Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové
Katedra analytické chemie

Universität Wien
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Nová HPLC metoda pro stanovení nafazolinu v očních přípravcích

Martina Dulavová **2011**

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A New Selective and Stability Indicating HPLC Assay for the Determination of Naphazoline in Preparations for Ocular Use

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Prohlášení

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato práce nebyla použita k získání jiného nebo stejného titulu.

Acknowledgement

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I would like to give my special thanks to Ass.Prof.Hannelore Kopelent and PharmDr. Lucie Havlíková, Ph.D.

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Abstract

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Department of Analytical Chemistry

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Title of Diploma Thesis: A New Selective and Stability Indicating HPLC Assay for the Determination of Naphazoline in Preparations for Ocular Use

Naphazoline is a 2-imidazolidine derivated drug and alpha-adrenergic agonist with vasoconstrictive and decongestive properties. Naphazoline is indicated for the therapy of rhinitis, sinusitis or allergic conjunctivitis. Naphazoline is used in liquid formulations for ophthalmic and nasal application.

Naphazoline is marketed in a number of commercially available products; for example, it is contained in Coldan® Augentropfen. Due to economic and therapeutic reasons, hospital pharmacies produce miscellaneous in-house preparations. For our study, two different preparations manufactured in the sterile production of a hospital pharmacy containing naphazoline are investigated. The first formulation is based on two commercially available products and it is a mixture of Coldan® Augentropfen and Okuzell® Augentropfen at a ratio of 1:9. The second formulation is Bor-Naphazolin Augentropfen and it is completely prepared in a hospital pharmacy. In addition to naphazoline, this product also contains other ingredients such as benzalkonium chloride and boric acid. The aim of this study was to investigate these two in-house preparations and prove their quality in order to increase medication safety.

The project included development and validation of an HPLC method for quantitation of naphazoline in the respective preparations. Photo- and thermo stability of these preparations were investigated as well. A new HPLC method was developed for selective and stability indicating determination of naphazoline. Mobile phase consisting of methanol : triethylamine 0.05M/pH 3 set up using phosphoric acid (30:70 v/v), and a BDS HYPERSIL C_{18} column (dim (mm) 150x4, particle size 5μm) were used for the

chromatography. The HPLC method was validated according to international guidelines and proved to fulfill all requirements.

The content of naphazoline hydrochloride in different production lots of both in-house eye drops are analysed by the proposed HPLC method. Moreover, a stability assessment for the formulations was carried out with special emphasis on the photodegradation upon light exposure.

The results of the testing were noted and consulted with representatives of the hospital pharmacy. Based on the results of the HPLC analysis, the production processes were evaluated and optimized, thus contributing to medication safety for in-house preparations.

Abstrakt

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Kandidát: Martina Dulavová

Školitel: PharmDr. Lucie Havlíková, Ph.D.; Ass.Prof. Hannelore Kopelent

Název diplomové práce: Nová HPLC metoda pro stanovení naphazolinu v očních přípravcích

Naphazolin je derivátem 2-imidazolinu a také alfa-adrenergní agonista s vasokonstrikčními a dekongescenčními vlastnostmi. Naphazolin je používán při terapii rinitidy, sinusitidy nebo alergické konjunktivitidy. Pro oční a nosní podání je používán ve formě vodných přípravků.

Naphazolin je prodáván ve velkém množství komerčně dostupných produktů, jako je například Coldan® Augentropfen (oční kapky). V nemocničních lékárnách se z ekonomických a tereapeutických důvodů dává přednost individuálně připravovaným přípravkům s naphazolinem. V naší práci byly analyzovány dva odlišné preparáty obsahující naphazolin připravené ve sterilních podmínkách v nemocniční lékárně. První přípravek je složen ze dvou komerčně dostupných přípravků, je to směs Coldan® Augentropfen a Okuzell® Augentropfen v poměru 1:9. Druhý přípravek je Bor-Naphazolin Augentropfen, který je kompletně připravován v nemocniční lékárně. Tyto přípravky obsahují kromě naphazolinu pomocné látky benzalkonium chlorid a kyselinu boritou. Cílem práce bylo testovat výše uvedené dva individuálně připravované přípravky a prokázat jejich kvalitu za účelem zvýšení jejich bezpečnosti.

Projekt zahrnoval vývoj a validaci HPLC metody pro kvantifikaci naphazolinu ve zmíněných přípravcích. Byla testována fotostabilita těchto přípravků a stabilita za zvýšené teploty. Pro selektivní a stabilitní studii naphazolinu byla vyvinuta nová HPLC metoda. Jako nejvhodnější byla zvolena mobilní fáze methanol : triethylamin 0,05M/pH 3 (pH bylo nastaveno kyselinou fosforečnou) (30:70 v/v) a BDS HYPERSIL C_{18} kolona s rozměry (mm) 150x4 a velikostí částic 5µm. Tato HPLC metoda byla validována a splnila všechny požadavky mezinárodních autorit.

U různých druhů přípravků obou očních kapek byl stanovován obsah naphazolinu pomocí navržené metody. Dále bylo provedeno stanovení stability těchto přípravků, speciální důraz byl kladen na fotodegradační procesy vlivem světla za použití Suntestu.

Výsledky tohoto testování byly zaznamenány a konzultovány s reprezentanty nemocniční lékárny. Na základě výsledků HPLC analýzy byly zhodnoceny a optimalizovány výrobní procesy, což přispělo ke zvýšení bezpečnosti těchto připravovaných přípravků.

Abbreviations

HPLC	High Performance Liquid Chromatography
Mr	Molecular weight
AT	Augentropfen
CE	Capillary electrophoresis
CD-MECK	Micellar electrokinetic chromatography with the addition of cyclodextrin
SIC	Sequential injection chromatography
NP-HPLC	Normal phase
RP-HPLC	Reversed phase
S/N´	Signal-to-noise ratio
LOD	Limit of detection
LOQ	Limit of quantitation
Rt	Retention time
DAD	Diode-array detector

1. INTRODUCTION

Naphazoline is a 2-imidazolidine derivated drug and alpha-adrenergic agonist with vasoconstrictive and decongestive properties. Naphazoline is indicated for the therapy of rhinitis, sinusitis or allergic conjunctivitis. Naphazoline is used in liquid formulations for ophthalmic and nasal application.

Naphazoline is marketed in a number of commercially available products; for example, it is contained in Coldan® Augentropfen. Due to economic and therapeutic reasons hospital pharmacies produce miscellaneous in-house preparations. For our study, two different preparations manufactured in the sterile production of a hospital pharmacy containing naphazoline are investigated. The first formulation is based on two commercially available products and is a mixture of Coldan® Augentropfen and Okuzell® Augentropfen at a ratio of 1:9. The second formulation is Bor-Naphazolin Augentropfen and is completely prepared in a hospital pharmacy. In addition to naphazoline, this product also contains other ingredients such as benzalkonium chloride and boric acid.

2. AIM AND DESCRIPTION OF THE WORK

The aim of this study is to investigate two in-house preparations - a mixture of Coldan® Augentropfen and Okuzell® Augentropfen and Bor-Naphazolin Augentropfen and prove their quality in order to increase medication safety.

The first task will be the development and validation of a new HPLC method for quantitation of naphazoline in the respective preparations. The assay will have to be selective, stability indicating and validated according to international guidelines.

The content of naphazoline hydrochloride in the different production lots of both in-house eye drops should be analysed utilizing the proposed HPLC method. Moreover, a stability assessment for the formulations is planned with special emphasis on the photodegradation upon light exposure. Based on the prospective results, the production processes should be evaluated and optimized to enhance medication safety for in-house preparations.

3. THEORETICAL PART

3.1. Characteristics

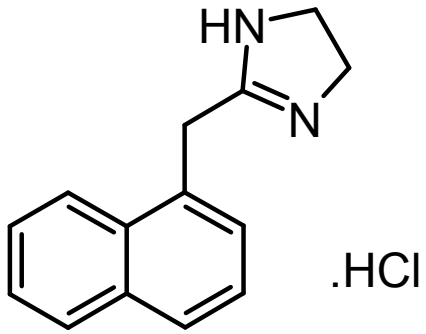
3.1.1 Naphazoline

3.1.1.1 Nomenclature

Molecular formula: $C_{14}H_{15}ClN_2$

Nomenclature: 2-(Naphtalen-1-ylmethyl)-4,5-dihydro-1*H*- imidazole hydrochloride

Structural formula:



Naphazoline HCl

CAS No: 550-99-2

Molecular weight (Mr): 246.7

3.1.1.2 Physical and chemical properties

Naphazoline hydrochloride is a white or almost white, odorless crystalline powder.

The melting point has been reported as 257°C with a range of 255 – 260°C (with decomposition).

pKa is 10.9 at 20 °C. [1]

Solubility at room temperature:	**solvent**	**solubility**
	Water	1 in 6
	Ethanol	1 in 15
	Chloroform	very slightly soluble
	Diethyl ether	practically insoluble

An aqueous solution (1 in 100 ml) of naphazoline hydrochloride in water is clear and colorless, and the pH value is between 5.0 and 6.6 [2].

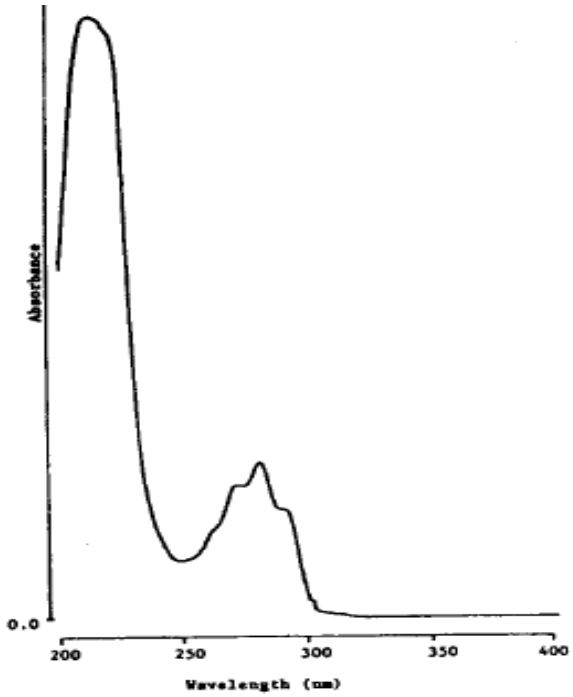


Fig 1: UV spectrum of naphazoline hydrochloride [2].

3.1.1.3 Pharmacology

Pharmacodynamics

Naphazoline is a derivate of 2-imidazoline. These compounds together with catecholamins belong to sympathomimetic drugs. Derivates of 2-imidazoline have a direct effect on α_1-receptors, which are mostly situated in blood vessels and the bronchus.
Naphazoline is a direct-acting sympathomimetic drug and has a direct vasopressor effect on vascularization of skin and mucous tissue. Therefore, naphazoline is used against

oedema of the nose and larynx mucosal tissue. It can also be used for ophthalmic therapy, especially for conjunctivitis.

Pharmacokinetics

After mucosal application, the effect of naphazoline begins after some minutes and lasts for 4 to 6 hours. Naphazoline can be resorbed through mucous tissue to the system circulation and thus can cause systemic side effects.

Indication

Naphazoline is indicated for the therapy of rhinitis, sinusitis, nasopharyngitis and nonspecific and allergic conjunctivitis, and it is also used in rhinoscopy in addition to local anesthetics.

Dosage

0.05 % solution:
For adults – 4 to 6 times a day, 2 to 4 drops into each nostril.
For children – the maximum permissible dose is 2 to 4 drops into each nostril 2 to 3 times a day.

