used for quantitative determination of ramipril from its formulations, mixtures with other drugs (diuretics or other antihypertensive agents) and from biological fluids.
VI. THE VALIDATION OF THE ANALYTICAL METHODS

A new analytical method created or acquired, must be validated before it can be applied. There are methodological steps to be taken for verification, confirming its scientific validity.

According to USP 28, the validation of analytical methods is a process which establishes through laboratory studies the necessary conditions needed for the method to be used [111].

EXPERIMENTAL PART

VII. QUANTITATIVE ASSAY OF LISINOPRIL THOUGHT HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

VII.1. Aim and objectives

We proposed the development and validation of a sensitive and rapid method for separation and quantitative determination of lisinopril by high performance liquid chromatography using a LiChrospher® 100 RP 8 chromatographic column [125, 126].

VII.3. The characteristics of the chromatographic method

Method development involved testing several mobile phases (varying the components and the proportions between them), their flow rate and establishing other chromatographic parameters, aimed at obtaining a symmetrical peak, with good resolution.
Table 25. The chromatographic Conditions

<table>
<thead>
<tr>
<th>Chromatographic mode</th>
<th>reversed phase chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution mode</td>
<td>isocratic</td>
</tr>
<tr>
<td>Detection</td>
<td>at $\lambda=215$ nm</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>0.125% sodium hexansulphonate in pH 1.2 phosphate buffer and acetonitrile (72:28, v/v)</td>
</tr>
<tr>
<td>Flow rate of mobile phase</td>
<td>1 mL/minute</td>
</tr>
<tr>
<td>Pressure</td>
<td>63 bar</td>
</tr>
<tr>
<td>Autosampler temperature</td>
<td>20°C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 µL</td>
</tr>
<tr>
<td>Column</td>
<td>LiChrospher® 100 RP 8 (125x4,6), 5µm</td>
</tr>
<tr>
<td>Column temperature</td>
<td>40°C</td>
</tr>
<tr>
<td>Stop time</td>
<td>4,5 minutes</td>
</tr>
<tr>
<td>Retention time for lisinopril</td>
<td>1,8 minutes</td>
</tr>
</tbody>
</table>

Figure 32. Chromatogram obtained for 30 µg/mL lisinopril solution
VII.4. Validation of chromatographic method

In order to establish the linear domain of the method five series of 20 µL calibration standards were injected for concentration range between 0.6 and 60 µg/mL. Calibration curve was drawn in Figure 34 for this range and the obtained data were statistically evaluated.

![Figure 34. Calibration curve](image)

The equation of the calibration curve is:

\[ \text{Area} = 15.198 \times \text{Concentration} - 0.188. \]

After studying the precision the method (repeatability of the analysis) has been established that the relative standard deviation (RSD = 0.50%) has a value of less than 2%, which demonstrates that the proposed analytical method is accurate.

Intermediate precision was evaluated by repeated analysis of samples of different concentrations in five different days. An analysis requires obtaining the calibration curve and analysis of three quality control samples of three concentrations 3, 30 and 45 µg/mL. RSD value ranged between 0.52 ÷ 0.89%, and recovery varied between 97.95 and 100.25%. According to experimental data obtained, the relative standard deviation has a value of less than 2%, which demonstrates that the proposed analytical method is precise.

Accuracy was assessed by comparing the response of three processed samples with analyte concentrations of 1.5, 15 and 22.5 µg/mL respectively with the response obtained for three solutions of
same concentrations obtained by diluting the stock solution. Average retrieval lisinopril values were 97.55, 100.14 and 100.18% respectively.

The lower limit of quantification (lowest concentration standard value of the coefficient of variance below 10%) was set of 0.6 microgram standard/mL.

Selectivity of the method was studied by analyzing a sample containing all the ingredients of *Lisinopril Atb*® tablets produced by Antibiotice Iași, in order to establish the interference of the inactive compounds on the results of analysis.

![Figure 35. Chromatogram obtained for the placebo solution](image)

According to the chromatogram in **Figure 35**, there is no peak at the retention time characteristic for lisinopril - 1.8 minutes, so the method is selective.

**VII.5. Stability study of lisinopril solution**

In this study we used two solutions of 3 µg/mL and 45 µg/mL lisinopril.

Lisinopril is stable at room temperature in pH 1.2 solution for 4 hours with changes in its concentration between 0.470 and -0.414% for those two solutions.