0.1 % solution:
For special therapeutical and diagnostic purposes, 2 times a day spraying, in addition to local anesthetics 2 to 4 drops/ml of anesthetic. In order to prevent overdosing, the solution of naphazoline cannot be used in children younger than 6 years. [1].

Long-term usage increases the danger of side effects and is therefore undesirable. A break in the therapy should last 10 days for adults and 5 days for children.

Side effects

A local application of naphazoline solution can induce systemic side effects such as an increase in blood pressure, tachycardia, arrhythmia, and anginous problems. Long-term usage can damage the mucous tissue in the nose, which will tumefy, so the next application of decongestant is needed. Dependence can develop. Chronic usage can cause necrosis as

well. Symptoms of poisoning can appear if the preparations are used by young children. These symptoms include nausea, problems with respiration and coma.

Contraindications

Glaucoma, rhinitis sicca, attention at hypertonia, hyperthyreosa and cardiovascular disorder.

Interactions

Simultaneous ingestion of an MAO-blocker and naphazoline can contribute to a hypertension crisis. The same crisis can appear if naphazoline is used sooner than 10 days after discontinuing the MAO-blocker. Therefore, naphazoline should not be used within 10 days after the MAO-blocker [1].

Stability

Naphazoline is stable in a solid state, but aqueous solutions are less stable, whereas acidic solutions are relatively stable [2]. Naphazoline in basic solution prone to hydrolysis (Fig 2). The hydrolysis can be increased by heat. That is why the solutions cannot be sterilized by heat but only sterile-filtered.

The first step in the hydrolytic reaction is the formation of 1-naphthylacetylethylenediamine, which further degrades to form 1-naphthylacetic acid and ethylenediamine.

Naphazoline is incompatible with heavy metals and aluminium.

The preparations have to be protected from light and should not be stored above 25°C. The eye drops should be used up within 4 weeks after first opening the containers.

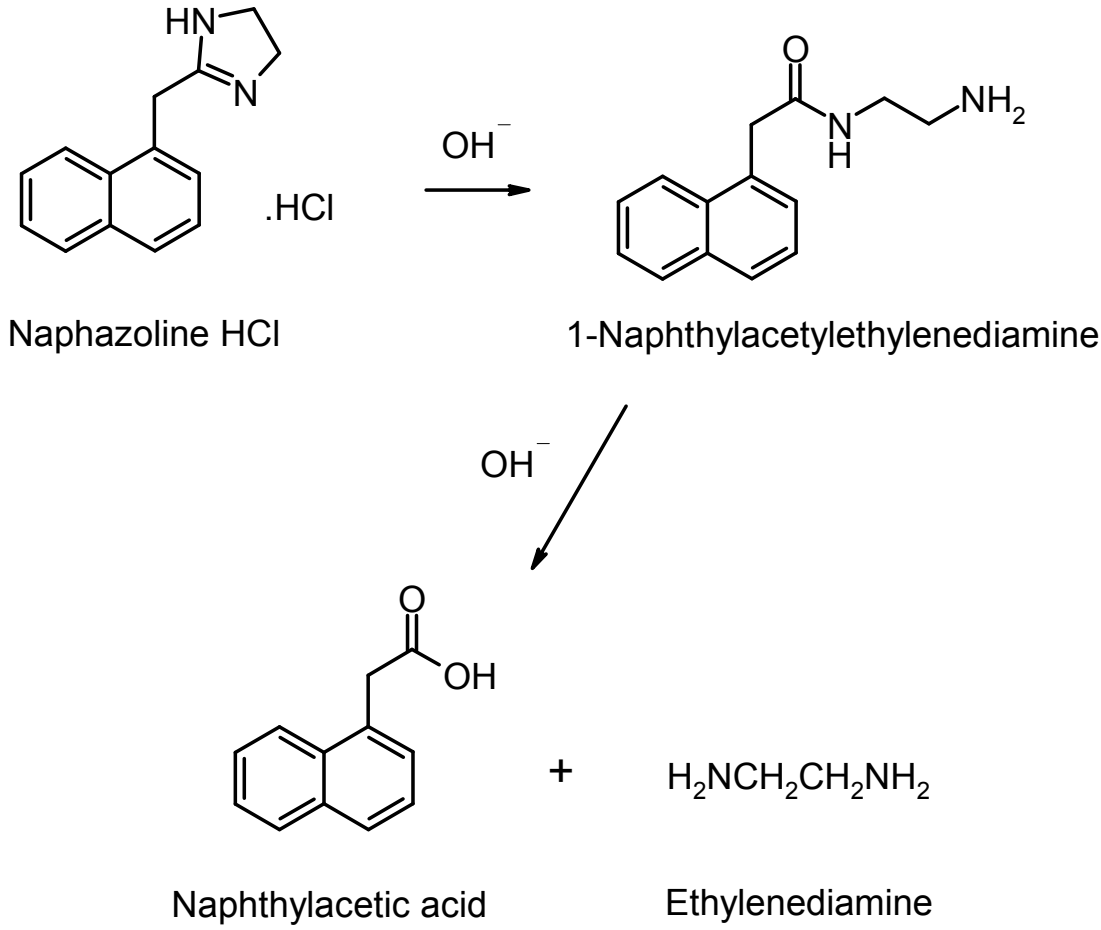


Fig 2: degradation products of naphazoline hydrochloride under alkaline conditions [2].

3.1.1.4 Formulation used in hospital pharmacy

3.1.1.4.1. Naphazolinhydrochlorid 0.01%, Augentropfen 5 ml

This preparation is a mixture of Coldan® Augentropfen and Okuzell® Augentropfen. These drops are produced in amounts of twenty containers, and each container contains 5 ml of eye drops. Usability is one year from the date of preparation and four weeks after opening the container.

10 ml of an isotonic water solution of Coldan Augentropfen contain 10 mg naphazolinehydrochloride and 6.7 mg methyl 4-hydroxybenzoate.

10 ml of a water solution of Okuzell Augentropfen contain 5 mg hypromellose and 0.2 mg benzalkoniumchloride.

Tab 1: ratio for Naphazolinhydrochlorid 0.01%, Augentropfen preparation

Substance	Weighing amount ml
Coldan AT	10
Okuzell AT	90

AT-Augentropfen

3.1.1.4.2. Bor-Naphazolinhydrochlorid 0.025%, Augentropfen 10ml.

These antiseptic, sterile preparations are completely prepared in hospital pharmacies. Each bottle contains 10 ml of the eye drops solution. Usability is one year from the date of preparation and four weeks after opening the container.

Tab 2: Bor-naphazolinhydrochlorid 0.025%, Augentropfen preparation

Substance	prescript amount g
Naphazoline HCl	0.3
Boratic acid	2.4
Natrium chloride	9.6
Benzalkonium chloride-EDTA 1% solution	12
Aqua ad inject.	1175.7

3.1.1.5. Naphazoline in literature

To obtain an overview of the existing literature, an online search was done in Chemical Abstracts with emphases on several high perfomance separation techniques (e.g. HPLC, CE) for quantitation of the compound and the drug formulations.

Capillary electrophoresis (CE) has been proposed for the analysis of naphazoline. Okamoto et al. used the technique of micellar electrokinetic chromatography with the addition of cyclodextrin (CD-MEKC) to simultaneously determine 11 active ingredients (including naphazoline) in ophthalmic solutions. The effect of five different cyclodextrin types on the separation was investigated. It was demonstrated that a system containing sodium dodecyl sulfate with dimethyl-β-cyclodextrin and tetrabutylammonium phosphate salts was optimal for the separation of ingredients. This method was confirmed to be applicable to the quantitative and also qualitative determination of ingredients in commercial ophthalmic solutions [3].

Inclusion complexes of naphazoline with β-cyclodextrin have been investigated. The first study presented by A. Dawoud Bani-Yaseen et al. was aimed at spectrofluorometric, thermal and mechanics studies of β-cyclodextrin inclusion complexes of antazoline, naphazoline and xylometazoline [4].

The same research team studied the photostability of antazoline, xylometazoline and naphazoline with inclusion complexation with β-cyclodextrins. They compared the photostability of pure active substances with substances in complexes with β-cyclodextrins. HPLC was used for the determination of analytes after photodegradation; the mobile phase consisted of 5mM sodium 1-heptanesulfonate in water : acetonitrile : acetic acid (74:25:1 v/v) pH 3.5. UV-detection of naphazoline was carried out at wavelengths 280 and 220 nm. It has been shown that the photostability of the respective compounds is enhanced by inclusion complexation with β-cyclodextrin [5].

The pH effect on the spectroscopic behavior of naphazoline has been demonstrated in an aqueous solution by combining steady state and time resolved spectroscopic experiments. This study provided insight into the overall degradation pathways of naphazoline upon excitation at different pH values [6].

Díaz et al. compared three phosphorescent methods for determining naphazoline in a solution. The first method used micelles to stabilize phosphorescence signals in a solution. Furthermore, heavy atom salt and sodium sulfite were used to obtain phosphorescence. In the last method, they used an optical sensor based on the phosphorescent properties of the analyte on a solid sensor phase [7].

A separation method based on reversed-phase sequential injection chromatography was performed with naphazoline nitrat and methylparaben. This SIC system was proved to be a convenient and efficient tool for the separation and determination of naphazoline nitrate and methylparaben in pharmaceutical preparations [8].

The most widely used method for quantitative and qualitative analysis of naphazoline is HPLC.

For ophthalmic and nasal solutions containing benzalkonium chloride and naphazoline nitrate or tetrahydrozoline hydrochloride, an HPLC method was proposed. The mobile phase contained acetonitrile-diluted acetic acid (80:20 v/v) with 6 mM tetramethylammonium bromide. A sample was injected onto a 5 mm RP C8 column [9].

An HPLC analysis of tetrahydrozoline and naphazoline in ophthalmic solutions was also published by Bauer and Krogh. They proposed the use of a μBondapak C_{18} column and a mobile phase containing 6g of sodium citrate dihydrate and 4g of citric acid in 700 ml of water; the pH was adjusted to 2.2 by the addition of perchloric acid, and 300 ml of methanol were added. The injection volume was 20μl, and the flow rate was 2.0 ml/min. The analytes were detected at the wavelength 265 nm [10].

A different assay for the identification of degradation products of naphazoline in nasal drops was performed by Akgul et al. They used a mobile phase consisting of a phosphate buffer and methanol (7:3 v/v), pH 2.2 [11].

In another study the content of naphazoline in nasal drops was detected by HPLC using a BDS C_{18} column and methanol : 0.05M ammonium sulphate (50:50 v/v) as a mobile phase [12].

Naphazoline hydrochloride and diphenhydramine hydrochloride in naphazoline eye drops were investigated. The mobile phase contained ammonium sulphate 0.05mol/l and methanol (30:70 v/v, pH5) [13], or a quarternary eluent containing methanol-water-acetic acid-triethylamine (50:48:1.5:0.5 v/v) as the mobile phase [14]. Another mobile phase was used by Rui-Ling et al. consisting of methanol : water : triethylamine (50:50:0.25 v/v), pH 4. The detection wavelength was set at 280 nm [15].

A 5-μm Supelcosil LC-8 column and a mobile phase of acetonitrile : water : triethylamine (40:59.75:0.25 v/v) adjusted to pH 4.5 was used for analysis of pharmaceuticals containing naphazoline nitrate and antazoline sulfate [16].

Akiyama et al. developed and used methoxy(3-morpholinopropyl)silanediyl modified silica gel for HPLC separation and determination of components (including naphazoline)

in eye drops. For the separation, the mobile phase consisted of 0.1M phosphate buffer (pH 2.3) [17].

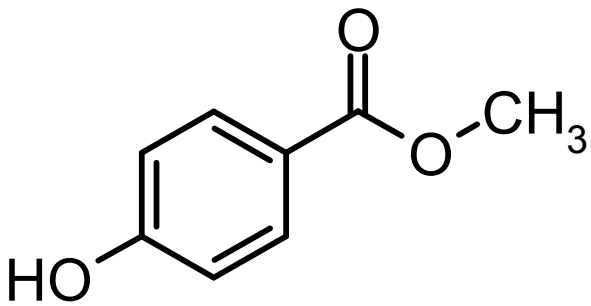
The degradation products of naphazoline and antazoline in an anti-infective ophthalmic solution have been elucidated by Ruckmick et al. The active drugs were hydrolyzed at a high pH. The products of hydrolysis were characterized by NMR, FT-IR and MS. The major degradation product was identified as N-[(N-benzylanilino)acetyl]ethylenediamine. To resolve this degradation product from the active drugs, an HPLC method was developed. The mobile phase was methanol : water, 57:43 containing 22mM heptanesulfonic acid, 0.1% dibutylamine and 1% acetic acid. A C18 column (250 x 4.6 mm) was used. 20 µl of the sample of the ophthalmic solution (dilution 1:10) with water were injected and detected at 280 nm [18].

None of the published high performance separation techniques have been found suitable for application to the objective of our project.

3.1.2. Methylparaben (methyl-4-hydroxybenzoate)

3.1.2.1. Nomenclature

Molecular formula: $C_8H_8O_3$
Structural formula:



Methylparaben

CAS No: 99-76-3
Molecular weight (Mr): 152.15 g/mol

3.1.2.2. Physical and chemical properties

Methylparaben is a white or almost white crystalline powder. The melting point has been reported as 131°C.

Solubility at room temperature:	**solvent**	**solubility (ml)**
	Water	1 in 500
	Boiling water	1 in 20
	Ethanol	1 in 3.5
	Acetone	1 in 3
	Boiling glycerole	1 in 60

Water solutions of methylparaben are highly stable at increased temperature [1].
This product is mainly used as an antiseptic in foodstuff, medicine and cosmetics. It can also be used in feed as an antiseptic.

3.2. HPLC

3.2.1. Introduction

HPLC (High Performance Liquid Chromatography) is the most widely used analytical separation technique, which can be used in all areas of drugs and substance analysis. HPLC can be applied to quantitative and qualitative analysis. The advantages of this method are for example speed of analysis, automation of instrument, minimal amount of the sample [19]. HPLC is also suitable for separating nonvolatile termally fragile species. HPLC is very good for analysis of pharmaceuticals, because major parts of remedies are non-volatile.

HPLC is a physical method of separation in which the components to be separated are distributed between two phases - a mobile and a stationary phase. The mobile phase is a liquid delivered under high pressure to ensure a constant flow rate. The stationary phase is packed into a column capable of withstanding the high pressures which are necessary. A separation occurs if the components of a mixture interact to different extents with the mobile and/or stationary phases and therefore take different times to move from the position of sample introduction to the position at which they are detected. If all analytes have total affinity for the mobile phase and do not interact with the stationary phase, all analytes move at the same time and are not separated. If all analytes have total affinity for the stationary phase and do not interact with the mobile phase, analytes are retained on the column. The desired separation is therefore achieved by modifying the properties and the content of the mobile and/or the stationary phase [20].

Qualitative analysis

This is based on comparison of the retention characteristic (retention time) of an analyte with reference material measured under the same condition.

Quantitative analysis

This involves comparison between the analyte peak area (or height) in the sample and the exact known amount of the same compound measured under identical conditions [21].

3.2.2. HPLC apparatus

The HPLC apparatus is composed of parts which secure the transport of eluents, samples dosing, separation of substances and their detection. HPLC apparatus is composed of solvents reservoir, high-pressure pump, sample injection device, chromatographic column and a detector which is connected to the computer for data collection and evaluation and for control of the chromatographic system.

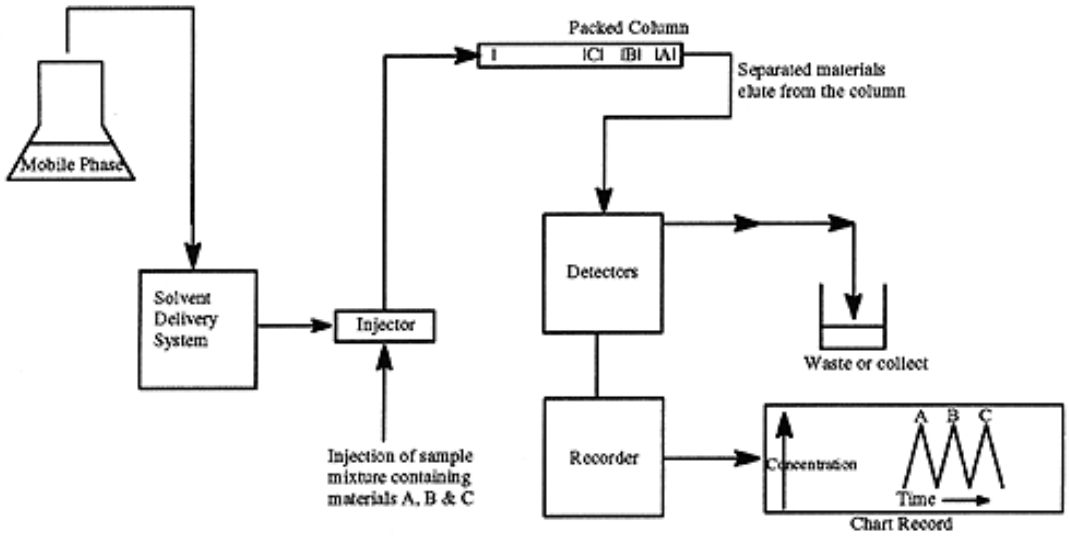


Fig 3: Block-scheme of HPLC-apparatus [22]

HPLC pump

It has to generate high pressure, which can be up to 350 or even 500 bar. The pump has to be made of inert material with regard to the composition of the eluent. It should secure a constant flow without pulsation in the range of 0.1ml/min to 5 or 10 ml/min. High pressure pumps are made from steel, teflon or ceramics. There are different sorts of HPLC pumps [20].

Sample injector

The most commonly used injector in HPLC is the loop injector. This injector introduces a liquid sample into a flowing stream [20].

HPLC columns

The HPLC columns are tubes filled with sorbent and closed off with steel frits. The majority of columns are 5, 10, 15, or 25 cm long and have a diameter of 1 to 5 mm. They are commonly made of 316-grade stainless steel. This material is austenitic chromium-nickel-molybdenum steel, is resistant to corrosion and has good properties in high pressure. The inside of the column must be highly polished without grooves or microporous structures. Other materials used for HPLC columns are glass or tantalum, which is used rarely. A special type of column system is formed with polyethylen tubes compressed by hydraulic fluid in a suitable casing [21].

Detector

The detector recognizes when an analysed substance is eluted from a chromatographic system. It can detect changes in eluent composition, and it then transforms these changes into an electric signal that is shown on a display as a deviation from the baseline (a peak). The detector should be sensitive with a quick response, should not interfere to changes in temperature or composition of the mobile phase and should be able to analyse small amounts of a compound.

The most frequently used detectors measure absorbance of ultraviolet light at specific wave lengths. UV-active molecules are, e.g. compounds with two conjugated double bonds, an aromatic ring, a carbonyl group, a nitro group and a molecule containing ions Br^-, I^-, NO_3^-, NO_2^-.

Diode array – detector:
In this type of detector, light goes through its cell and is divided spectrally in a polychromator. Then, the spectral light is detected by diodes, and each diode obtains a fraction of the information which is interpreted. So this detector scans the whole absorbtion spectrum of an eluent in time. The result is three-dimensional chromatogram-dependence on absorbance, wave-length and time [21].

3.2.3. HPLC methods

In the HPLC system, NP-HPLC (normal phase) and RP-HPLC (reversed phase) can be used.

In NP-HPLC, the sorbent in a column is usually unmodified silicagel, which has a free OH group, and polar substances are absorbed through a hydrogen bond. With these phases, unpolar eluents are used.

The most often used is RP-HPLC. In this HPLC system unpolar stationary phases as chemically modified silicagel are used. The silica has an OH group on its surface and may interact with another compound to form a stationary phase, whose properties depend on a sort of substituent. The most often are used octadecylsilan (C_{18})- alkan with 18 C-atoms. This stationary phase is unpolar. Other often used substituents are C_8 and shorter alkyl chains or cyclohexyl or phenyl [21].

3.2.4. Validation of the HPLC system

Validation can be defined as a procedure with the aim to demonstrate and document the quality of an analytical method. Validation is requested for a new method, for transfer of the method and for system suitability testing.

Validation is used for analytical procedures:

- Identification tests
- Quantitative tests for impurities content
- Limit tests for the control of impurities

Validation characteristics that are considered:

- Specificity
- Linearity

- Precision
- Accuracy
- Detection limit
- Quantitation limit
- Range
- Robustness

3.2.4.1. Specificity

Specificity is the first step in the validation of a method. Specificity is the ability to accurately evaluate the sample in the presence of other components which are expected to be present. These can be impurities, degradation products and solvent.

Identification

For identification tests, it is necessary to discriminate between an analysed compound and compounds with a similar structure. For this purpose are confirmed by positive results from samples containing the analyte coupled with negative results from samples without this analyte.

Impurity tests

These tests are done to ensure that all analytical procedures provide exact information about the content of impurities in an analyte.

If impurities are available:

For an assay, appropriate levels of impurities are added to the sample with the analyte. In this case, it should be demonstrated that the assay results are unaffected by impurities.

Impurity tests are established by spiking a substance with appropriate levels of impurities. The separation of these impurities individually from other components should be demonstrated.

If impurities are not available:

In this case, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterised procedure

e.g.: pharmacopoeial method. Peak purity can be useful to demonstrate that the analyte chromatographic peak does not contain more than one component [23].

3.2.4.2. Linearity

It can be proved by measuring of several analyte concentrations, which are made by diluting of a standard stock solution or by weighting. A minimum of 5 different concentration levels are required. Linearity is established by evaluating a plot of signals as a function of analyte concentration or content. The results should be evaluated by statistical methods, for example, by calculation of a regression line. A correlation coefficient, y-intercept and slope of the regression line should be noted. A correlation coefficient above 0.99 is sufficient, but poorer correlation coefficients are not acceptable [24].

3.2.4.3. Precision

This can be defined as the degree of agreement among individual test results when multiple samplings of the same sample are measured under the same conditions. Precision is divided into three types: repeatability, intermediate precision and reproducibility.

Repeatability is the precision of the method under the same conditions over a short interval of time. It should be conducted by measuring at least 9 determinations of samples corresponding to the specified range for the procedure (for example, 3 concentrations/ 3 replicates of each). This is followed by averaging the peak-area and calculating of relative standard deviation [23].

Intermediate precision
Within the same laboratory. Analysis of multiple preparations of samples on different days and equipment.

Reproducibility examines inter-laboratory precision. It is assessed in collaborative studies and method transfer experiments.