It was also found that the variation of lisinopril concentration after storage for 6 days at 5±3°C was 3.466% for 3 µg/mL solution and 1.686% for 45 µg/mL solution.
**VII.6. Evaluation through dissolution test of Lisinopril Atb® 20 mg and Zestril® 20 mg tablets**

Dissolution test was conducted with a number 2 device Erweka LH equipped with DT 808 blades. The used dissolution medium was that recommended by the United States Pharmacopoeia - Edition 29 - 0.1 M HCl solution, and three buffer solutions with pH values of 1.2, 4.5 and 6.8. The dissolution profiles were studied using 900 mL of each of the different dissolution media at a temperature of 37°C and using a stirring speed of 50 or 75 rotations per minute. Samples were collected after 10, 15, 20 or 30 minutes.

**Figur3 36.** Dissolution profile of the studied pharmaceutical products in HCl 0.1M solution

**Figure 37.** Dissolution profile of the studied pharmaceutical products in pH 1.2 buffer

**Figure 38.** Dissolution profile of the studied pharmaceutical products in pH 4.5 buffer

**Figure 39.** Dissolution profile of the studied pharmaceutical products in pH 6.8 buffer
According to the obtained results and graphics, both pharmaceuticals released over 85% of the declared dosage of lisinopril, and their dissolution profiles are almost identical. So using the new method based on the determination of dissolution through high performance liquid chromatography, the bioequivalence of 

*Lisinopril Atb®* 20 mg tablets produced by Antibiotics SA, Romania and *Zestril®* 20 mg tablets produced by Astra Zeneca - Great Britain was demonstrated in vitro.

**VIII. SPECTROPHOTOMETRIC ASSAY OF LISINOPRIL USING 2,4-DINITROPHENOL**

**VIII.1. Aim and objectives**

The researches have pursued the development and validation of a new spectrophotometric quantitative evaluation method using 2,4-dinitrophenol and its application in the analysis of lisinopril tablets.

**VIII.3. Establishing optimum working conditions**

− establishing the reaction medium;
− establishing the detection wavelength;
− establishing reagent concentration;
− sample stability study.

**VIII.4. Procedure**

The conducted research established the following working procedure: an aliquot of 1 mL methanol solution of 10÷70 µg/mL lisinopril solution was mixed with 1mL 0.5% 2,4-dinitrofenol methanol solution and brought to 5 mL with the same solvent, thus obtaining samples of equivalent concentration of lisinopril between 2 and 14 µg/mL. After 15 minutes the absorbance was measured at 400 nm in 1 cm cuvette against a blank prepared under the same conditions.
VIII.5. Validation of analysis method

In order study the linearity of the method, working solutions were prepared by diluting stock lisinopril solution with methanol. Aliquots of the resulting solutions are processed according to the method, so that after adding all reagents (final volume of 5 mL), the equivalent concentration of lisinopril, respectively of reaction product, are in the range 2-100 µg/sample or 0.4-20 µg/mL, respectively.

After statistically processing the experimental data, the following results were obtained:
- the method is linear in the concentration range 2-14 µg/mL;
- the equation of the calibration curve is: absorbance = 0.0459 x concentration + 0.0057;
- for this range, the regression coefficient ($r^2$) was 0.9991 and standard error (SE) was 0.0065.
In order to evaluate the precision there were determined:
- detection repeatability (precision of the system) for a total of 10 determinations, the relative standard deviation (RSD) was 0.34%;
- repeatability of the method (precision of the method) for three independent solutions of three different concentration levels the relative standard deviation (RSD) value was 1.43% with a confidence interval of the average value contained in the 105.51\(\div\)96.77 %;
- intermediate precision for three independent solutions at three different concentration levels for which the value of relative standard deviation (RSD) is 1.79% with a confidence interval of the average value contained in the 101.93 \(\div\) 96.78%.

To estimate the accuracy, the recovery was measured for a total of three samples at three different concentration levels, thus obtaining BIAS = 100.39% for the interval 97.40% - 102.83% and relative standard deviation was RSD = 1.72%.

There were calculated the detection limit of 0.42 \(\mu\)g/mL and the limit of quantification 1.41 \(\mu\)g/mL.

**VIII.6. Spectrophotometric determination of lisinopril in Lisinopril Tablets USP 5mg\(^\circ\) produced by Mylan Pharmaceuticals Inc., United States of America**

The spectrophotometric assay method has been applied to study Lisinopril tablets USP 5mg\(^\circ\) produced by Mylan Pharmaceuticals Inc., United States of America. The experimental data shows that the average content of 99.83% fall within the limits...
imposed by Romanian Pharmacopoeia Xth Edition for the declared content of active substance per tablet: for 10 mg active substance a deviation of ±10% of declared value is allowed [139].

IX. SPECTROPHOTOMETRIC ASSAY OF LISISNOPRIL USING POTASSIUM PERMANGANATE IN ALKALINE MEDIUM

IX.1. Aim and objectives

The purpose of the undertaken study was to provide a new sensitive and rapid method for quantitative determination of lisinopril, based on the reduction of potassium permanganate in alkaline medium and spectrophotometric analysis of manganese ion results in an amount equivalent to lisinopril. Manganese ion is green and has an absorption maximum at 608 nm.

IX.3. Establishing optimum working conditions:
- establishing the detection wavelength;
- establishing reagent concentration;
- establishing the optimum pH value;
- establishing the reagent adding order;
- establishing the optimum heating period;
- sample stability study.

IX.4. Procedure

As a result of the described research the following working procedure was established: to a volume of 1 mL solution containing 10-70 µg/mL lisinopril, there are added 1 mL 1M sodium hydroxide solution and 1 mL 0.005 M potassium permanganate solution and supplemented with distilled water up to 5 mL. Samples are heated for 15 minutes at 90°C using a water bath, allowed to cool back to room temperature and the absorbance is measure at 609 nm in 1 cm cuvette against a blank prepared under the same conditions.
IX.5. Validation of analysis method

In order to study the linearity of the method, working solutions were prepared by diluting stock lisinopril solution with distilled water. Aliquots of the resulting solutions are processed according to the method, so that after adding all reagents (final volume of 5 mL), the equivalent concentration of lisinopril, respectively of reaction product, are in the range 5-80 µg/sample or 1-16 µg/mL, respectively.

Figure 55. Calibration curve

Regarding the linearity of the assay method for lisinopril, after statistically processing the experimental data the following results were obtained:
- the method is linear in the concentration range 2-14 µg/mL;
- the equation of the calibration curve is: absorbance = 0.0567 x concentration + 0.0549;
- for the studied linear domain, the regression coefficient \( r^2 \) was 0.9991 and the standard error (SE) was 0.0103.

In order estimate the precision, there were studied:

- detection repeatability (precision of the system) for a total of 10 determinations, the relative standard deviation (RSD) was 0.39%;
- repeatability of the method (precision of the method) for three independent solutions at three different concentration levels for which the relative standard deviation value (RSD) was 1.50% with a confidence interval of the average value of 100.97 ÷ 98.88 %;
- intermediate precision for three independent solutions at three different concentration levels for which the value of relative standard deviation (RSD) was 1.12% with a confidence interval of the average value of 100.74 ÷ 99.18%.

To estimate the accuracy, recovery was measured for a total of three samples at three different concentration levels, thus obtaining Bias = 100.32% for the interval 97.19% - 101.83% and relative standard deviation was RSD = 1.35%.

There were calculated the limit of detection of 0.54 µg/mL and the limit of quantification of 1.83 µg/mL.

**IX.6. Spectrophotometric assay of lisinopril in Lisihexal® 20 mg tablets**

The spectrophotometric method for the determination of lisinopril using alkaline potassium permanganate has been applied to study *Lisihexal®* 20 mg tablets produced by Hexal Pharma GmbH, Austria. The results in terms of recovery for the declared amount of lisinopril meet the requirements of the Romanian Pharmacopoeia Xth Edition [139].
X. TURBIDIMETRIC ASSAY OF LISISNORPRIL USING FOSFOMOLIBDENIC ACID

X.1. Aim and objectives

For the quantitative assay of lisinopril we have developed an analytical method based on the reaction with phosphomolibdenic acid when a poorly soluble compound is formed, whose absorbance measured at 369 nm is proportional to the concentration of lisinopril [156].

X.3. Establishing optimum working conditions

− establishing the detection wavelength;
− establishing optimum reagent concentration;
− establishing optimum pH value;
− establishing optimum surfactant concentration;
− sample stability study.

X.4. Procedure

As a result of the described research the following working procedure was established: to aliquots of 2 mL solution containing 20÷80 µg/mL lisinopril, there are added 1 mL 1% phosphomolibdenic acid solution, 1 mL of 0.2 M hydrochloric acid solution and 1 mL 0.01% sodium lauryl sulfate solution. After 20 minutes measure the absorbance at wavelength of 369 nm in 1 cm cuvette against a blank prepared under the same conditions.
X.5. Validation of analysis method

In order study the linearity of the method, working solutions were prepared by diluting stock lisinopril solution with distilled water. Aliquots of the resulting solutions are processed according to the method, so that after adding all reagents (final volume of 5 mL), the equivalent concentration of lisinopril, respectively of reaction product, are in the range 10-200 µg/sample or 2-40 µg/mL, respectively.
Regarding the linearity of the assay method for lisinopril, after statistically processing the experimental data the following results were obtained:

- the method is linear in the concentration range 8-32 µg/mL;
- the equation of the calibration curve is: absorbance = 0.043 x concentration - 0.302;
- for the studied linear domain, the regression coefficient ($r^2$) was 0.9990 and the standard error (SE) was 0.0399.