3.2.4.4. Accuracy

Accuracy should be established across the specified range of the analytical procedure, e.g. comparison between results of the analyte and reference material measured under the same conditions or comparison of the results of the analytical procedure with a second well-characterised procedure.
For expression a minimum of 9 determinations over a minimum of 3 concentrations (e.g. 3 concentrations/ 3 replicates).

3.2.4.5. Detection and quantitation limits

The limit of detection (LOD) is defined as the lowest amount of analyte that gives a measurable response. It is based on recognition of the signal-to-noise ratio (S/N´). Determination of the S/N´ ratio is performed by comparing measured signals from samples and establishing the minimum concentration at which the analyte can be reliably detected. For HPLC methods, an S/N´ ratio of 3:1 is recommended.

The limit of quantitation (LOQ) is defined as the lowest concentration of analyte which can be quantified with suitable precision and accuracy. LOQ is particularly used for the detection of impurities and degradation products. In this case determination of the S/N´ ratio is performed by comparing measured signals from samples and establishing the minimum concentration at which the analyte can be reliably quantified. An S/N´ ratio of 10:1 is recommended [23].

3.2.4.6. Range

The range is an interval between upper and lower amounts of an analyte for which the analytical method has adequate accuracy, precision and linearity. The concentration ranges of standard solutions should encompass values expected in measured samples. The concentration ranges should be at intervals from 50 to 120 per cent.

3.2.4.7. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. Robustness is practised by systematically changing important parameters in the method and measuring their effect on separation.

The aim is to know to which changes the analytical system is susceptible. The analytical conditions should be controlled accordingly [24].

3.2.4.8. System suitability testing

This is defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The tests are based on the concept that the equipment, electronics, analytical operation and samples to be analysed constitute an integral system that can be evaluated as such [23].

4. EXPERIMENTAL PART

4.1. Material and Methods

4.1.1. Compounds

Naphazolin hydrochlorid

Produced by: FAGRON GmbH et Co. KG

Charge: 10C23-N24

Content: 100.3 %

Methyl 4-hydroxybenzoate

Produced by: Sigma-Aldrich Production GmbH

Charge: 1405462

Content: 99.8 %

Triethylamine buffer substance

Produced by: E. Merck

Charge: L935817

Acetic acid 96%

Produced by: Merck KgaA

Charge: K24415062

1-Heptane sulfonic acid sodium salt

Produced by: Fisher Scientific

Charge: H/0168/48

Coldan®-Augentropfen 10ml Lösung

Produced by: Sigmapharm, Wien

Charge:3103

Expiration date: 03/2015

10 ml of an isotonic water solution contain 10 mg naphazoline hydrochloride and 6.7 mg methyl- 4-hydroxybenzoate

Okuzell®-Augentropfen 10ml
Produced by: Penta Arzneimittel GmbH
Charge: 100527
Expiration date: 05/2012
10 ml of a water solution contain 5 mg hypromellose and 0.2 mg benzalkonium chloride.

4.1.2. Preparations

The names of the preparations are used as given on the labels of the in-house preparations.

Bor-Naphazolinhydrochlorid 0.025%, Augentropfen 10ml

Antiseptic, sterile
Preparation dates: 22.11.2010
16.09.2010
05.01.2011
19.01.2011

In-house preparation, 1200g of a drops solution contain 0.3g of naphazoline hydrochloride, 2.4g of boric acid, 9.6g of sodium chloride, 12.0g of a benzalkonium chloride-EDTA 1% solution and 1175.7g of aqua ad inject.

Naphazolinhydrochlorid 0.01%, Augentropfen 5 ml

Conservated, antiseptic
Preparation dates: 12.05.2010
01.10.2010
05.01.2011

In-house preparation, mixture of 10ml of Coldan® Augentropfen and 90ml of Okuzell® Augentropfen, subdivided and bottled into 20x5ml.

4.1.3. Apparatus

4.1.3.1. HPLC

For the preliminary test:

HPLC-apparatus

Pump: SHIMADZU LC 10AS

Autosampler: SHIMADZU SIL-10AD

Diode array detector: SHIMADZU SPD-M20A

Column oven: SHIMADZU CTO-10AC

HPLC-column:

EcoCART® 125-3 HPLC Cartridge LiChrospher® 100 RP-18 endcapped, particle size 5µm

Validation and quantitative assays:

HPLC-apparatus

Pump: SHIMADZU LC-20AD

Autosampler: SHIMADZU SIL-20A/20AC

Diode array detector: SHIMADZU SPD-M20A

Column oven: SHIMADZU CTO-20A/20AC

HPLC-column:

Thermo, electron corporation, BDS HYPERSIL C_{18}, Dim (mm) 150x4, particle size 5µm

Part No 28105-154030

4.1.3.2. Ultrasonic bath

Ultrasonic bath BRANSON 5200

4.1.3.3. Suntest

Suntest CPS Accelerated Exposure Machine (Heraeus, Hanau, Germany; Art.No. 55007014). It has a xenon burner NXE 1500 with black panel temperature 49°C,

windowglass filter (Art.No. 56009562). The distance from the source to the specimen table is 22 cm.
The test was performed at radiation intensity 1300 W/m^2;. Time factor:15 means that 1 min irradiation in the Suntest corresponds to 15 min of bright sunlight.

4.1.3.4. pH-Meter

pH-values were determined by an Orion 420 A pH-Meter. Electrode- Hamilton Slimtrode- was calibrated with Merck buffer-solutions with pH 4 and pH 7.

4.1.3.5. Pipette

TREFFLAB micropipette volume 200 - 1000µl
NICHIRYO micropipette model 5000 volume 1000, 500, 100 µl

4.1.3.6. Analytical balance

Sartorius A200S

4.1.3.7. Glassware

Beakers, volumetric flasks, pipettes.

4.1.3.8. Solvent for sample preparation

Water HPLC Gradient grade – Fisher Scientific

4.1.4. Eluent

Methanol : Methanol HPLC Gradient grade, Fisher Scientific

Triethylamine buffer 0.05M/ pH 3**:**

5.06g triethylamine were mixtured with HPLC water in a beaker. The pH was set to pH 3 with phosphoric acid. This mixture was transferred to a 1000ml volumetric flask, were added 100ml of HPLC methanol, and this mixture was diluted to 1000ml with HPLC water.

Acetic acid 2%:

20.83ml of acetic acid were diluted with HPLC water to a 1000ml volumetric flask.

Heptane sulfonic acid 5mM + 2% acetic acid, pH=2.47:

1.00g 1-heptane sulfonic acid sodium salt was dissolved with HPLC water in a beaker. This mixture was transferred to a 1000ml volumetric flask, were added 20ml of acetic acid and this mixture was diluted with HPLC water to 1000ml in a volumetric flask.

Before being used, eluents were sonicated for 20 minutes.

4.2. Method development

For quantitative and qualitative analysis, it is necessary to achieve separation of the components of the samples. Based on knowledge of the analytes under investigation, we modify the properties and content of the mobile and/or the stationary phase to achieve the desired separation. It is also important to have main peaks without tailing and fronting. For quantitative analysis, areas of each component of a mixture are then calculated.

In the current project, the separation of naphazoline hydrochloride and methylparaben as well as their degradation products is necessary. Knowledge of the retention characteristics of naphazoline and methylparaben enables us to determine if there is some interference between these compounds with other components of the sample.

To determine suitable conditions of the chromatographic system, naphazoline hydrochloride was analysed with a different constitution of the mobile phase and also with a different pH of the mobile phase. In literature, published methods used C_{18} or C_8 columns and mobile phase containing acetonitrile : diluted acetic acid (80:20 v/v) with 6 mM tetramethylammonium bromide or 1-heptanesulfonate. Other published methods used mobile phase containing sodium nitrate dihydrate and citric acid or methanol and amonium sulphate. The pH of published methods was always in acid range (between 2-3.5).

4.2.1. Preliminary tests

4.2.1.1. Mobile phase determination

The first experiments were performed with naphazoline hydrochloride solution (100µg/ml).
Three buffer solutions were prepared (water phase) and combined with methanol (organic phase). EcoCART® 125-3 HPLC Cartridge LiChrospher® 100 RP-18 endcapped, particle size 5µm column was used.

Tab 3: The determination of mobile phase, 12.5 cm RP-18 endcapped, sample: naphazoline hydrochloride solution 100 µg/ml

system	%	pH	naphazoline peak
methanol : acetic acid 2%	50:50	2	peak tailing
methanol : heptanesulpfonic acid 5 mM + acetic acid	50:50	2.47	peak tailing
methanol : triethylamine 0.05M	50:50	3	Rt 1.7

The best peak shape was obtained with the system methanol : triethylamine 0.05M set to pH 3; 50:50 (v/v). But the retention time is nearly at the start time of chromatogram. It is necessary to modify the content of the mobile phase.

Tab 4: The determination of mobile phase, 12.5 cm RP-18 endcapped column, sample: naphazoline hydrochloride solution 100 µg/ml

system	%	pH	retention time
methanol : triethylamine 0.05M	50:50	3	1.7
methanol : triethylamine 0.05M	40:60	3	2.5
methanol : triethylamine 0.05M	30:70	3	4.5
methanol : triethylamine 0.05M	20:80	3	7.9

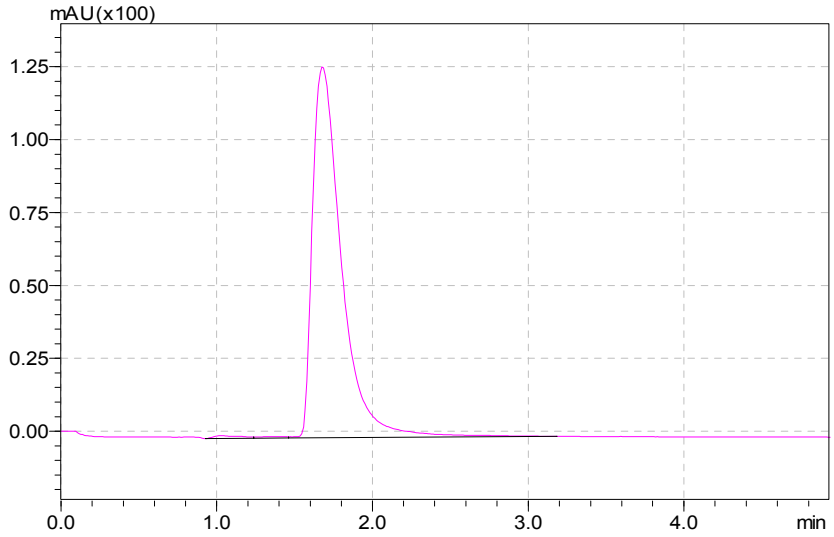


Fig 4: naphazoline hydrochloride solution 100 µg/ml in HPLC- system-12.5 cm RP-18 endcapped column, mobile phase methanol : triethylamine, 50:50 (v/v), pH3