In order estimate the precision, there were studied:
- detection repeatability (precision of the system) for a total of 10 determinations, the relative standard deviation (RSD) was 0.22%;
- repeatability of the method (precision of the method) for three independent solutions at three different concentration levels for which the relative standard deviation value (RSD) was 1.64% with a confidence interval of the average value of 96.23 ÷ 103.82 %;
- intermediate precision for three independent solutions at three different concentration levels for which the value of relative standard deviation (RSD) was 1.41% with a confidence interval of the average value of 96.75 ÷ 103.26%.

To estimate the accuracy, recovery was measured for a total of three samples at three different concentration levels, thus obtaining Bias = 100.59% for the interval 98.51% - 102.39% and relative standard deviation was RSD = 1.42%.

There were calculated the limit of detection of 2.33 µg/mL and the limit of quantification of 7.79 µg/mL.

**X.6. Spectrophotometric determination of lisinopril in Ranolip® 20mg tablets**

The turbidimetric method for the assay of lisinopril using phosphomolibdenic acid was applied to the study of Ranolip® 20 mg tablets produced by Ranbaxy Laboratories Limited India. The results in terms of recovery for the declared amount of lisinopril meet the requirements of the Romanian Pharmacopoeia Xth Edition [139].
XI. TURBIDIMETRIC ASSAY OF RAMIPRIL USING PHOSPHOMOLIBDENIC ACID

XI.1. Aim and objectives
For the quantitative assay of ramipril we have developed an analytical method based on the reaction with phosphomolibdenic acid when a poorly soluble compound is formed, whose absorbance measured at 361 nm is proportional to the concentration of ramipril.

XI.3. Establishing optimum working conditions
– establishing the detection wavelength;
– establishing optimum reagent concentration;
– establishing optimum pH value;
– establishing optimum surfactant concentration;
– sample stability study.

![Absorption spectrum for 32 µg/mL ramipril sample](image)

Figure 65. Absorption spectrum for 32 µg/mL ramipril sample

XI.4. Procedure
As a result of the described research the following working procedure was established: to aliquots of 2 mL solution containing 20–90 µg ramipril, there are added 1 mL 2% phosphomolibdenic acid solution, 1 mL of 1 M hydrochloric acid solution and 1 mL 0.01% sodium lauryl sulfate solution. After 15 minutes measure the
absorbance at wavelength of 361 nm in 1 cm cuvette against a blank prepared under the same conditions.

**XI.5. Validation of analysis method**

In order to study the linearity of the method, working solutions were prepared by diluting stock ramipril solution with distilled water. Aliquots of the resulting solutions are processed according to the method, so that after adding all reagents (final volume of 5 mL), the equivalent concentration of ramipril, respectively of reaction product, are in the range 20-200 µg/sample or 4-40 µg/mL, respectively.

![Figure 71. Calibration curve](image_url)

Regarding the linearity of the assay method for ramipril, after statistically processing the experimental data the following results were obtained:
- the method is linear in the concentration range 8-36 µg/mL;
- the equation of the calibration curve is: absorbance = 0.027 x concentration - 0.095;
- for the studied linear domain, the regression coefficient ($r^2$) was 0.9992 and the standard error (SE) was 0.0079.

In order to estimate the precision, there were studied:
- detection repeatability (precision of the system) for a total of 10 determinations, the relative standard deviation (RSD) was 0.35%;
- repeatability of the method (precision of the method) for three independent solutions at three different concentration levels for
which the relative standard deviation value (RSD) was 1.03% with a confidence interval of the average value of 97.22 \pm 101.97 \%;

- intermediate precision for three independent solutions at three different concentration levels for which the value of relative standard deviation (RSD) was 1.20% with a confidence interval of the average value of 98.28 \pm 101.94\%.

To estimate the accuracy, recovery was measured for a total of three samples at three different concentration levels, thus obtaining Bias = 98.90\% for the interval 97.13\% - 101.03\% and relative standard deviation was RSD = 1.15\%.

There were calculated the limit of detection of 0.86 \mu g/mL and the limit of quantification of 2.88 \mu g/mL.

**XI.6. Spectrophotometric determination of ramipril in Tritace\textsuperscript{®} 5mg tablets**

The turbidimetric method for the assay of ramipril using phosphomolibdenic acid was applied to the study **Tritace\textsuperscript{®} 5mg tablets** produced by Aventis, Turkey. The results in terms of recovery for the declared amount of lisinopril meet the requirements of the Romanian Pharmacopoeia Xth Edition [139].

**XII. TURBIDIMETRIC ASSAY OF LISINOPRIL USING PHOSPHOTUNGSTIC ACID**

**XII.1. Aim and objectives**

For the quantitative assay of lisinopril we have developed an analytical method based on the reaction with phosphotungstic acid when a poorly soluble compound is formed, whose absorbance measured at 335 nm is proportional to the concentration of lisinopril.

**XII.3. Establishing optimum working conditions**

- establishing the detection wavelength;
- establishing optimum reagent concentration;
- establishing optimum pH value;
- establishing optimum surfactant concentration;
- sample stability study.

![Absorption spectrum for 24 μg/mL lisinopril sample](image)

**Figure 73. Absorption spectrum for 24 μg/mL lisinopril sample**

**XII.4. Procedure**

As a result of the described research the following working procedure was established: to aliquots of 2 mL solution containing 20-80 μg/mL lisinopril, there are added 1 mL 2% phosphotungstic acid solution, 1 mL of 0.5 M hydrochloric acid solution and 1 mL 0.01% sodium lauryl sulfate solution. After 15 minutes measure the absorbance at wavelength of 335 nm in 1 cm cuvette against a blank prepared under the same conditions.

**XII.5. Validation of analysis method**

In order to study the linearity of the method, working solutions were prepared by diluting stock lisinopril solution with distilled water. Aliquots of the resulting solutions are processed according to the method, so that after adding all reagents (final volume of 5 mL), the equivalent concentration of ramipril, respectively of reaction product, are in the range 15-180 μg/sample or 3-36 μg/mL, respectively.

Regarding the linearity of the assay method for lisinopril, after statistically processing the experimental data the following results were obtained:
- the method is linear in the concentration range 9-33 μg/mL;
- the equation of the calibration curve is: absorbance = 0.0369 x concentration - 0.1659;
for the studied linear domain, the regression coefficient ($r^2$) was 0.9995 and the standard error (SE) was 0.02753.

**Figure 79. Calibration curve**

In order estimate the precision, there were studied:
- detection repeatability (precision of the system) for a total of 10 determinations, the relative standard deviation (RSD) was 0.42%;
- repeatability of the method (precision of the method) for three independent solutions at three different concentration levels for which the relative standard deviation value (RSD) was 0.673% with a confidence interval of the average value of 98.69 ±101.78 %;
- intermediate precision for three independent solutions at three different concentration levels for which the value of relative standard deviation (RSD) was 1.11% with a confidence interval of the average value of 99.15 ±100.97%.

To estimate the accuracy, recovery was measured for a total of three samples at three different concentration levels, thus obtaining Bias = 99.54% for the interval 98.27% - 100.96% and relative standard deviation was RSD = 0.81%.

There were calculated the limit of detection of 2.24 µg/mL and the limit of quantification of 7.47 µg/mL.

**XII.6. Spectrophotometric determination of lisinopril in Ranolip® 20mg tablets**

The turbidimetric method for the assay of lisinopril using phosphotungstic acid was applied to the study of Ranolip® 20 mg
tablets produced by Ranbaxy Laboratories Limited India. The results in terms of recovery for the declared amount of lisinopril meet the requirements of the Romanian Pharmacopoeia Xth Edition [139].

XIII. POTENTIOMETRIC ASSAY OF LISISNORPRIL USING A SELECTIVE MEMBRANE ELECTRODE BASED ON LISINOPRIL SILICOTUNGSTATE

XIII.1. Aim and objectives
The starting point of the study was the ability of lisinopril to form poorly soluble combinations with various reagents. Lisinopril is characterized by a large molecule and it has the ability to form very sparingly soluble in water complexes with high molecular weight by ionic association (salt type), when reacting with reagents, containing voluminous complex anions such as silicotungstic acid [179-182].

The purpose of the undertaken study was to provide a new method for quantitative evaluation using a selective membrane electrode that embeds as electroactiv material, lisinopril silicotungstate [168, 178].

XIII.2. The construction of the selective membrane electrode with polyvinyl chloride matrix
For the electrode, the selective membrane was obtained through evaporation of a tetrahydrofuran suspension containing 1% electroactiv material - lisinopril silicotungstate, 68% dioctilftalat as a plasticizer and 31% of polyvinyl chloride powder brought as 5% tetrahydrofuran solution [153, 179].

In order obtain the membrane 1.5 mL of mixture was brought in a glass ring with inner diameter of 3 cm and 3 cm in height, placed on a glass plate as shown in Figure 81. It was left at room temperature for 24 hours for complete evaporation of tetrahydrofuran, covered with a filter paper in order to protect the membrane.
Figure 81. The process for obtaining the selective membrane

The obtained membrane was applied at one end of a polyvinyl chloride tube of 60 mm in diameter and 5 cm length, using as an adhesive a 5% polyvinyl chloride solution in tetrahydrofuran and allowed to dry for 24 hours at room temperature.