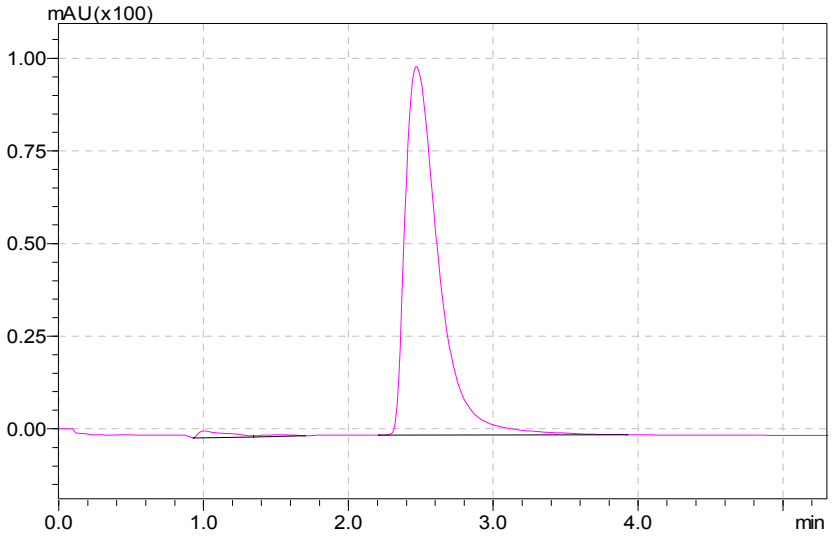


Fig 5: naphazoline hydrochloride solution 100 µg/ml in HPLC- system-12.5 cm RP-18 endcapped column, mobile phase methanol : triethylamine, 40:60(v/v), pH3

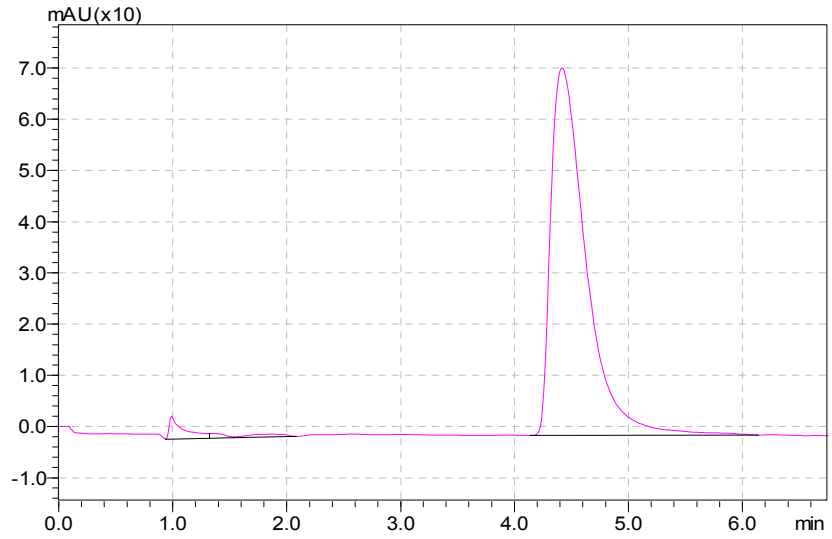


Fig 6: naphazoline hydrochloride solution 100 µg/ml in HPLC- system-12.5 cm RP-18 endcapped column, mobile phase methanol : triethylamine, 30:70(v/v), pH3

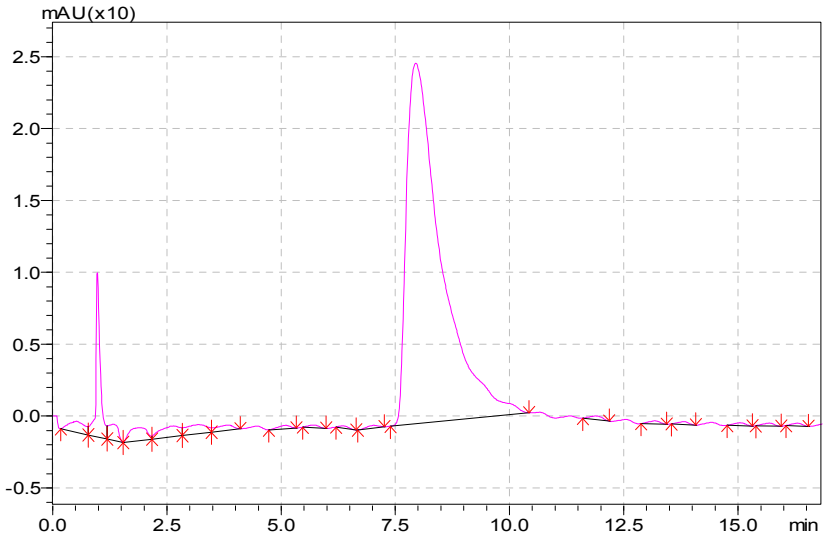


Fig 7: naphazoline hydrochloride solution 100 µg/ml HPLC- system-12.5 cm RP-18 endcapped column, mobile phase methanol : triethylamine, 20:80(v/v), pH3

The system with mobile phase consisting of methanol : triethylamine 0.05M; 30:70(v/v) was selected. The retention time of naphazoline at about 4.5min was optimal

In this system, solutions of methylparaben, benzalkonium chloride and a solution of boric acid were measured as well to investigate for co-eluting peaks.

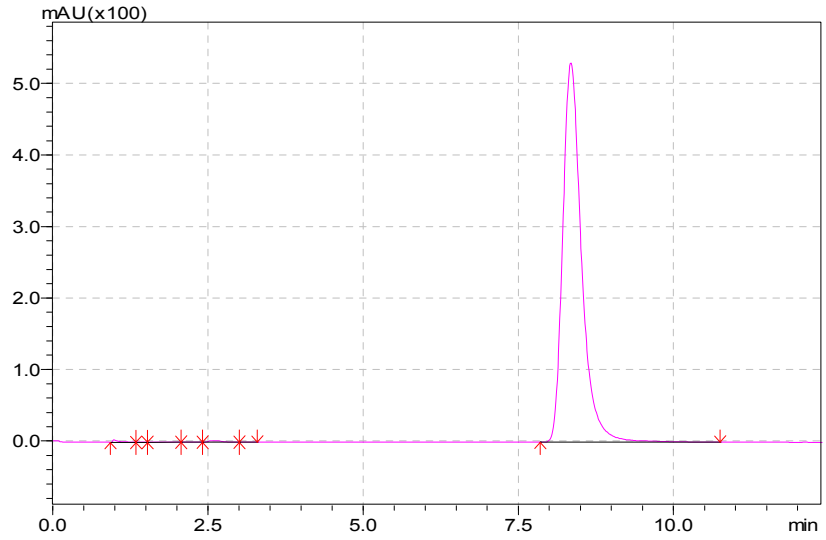


Fig 8: solution of methylparaben HPLC- system-12.5 cm RP-18 endcapped column, mobile phase- methanol : triethylamine 30:70(v/v), pH 3, Rt about 8.4 min.

The retention time of naphazoline in mobile phase contains methanol : triethylamine 30:70(v/v), was about 4.5min, there was no co-elution of naphazoline and methylparaben.

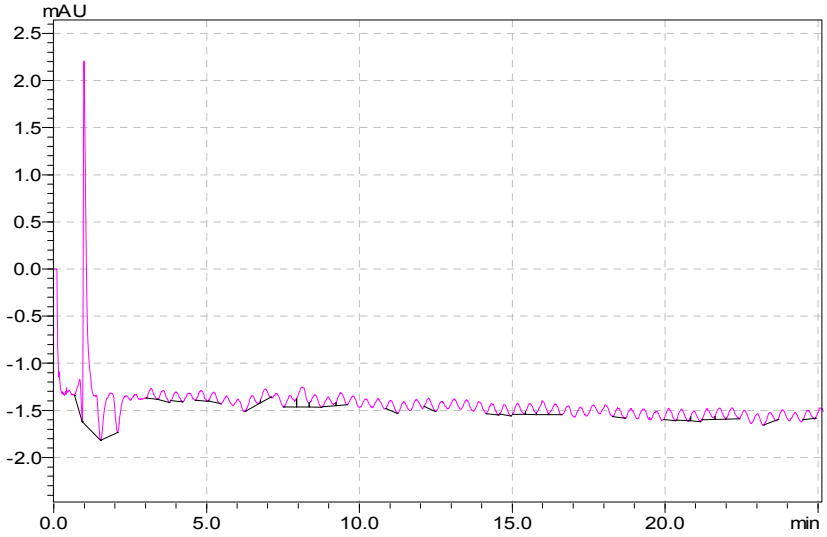


Fig 9: benzalkonium chloride HPLC- system-12.5 cm RP-18 endcapped column, mobile phase- methanol : triethylamine 30:70, pH 3.

The peak of benzalkonium chlorid was at the beginning of chromatogram. Boric acid had no signal after analysis by the proposed HPLC system. Therefore, the determination of naphazoline and methylparaben is not affected by these two ingredients.

4.2.1.2. Degradation products separation:

To determine the separation of naphazoline and its degradation products, the naphazoline drug was hydrolyzed by refluxing at a high pH and sunlight.

1 mg of naphazoline hydrochloride was diluted with HPLC water to 10 ml volumetric flask. This solution was alkalized with NH_4OH solution (to pH 9.9) and placed in the sun test machine for one hour. After the sun test, the pH had changed (resulting in a sample pH of 7.4). A solution of methylparaben was added to this sample The sample was measured by HPLC under proposed conditions.

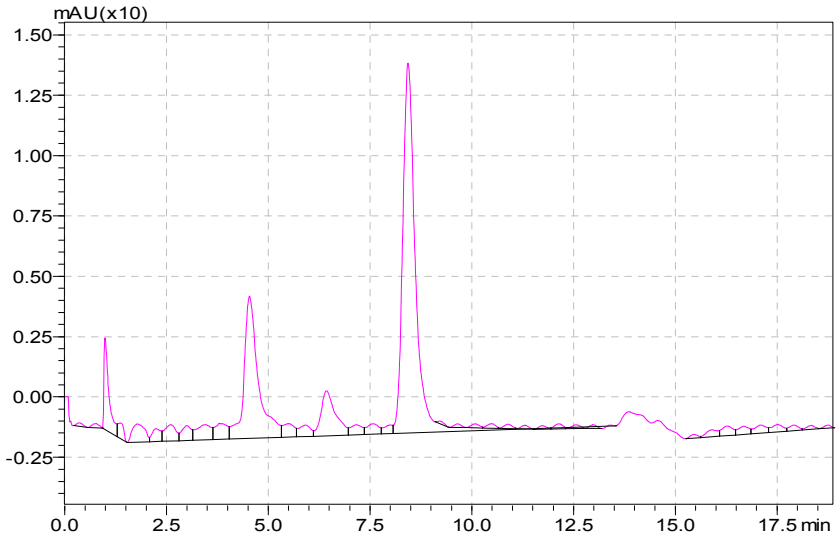


Fig 10: mixture of naphazoline hydrochloride after the sun test (with NH_4OH) and methylparaben, HPLC- system-12.5 cm RP-18 endcapped column, mobile phase- methanol : triethylamine 30:70 (v/v), pH 3. Retention times: naphazoline about 4.5 min, degradation product about 6.5 min and methylparaben 8.5 min.

The system methanol : triethylamine 0.05M 30:70 (v/v), pH 3, flow rate 0.8ml/min, 12.5 cm RP-18 endcapped column was optimal for the separation of naphazoline hydrochloride and the degradation product and methylparaben. This method was transferred to a second HPLC apparatus for the quantitative assays and validation.

4.2.2. Validation and quantitative assays:

For the quantitative assay and validation, a BDS HYPERSIL C_{18}, Dim (mm) 150x4, particle size 5µm column was used.

The eluent was modified by adding 10% of methanol to the triethylamine solution.

The sample from the sun test (see 4.2.1.2.) was measured under proposed conditions.