In the selective membrane electrode the internal reference solution was introduced in which was immersed the internal reference electrode. It was used as internal reference solution a $10^{-5}$ M lisinopril solution prepared in a saturated solution of silver chloride in order to increase the lifetime of the internal reference electrode by reducing the solubility of silver chloride deposited on the silver wire. The construction of the selective membrane electrode is shown in Figure 82.

Figure 82. Selective membrane electrode
XIII.3. Functional characteristics of selective membrane electrode

The internal reference electrode was a Ag-AgCl electrode and external reference electrode was the saturated calomel electrode. Before measurements, the selective membrane electrode was conditioned in a $10^{-5}$ M solution of lisinopril for 30 minutes and then quickly washed with distilled water. Recording electrode potential values was done with a large internal resistance electronic millivoltmeter (pH/mV meter, Hanna Instruments 300), at constant temperature and gentle stirring using a magnetic stirrer.

Optimum conditions were established for use of selective membrane electrode, studying:
- optimum pH;
- ionic strength;
- response time;
- life span;
- selectivity of the electrode.

To study the function of electrode, all potential measurements were made using the electrochemical cell:

\[
\begin{array}{c|c|c}
\text{lisinopril} & [\text{lisinopril}^+] = 10^{-9} - 10^{-2} \text{ M} & \text{saturated calomel electrode} \\
\text{selective electrode} & \mu = 1 (\text{KNO}_3) & \text{pH} = 4.0 \\
\end{array}
\]

Electromotive force of the cell is given by the formula:
\[
E_{(I)} = E_0' + S \lg [\text{lisinopril}^+]
\]
where: $E_0'$ = apparent conditional standard potential of the electrode; it has a constant value under certain working conditions;
$S$ = slope of the linear portion of the calibration curve.

The calibration curve was drawn representing the electrode potential (electromotive force) by $pC = - \lg [\text{lisinopril}^+]$ for lisinopril concentrations in the range $10^{-2}$ M - $10^{-9}$ M (Figure 88).
Using the calibration curve there are established the domain of linear response, detection limit, the slope of the electrode and the conditional standard potential of the electrode:

- the linear response domain of the electrode is area of concentration in which the electrode has a nernstian response; so the concentration in which the electrode can be used with good results. The electrode shows a linear response for concentrations between $10^{-7}$ M and $10^{-2}$ M lisinopril.

- limit of detection is the minimum concentration for which the electrode has a response. The limit of detection was $7.6779 \cdot 10^{-7}$ M lisinopril.

- slope of the electrode in the linear response region is marked by P and is determined by linear regression; the equation of the calibration curve and the coefficient of regression are established. The slope of the linear electrode is $P = -20.75 \text{ mV/ concentration decade}$.

- by extrapolating calibration curve [lisinopril$^{+}$] = 1 the value of the conditional standard potential of the selective membrane electrode $E_0$ compared to saturated calomel electrode is obtained. For this electrode it was set to 378.5 mV.

**XIII.4. Procedure**

The $10^{-2}$ M lisinopril stock solution is used in order to obtain working solutions for the concentrations in the range $10^{-2}$-$$10^{-7}$$ M by successive dilutions with distilled water. For each lisinopril solution
a volume of 10 ml is measured and transferred to 25 mL Berzelius glasses. For these solutions of lisinopril mixed with 1.5 mL pH = 4 buffer solution and 1.5 mL 1M KNO₃ solution the electrode potential values were measured using a millivoltmeter with high internal resistance.

**XIII.5. Validation of analysis method**

Linearity of the response function has been studied and it was found that the response function is linear on the studied domain 10⁻⁷ - 10⁻² M. The equation of the obtained regression line was calculated by the method of minimum squares (correlation coefficient \( r = 0.9991 \)).

![Figure 89. Calibration curve](image)

The linearity of results was evaluated by plotting the variation of concentration values based on the regression line equation depending on the assessed lisinopril concentration. The calculated values show a linear dependence, the slope of the line being equal to 1, intercept is equal to 0 and correlation coefficient is \( r = 0.9983 \).

There were calculated the limit of detection \( 8.66 \cdot 10⁻⁸ \) M and the limit of quantification \( 7.8 \cdot 10⁻⁸ \) M by using the graphic method.

For the estimation of precision there were determined:

- precision of the method: for three independent solutions at three different concentration levels the value of relative standard deviation (RSD) is 1.73% with a confidence interval of the average value contained in the 101.51 ÷ 98.84% interval;
intermediate precision: for three independent solutions at three different concentration levels for which the value of relative standard deviation (RSD) 1.83% with a confidence interval of the average value contained in the 104.25 ÷ 96.22% interval.

For the estimation of the accuracy, lisinopril recovery was measured for three samples at three different concentration levels resulting an average recovery of 99.92% in the range 98.05%-101.93%.

XIII.6. Potentiometric assay of lisinopril in Tonolysin® 20 mg tablets

The assay method for lisinopril by direct potentiometry using the selective membrane electrode that embeds as active material lisinopril silicotungstate was applied for the study of Tonolysin® 20 mg tablets produced by Gedeon Richter Romania, with results that correspond to Romanian Pharmacopoeia Xth edition [139].

GENERAL CONCLUSIONS

The doctoral thesis “The Establishment Of New Methods For Quality Control Of A Few Drugs Of The Angiotensin I Converting Enzyme Inhibitor Class” was modeled in a traditional manner by comprising a theoretical part and an experimental part.

In the theoretical part, hypertension and heart failure are reviewed in correlation to their treatment using angiotensin I converting enzyme inhibitors. Analytical profile of lisinopril is presented also, and it includes the description, synthesis and physico-chemical characterization. There is a review of the analytical techniques published so far, regarding the main physico-chemical methods for the quantitative evaluation of lisinopril with emphasis on spectrophotometric and high performance liquid chromatographic methods. There are some general considerations related to the
evaluation of analytical information on the criteria of validation, the main parameters being reviewed.

The experimental part (personal contributions) of the thesis is structured in seven chapters; each chapter is devoted to a quantitative evaluation method.

A high performance liquid chromatographic method for lisinopril by has been devised and validated.

A spectrophotometric method was developed for the assay of lisinopril using 2,4-dinitrophenol, which is based measuring the maximum absorbance at 400 nm of the resulting compound.

Another developed spectrophotometric method is an indirect one based on the reaction of lisinopril with permanganate in alkaline medium, when the latter suffers a reduction to manganese ion which has a maximum absorbance peak at 608 nm.

Two more methods are presented based on the formation of insoluble compounds of lisinopril with phosphomolibdenic acid and phosphotungstic acid with maximum absorbance at 369 nm, and 335 nm, respectively.

We have established optimal working conditions for each method: the wavelength of detection, optimal concentrations of reagents added and the stability of reaction products.

It presents a direct potentiometric method which is based on a selective membrane electrode that includes as electroactiv material insoluble compound produced by the reaction between acid and silicotungstic acid. After construction, the selective membrane electrode was characterized by studying the function of the electrode, optimal pH value, ionic strength, response time and duration of use. It was found that the optimum pH is in the acidic domain, optimum ionic strength is achieved by adding 1M KNO₃ solution, its response time is 20 seconds, and the electrode can be used with good results for about two months.

The quantitative evaluation methods for lisinopril have been validated by establishing the linearity domain, the calibration curve equation, the correlation and regression coefficients, the precision and accuracy and the limits of detection and quantification. The most important characteristics regarding the linearity of the methods are presented in Table 121.
Table 121. Evaluation of the linearity of the assay methods for lisinopril

<table>
<thead>
<tr>
<th>№</th>
<th>Method</th>
<th>Linearity domain</th>
<th>Correlation coefficient (r)</th>
<th>Coefficient de regression (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>high performance liquid chromatography</td>
<td>0.6-60 µg/mL</td>
<td>0.9999</td>
<td>0.9999</td>
</tr>
<tr>
<td>2</td>
<td>spectrophotometry using 2,4-dinitrophenol</td>
<td>2 – 14 µg/mL</td>
<td>0.9995</td>
<td>0.9991</td>
</tr>
<tr>
<td>3</td>
<td>spectrophotometry using potassium permanganate</td>
<td>1.0– 7.0 µg/mL</td>
<td>0.9995</td>
<td>0.9990</td>
</tr>
<tr>
<td>4</td>
<td>turbidimetry using phosphomolibdenic acid</td>
<td>8–32 µg/mL</td>
<td>0.9990</td>
<td>0.9995</td>
</tr>
<tr>
<td>5</td>
<td>turbidimetry using phosphotungstic acid</td>
<td>9-33 µg/mL</td>
<td>0.9995</td>
<td>0.9994</td>
</tr>
<tr>
<td>6</td>
<td>potentiometry using selective membrane electrode</td>
<td>10⁻⁷ – 10⁻² M</td>
<td>0.9991</td>
<td>0.9983</td>
</tr>
</tbody>
</table>

It appears that all methods have correlation and regression coefficients very close to 1, as required by european regulations which demonstrates that the described methods are linear.

For the all six methods the linearity of the results was evaluated noticing that the calculated values show a linear proportionality with the found concentration values according to the calibration.