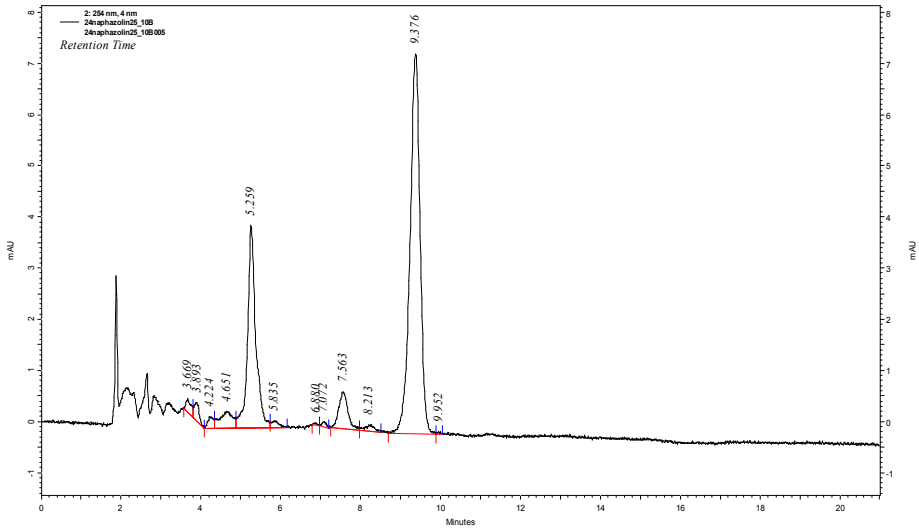


Fig 11: mixture of naphazoline hydrochloride in sun test (with NH_4OH) and methylparaben, HPLC- system-15 cm BDS C18 column, mobile phase- methanol : triethylamine 30:70 (v/v), pH 3. Retention time (Rt) 5.2-naphazoline; Rt 5.8-degradation product; Rt 7.5-degradation product; Rt 9.3-methylparaben

To decrease the retention time, the same sample (see 4.2.1.2..) was analysed with a different content of mobile phase, HPLC-15 cm BDS C18 column, mobile phase methanol : triethylamine 0.05M, 35:65 (v/v), pH 3

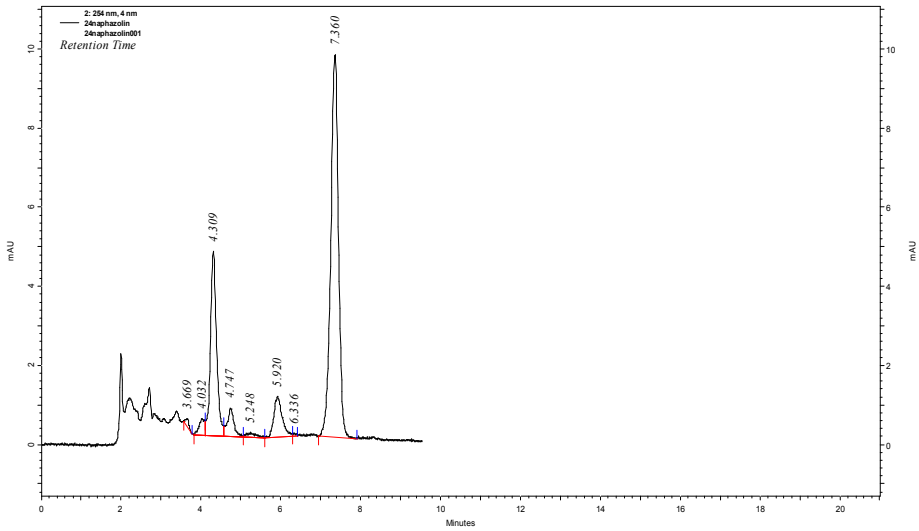


Fig 12: mixture of naphazoline hydrochloride after the sun test (with NH_4OH) and methylparaben, HPLC-15 cm BDS C18 column, mobile phase methanol : triethylamine 0.05M, 35:65 (v/v), pH 3. Rt 4.3-naphazoline, Rt 4.7 degradation product, Rt 5.9 degradation product, 7.3 methylparaben.

To increase the retention time, the same sample (see 4.2.1.2.) was analysed with a different content of mobile phase was analysed, HPLC-15 cm BDS C18 column, mobile phase methanol : triethylamine 0.05M, 25:75 (v/v), pH3

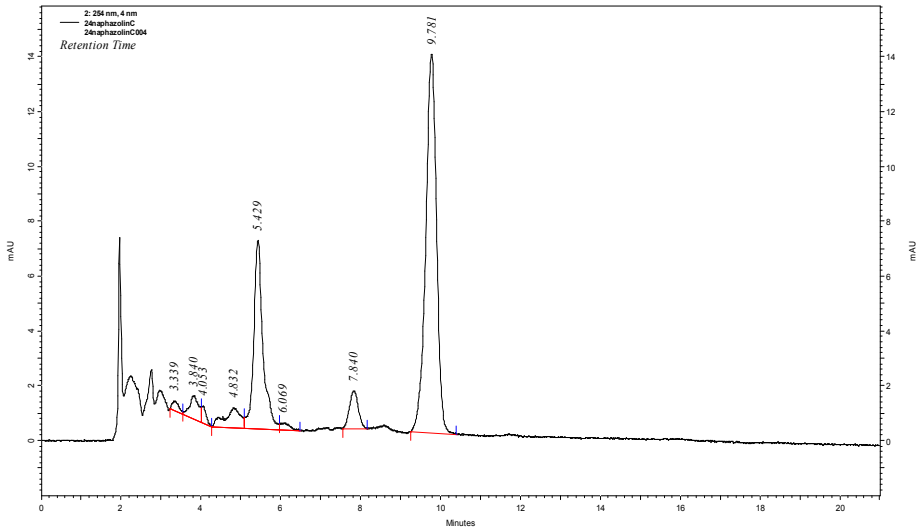


Fig 13: mixture of naphazoline hydrochloride in the sun test (with NH_4OH) and methylparaben, HPLC-15 cm BDS C18 column, mobile phase methanol : triethylamine 0.05M 25:75 (v/v), pH3. Rt 5.4 – naphazoline; Rt 6.09 – degradation product; Rt 7.8 – degradation product; Rt 9.7 methylparaben

Finally, the system with 65% of triethylamine 0.05M set to pH 3 was selected.

The pH of the mobile phase was modified to find the suitable conditions.

Tab 5: pH changes in mobile phase methanol : triethylamine, 35:65 (v/v)

system	(v/v)	pH	Rt min		
			naphazoline	degradation products	methylparaben
methanol : triethylamine 0.05M	35:65	7.5	no separated peak		
methanol : triethylamine 0.05M	35:65	5	2.7	3.02 ; 3.74	4.35
methanol : triethylamine 0.05M	35:65	3	4.3	4.77 ; 5.95	7.38

Due to technical problems with the column, the mobile phase had to be adapted and therefore, an eluent composition of methanol : triethylamine 0.05M, **30:70** (v/v), pH 3 was retested.

Since changes had been made to the mobile phase, selectivity tests were repeated with samples of pure naphazoline, methylparaben, benzalkonium chloride, boric acid, and a degradation product.

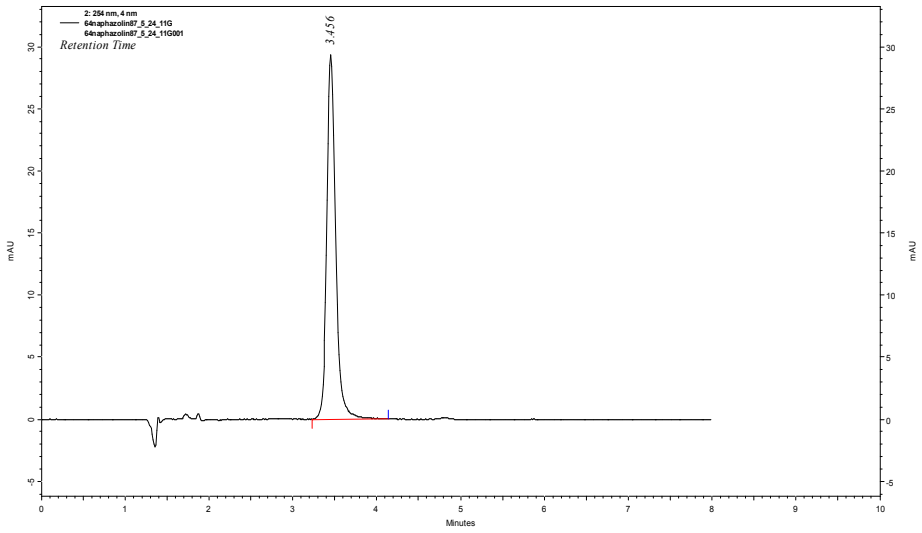


Fig 14: naphazoline hydrochloride in aqueous solution, HPLC- system-15 cm BDS C18 column, mobile phase- methanol : triethylamine 30:70 (v/v), pH 3, retention time (Rt) about 3.45

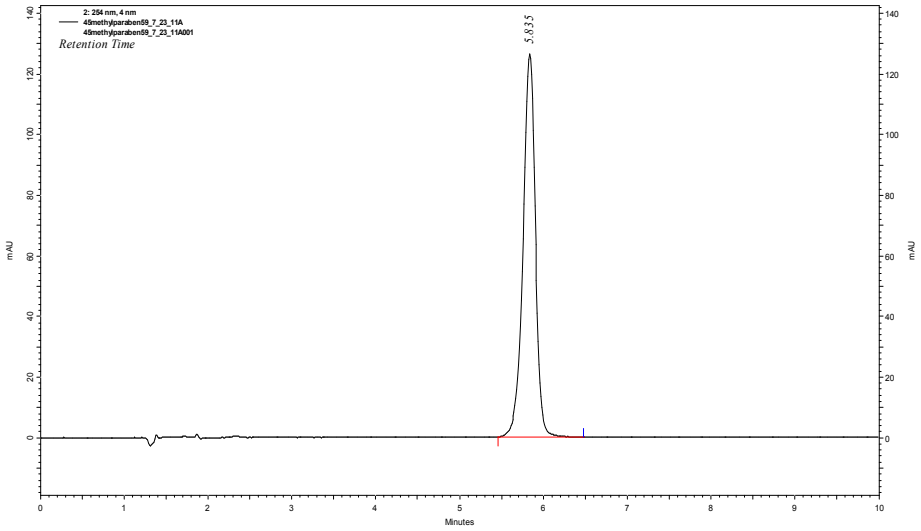


Fig 15: methylparaben in aqueous solution, HPLC- system-15 cm BDS C18 column, mobile phase- methanol : triethylamine 30:70 (v/v) , pH 3, Rt about 5.8

Analysis of boric acid solution; there was no peak on the chromatogram.

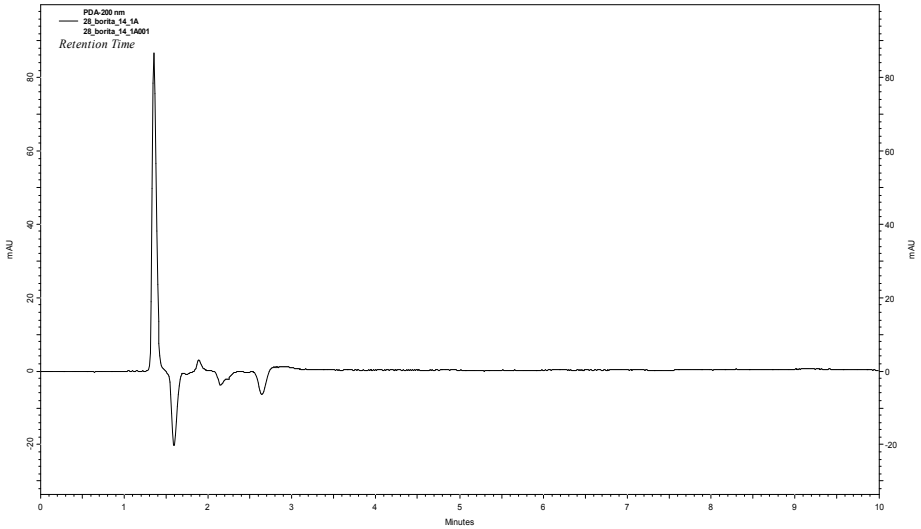


Fig 16: boric acid in aqueous solution, HPLC- system-15 cm BDS C18 column, mobile phase- methanol : triethylamine 30:70 (v/v) , pH 3

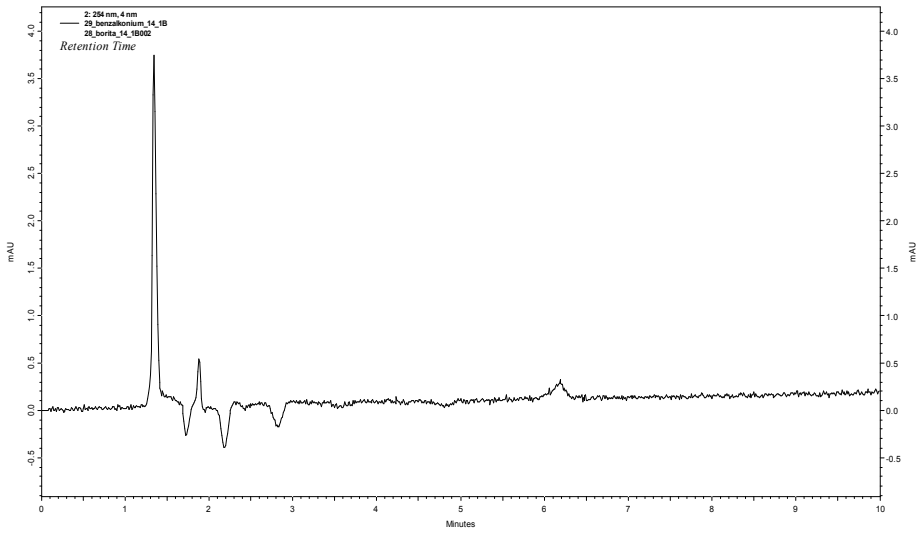


Fig 17: benzalkonium chloride, HPLC- system-15 cm BDS C18 column, mobile phase- methanol : triethylamine 30:70 (v/v), pH 3, Rt about 1.9

Analysis of sample of naphazoline hydrochloride after the sun test (with NH_4OH) with addition of methylparaben (4.2.1.2.).

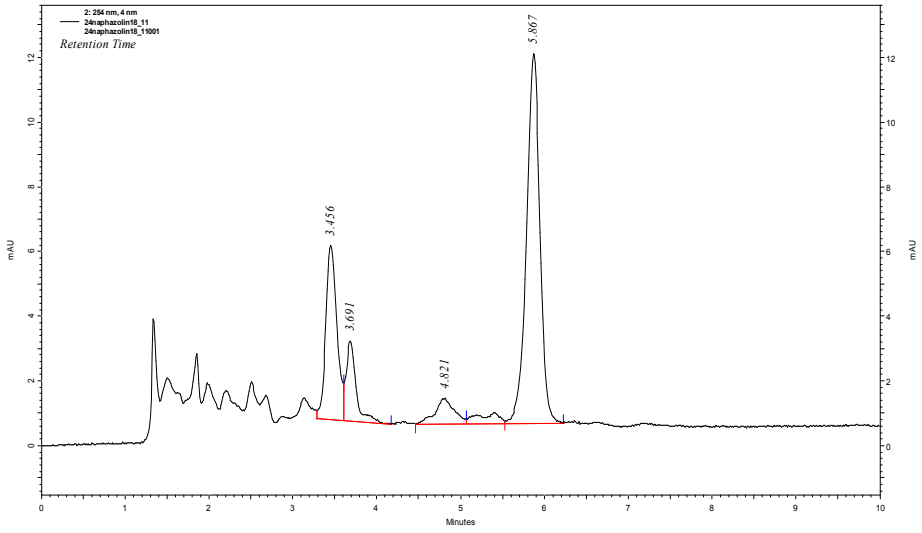


Fig 18: mixture of naphazoline hydrochloride in the sun test (with NH_4OH) and methylparaben, HPLC- system-15 cm BDS C18 column, mobile phase- methanol: triethylamin 30:70 (v/v), pH 3. Rt 3.4-naphazoline, Rt 3.6-degradation product, Rt 4.8-degradation product, Rt 5.8-methylparaben

4.2.3. Proposed HPLC system

Column:	BDS HYPERSIL C18, Dim (mm) 150x4, particle size 5 µm
Oven temperature:	30 °C
Flow rate:	1 ml/min
Injection volume:	10 µl
Detection:	λ (wave-length) = 254 nm and 280 nm
Mobile phase:	methanol : triethylamine 0.05M + 10 % methanol, 30:70 (v/v), pH 3
Time of analysis:	10 minutes

4.3. Validation

Validation is a procedure with the aim to demonstrate and document the quality of an analytical method.

4.3.1. Specificity

Specificity is the ability to accurately evaluate the sample in the presence of other components, which are expected to be present. These can be impurities, degradation products and solvent.

We would like to analyse in-house preparations of eye drops – Bor-Naphazolin 0.025% Augentropfen, Naphazolinhydrochlorid 0.01% Augentropfen (see 3.1.1.4.) and mass-manufactured eye drops Coldan Augentropfen and Okuzell Augentropfen. (see 4.1.2.)
For quantitative and qualitative analysis, the base line separation of each compound in a sample is required. Determination of retention times of each compound is significant.

Table 6: HPLC condition of the proposed method

HPLC system	
temperature	30 °C
flow rate	1 ml/min
injection volume	10 µl
eluents	70B+30A(v/v)
pH	3

A-methanol; B-triethylamine 0.05M

To determine the retention characteristics of naphazoline and methylparaben, the pure substances of naphazoline and methylparaben were measured under proposed conditions.

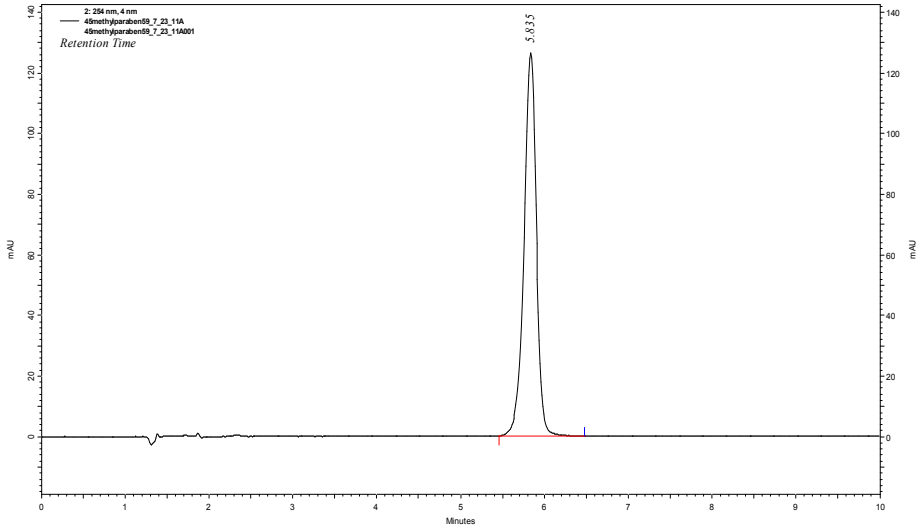


Fig 19: pure methylparaben, measured under proposed conditions (tab.6), retention time of methylparaben about 5.8min

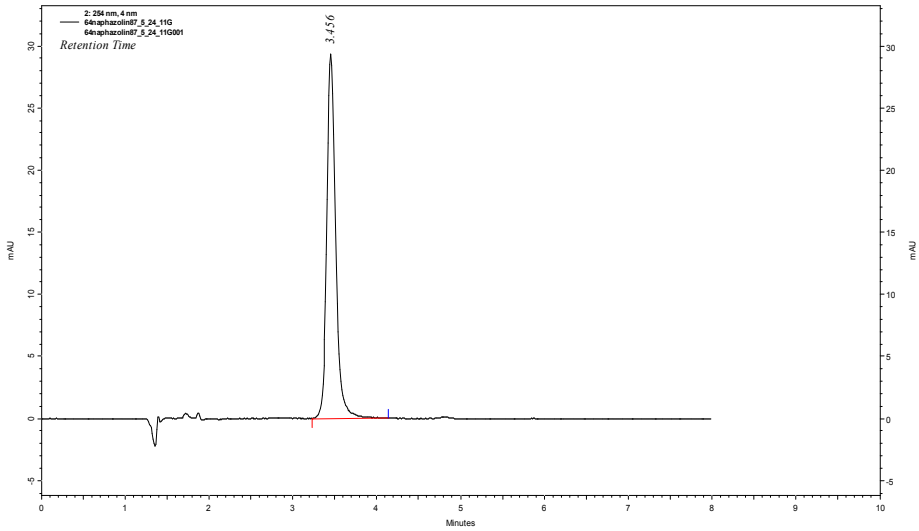


Fig 20: pure naphazoline hydrochloride, measured under proposed conditions (tab.6), retention time of naphazoline hydrochloride about 3.45

The retention time of methylparaben is significantly different from the retention time of naphazoline hydrochlorid; therefore, both compounds can be in one sample without interference. This was proved by analysing a mixture of naphazoline hydrochloride and methylparaben (Coldan drops).

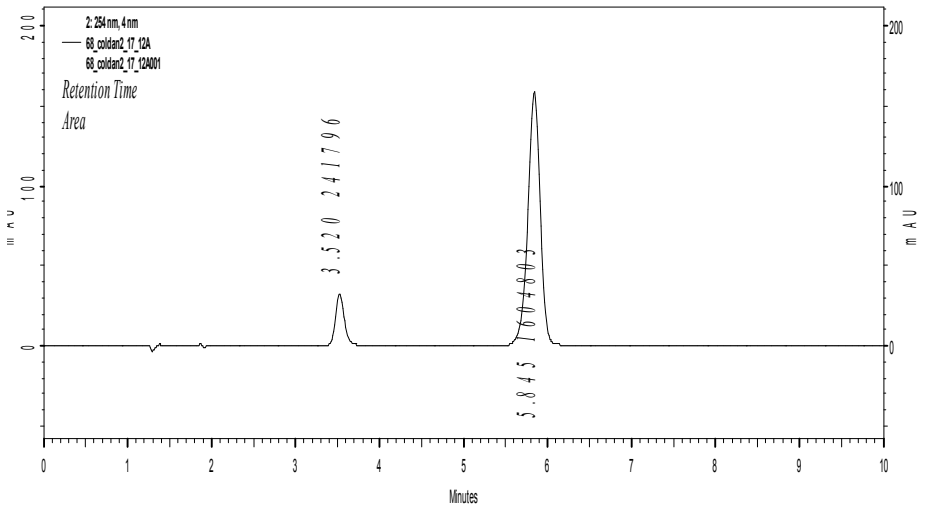


Fig 21: mixture of naphazoline hydrochloride and methylparaben measured under proposed conditions (tab.6)

Okuzell Augentropfen contains hypromellose (see 4.1.2.) and, for reasons of viscosity, was added to the in-house preparation. To determine if hypromellose is detected by HPLC or if there is some interference between naphazoline, methylparabene and hypromellose the aqueous solution of Okuzell was measured.