Regarding the precision study, there were evaluated the precision of the system, the precision of the method and the intermediate precision. Comparative results for the six methods of analysis the lisinopril are shown in Table 122.
Table 122. Evaluation of method for determining the precision and accuracy for lisinopril

<table>
<thead>
<tr>
<th>№</th>
<th>Method</th>
<th>Precision of the system</th>
<th>Repeatability</th>
<th>Intermediate precision</th>
<th>Accuracy (Bias)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>high performance liquid chromatography</td>
<td>-</td>
<td>RSD=0.506%</td>
<td>RSD=0.662%</td>
<td>100.18%</td>
</tr>
<tr>
<td>2</td>
<td>spectrophotometry using 2,4-dinitrophenol</td>
<td>RSD=0.34%</td>
<td>RSD=1.43%</td>
<td>RSD=1.79%</td>
<td>100.39%</td>
</tr>
<tr>
<td>3</td>
<td>spectrophotometry using potassium permanganate</td>
<td>RSD=0.39%</td>
<td>RSD=1.50%</td>
<td>RSD=1.12%</td>
<td>100.32%</td>
</tr>
<tr>
<td>4</td>
<td>turbidimetry using phosphomolibdenic acid</td>
<td>RSD=0.22%</td>
<td>RSD=1.64%</td>
<td>RSD=1.41%</td>
<td>100.59%</td>
</tr>
<tr>
<td>5</td>
<td>turbidimetry using phosphotungstic acid</td>
<td>RSD=0.42%</td>
<td>RSD=1.67%</td>
<td>RSD=1.11%</td>
<td>99.54%</td>
</tr>
<tr>
<td>6</td>
<td>potentiometry using selective membrane electrode</td>
<td>-</td>
<td>RSD=1.73%</td>
<td>RSD=1.83%</td>
<td>99.92%</td>
</tr>
</tbody>
</table>

It appears that for all six methods, the standard deviation has a value of less than 2% so all the methods are accurate. The bias of the methods is in the range 95-105% and we can say that the established methods are accurate.

The limits of detection and of quantification were calculated and their values are listed in Table 123. For the spectrophotometric methods this was done using the formula which used the slope and standard error values, but for the high performance liquid chromatography method, the quantification limit was determined based on signal to noise ratio by comparing the measured signals for samples with low known concentrations of the analyte to the blank sample followed by establishing the minimum concentration at which the analyte can be detected faithfully. For the potentiometric method the graphical method was used.
For all six methods the value for the limits of detection and of quantification are very low which show that the established methods are sensible. Of the six, the most sensitive assay method is the high performance liquid chromatography method.

The assay method for lisinopril by high performance liquid chromatography was used to perform dissolution tests on two pharmaceutical products *Lisinopril Atb®* 20 mg tablets produced by Antibiotice SA, Romania and *Zestril®* 20 mg tablets produced by Astra Zeneca, Great Britain. Both pharmaceuticals products have issued over 85% of the declared lisinopril, and their dissolution profiles were almost identical.

The other five methods have been applied experimentally for the quantitative determination of lisinopril from the following pharmaceutical products: *Lisinopril Tablets USP®* 5mg (Mylan Pharmaceuticals Inc. United States of America), *Lisihexal®* 20 mg (Hexal Pharma GmbH, Austria) *Ranolip®* 20mg (Ranbaxy Laboratories Limited, India) and *Tonolysin®* 20 mg (Gedeon Richter SA, Romania).

The results are comparable and they are in accordance with the Romanian Pharmacopoeia Xth edition referring to the permitted deviations in the composition of active substances in tablets.
There is a seventh method for quantitative determination of another inhibitor of angiotensin I converting enzyme: ramipril. It is a turbidimetric method based on an insoluble compound with maximum absorbance at 361 nm obtained in reaction with phosphomolibdenic acid. We have established the optimum conditions: wavelength of detection, optimum concentration of reagents and stability of reaction product. The method was validated and applied for quantitative determination of ramipril from *Tritace*® 5mg tablets produced by Aventis, Turkey.

While elaborating this thesis, diverse references have been covered, selecting from the multitude of data in the literature as significant 190 references for the intended purpose in this paper, namely the development, validation and application of practical analytical methods for quantitative determination of lisinopril.

We believe that through this study we managed to achieve the original objectives and through the results we managed to bring a real contribution to the analysis of lisinopril and ramipril in dosage forms, with the possibility of extending their applicability to biological fluids.

The developed methods for the two drugs of the angiotensin I converting enzyme inhibitors class of and taken into the study, are modern, precise and sensitive, economical and easy to apply, which entitles us to assert that these methods can successfully replace traditional methods of analysis.
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