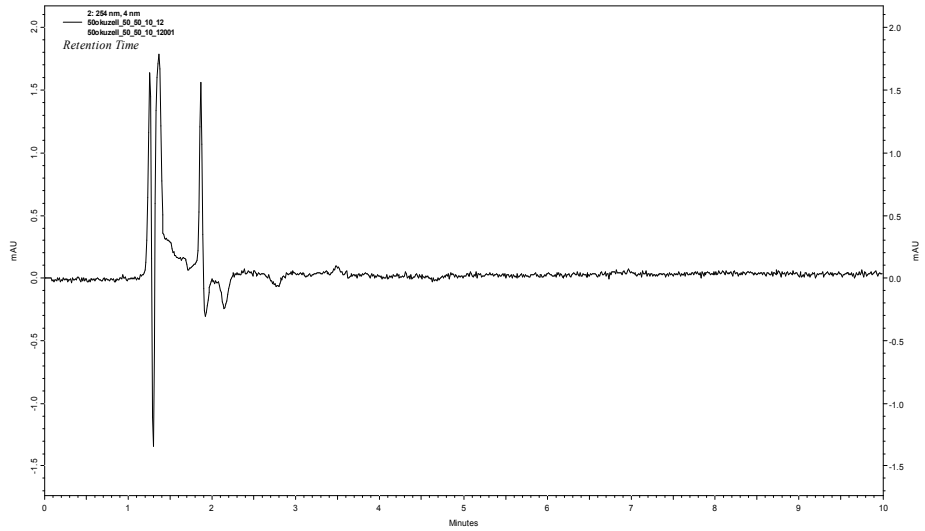


Fig 22: Okuzell Augentropfen diluted with HPLC water (50/50 v/v), measured under proposed conditions (tab.6)

There is no signal of hypromellose on the chromatogram. Naphazoline and methylparaben in the same sample with hypromellose are therefore unaffected by hypromellose.

Another analysed sample was Bor-naphazolin Augentropfen. Bor-naphazolin Augentropfen contains naphazoline hydrochloride together with other compounds (see 3.1.1.4.) which can interfere with naphazoline (mainly boric acid and benzalkonium chloride). To recognize if there is a co-elution of these compounds with naphazoline hydrochloride, boric acid and benzalkonium chloride were measured under proposed conditions.

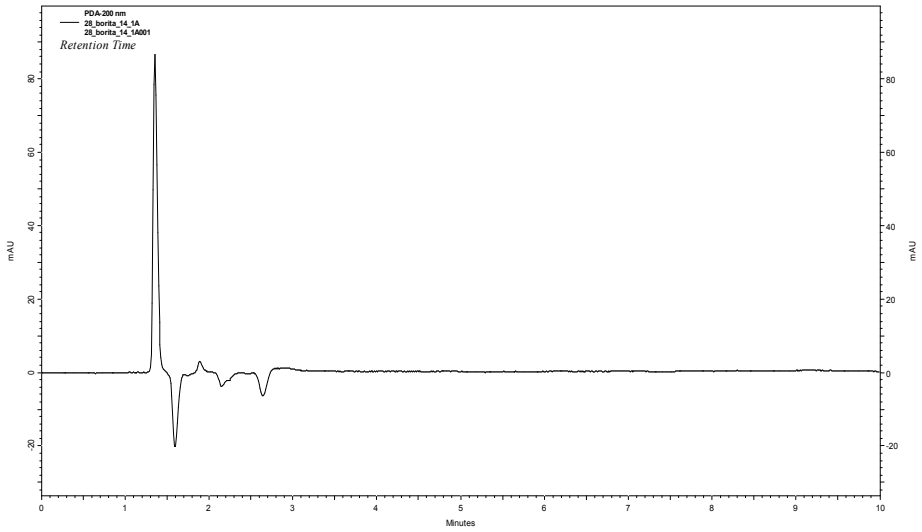


Fig 23: boric acid measured under proposed conditions (tab.6)

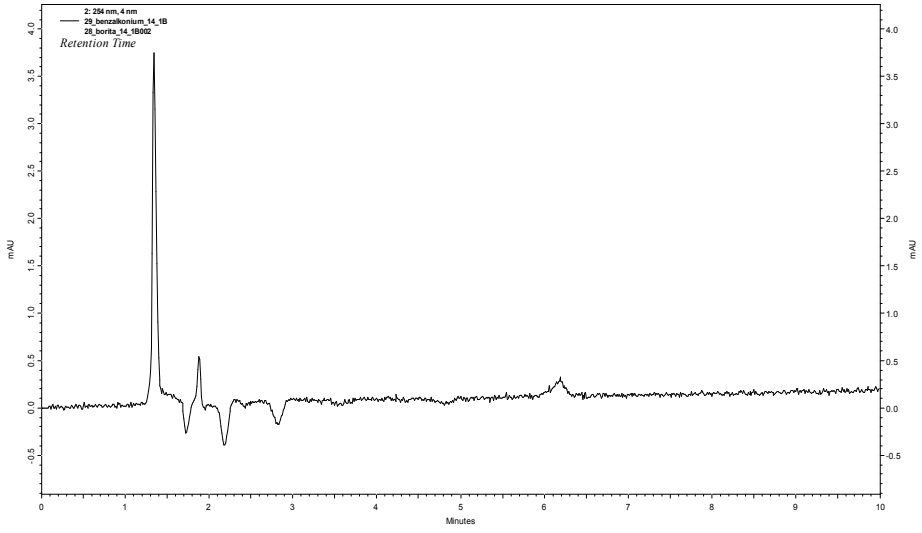


Fig 24: benzalkonium chloride measured under proposed conditions (tab.6)

There is no signal of boric acid in the chromatogram. The retention time of benzalkonium is at the beginning of the chromatogram. The components of Bor-Naphazolin Augentropfen did not interfere with naphazoline, which was proved by analysing of the solution of Bor-Naphazolin Augentropfen.

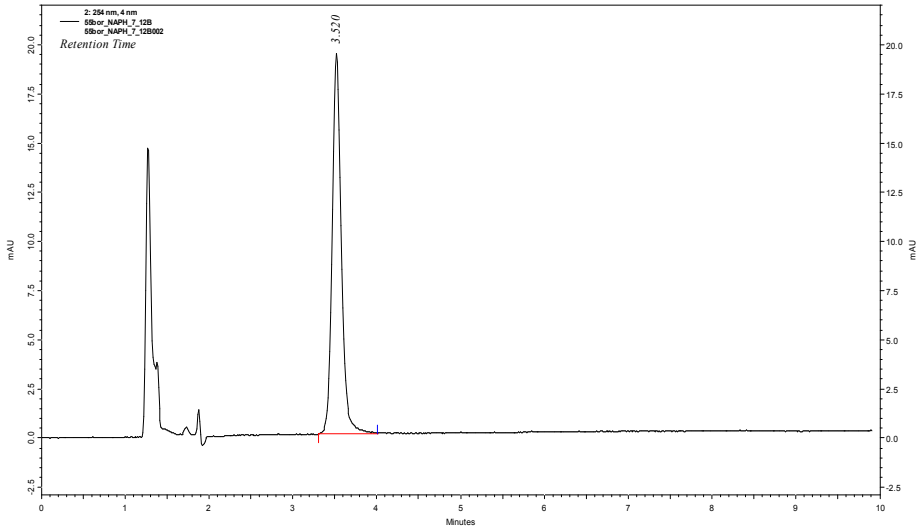


Fig 25: water solution of Bor-Naphazolin Augentropfen 25 µg/ml, measured under proposed conditions (tab.6)
Rt about 1.3-benzalkonium chloride; Rt 3.5-naphazoline

To demonstrate the separation of naphazoline and its degradation products, the naphazoline drug was hydrolyzed (see 4.4.1.2.)

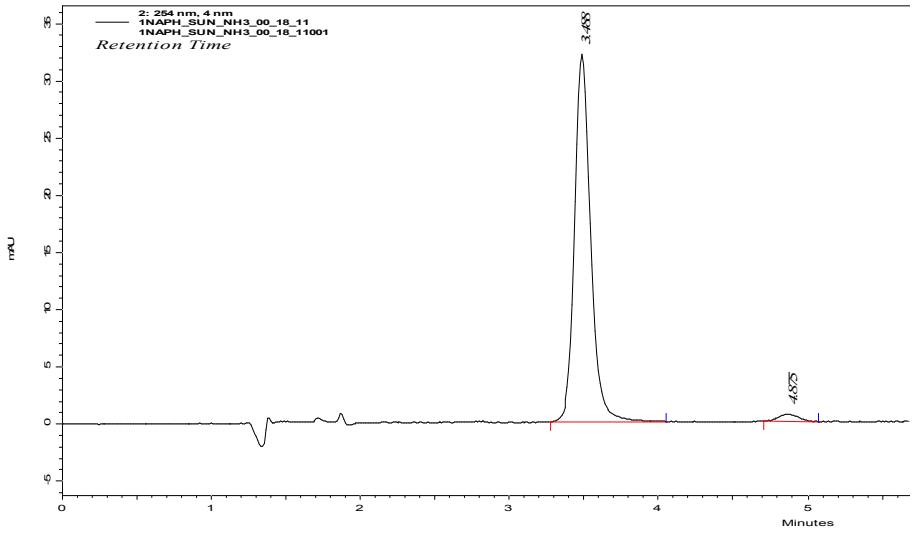


Fig 26: naphazoline hydrochloride 40µg/ml with NH_4OH (pH 10.5) at the beginning of the sun test (t=0 min), before injection to HPLC the solution was acidified to pH 5.5
Rt 3.5-naphazoline; Rt 4.8-degradation product

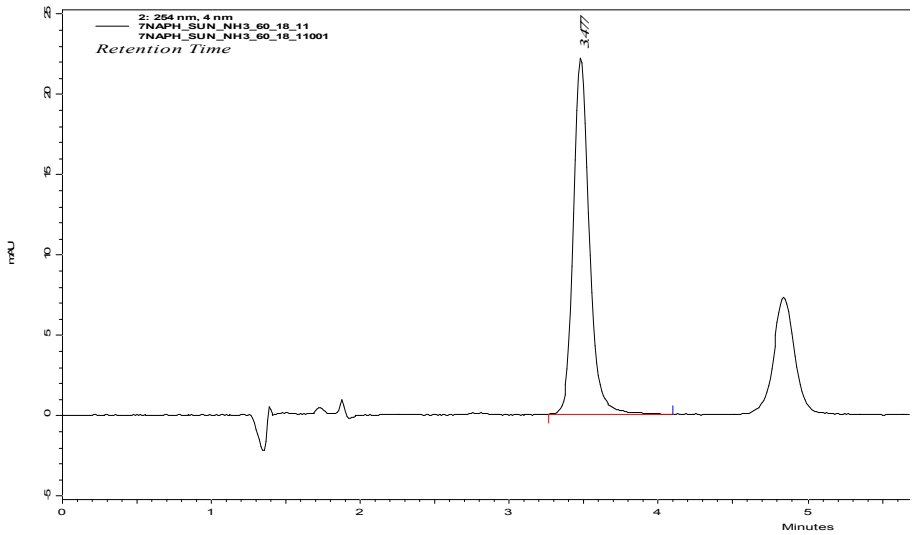


Fig 27: naphazoline hydrochloride 40µg/ml with NH_4OH (pH 10.5) in the sun test (t=60 min), before injection to HPLC the solution was acidified to pH 5.5
Rt 3.5-naphazoline; Rt 4.8-degradation product

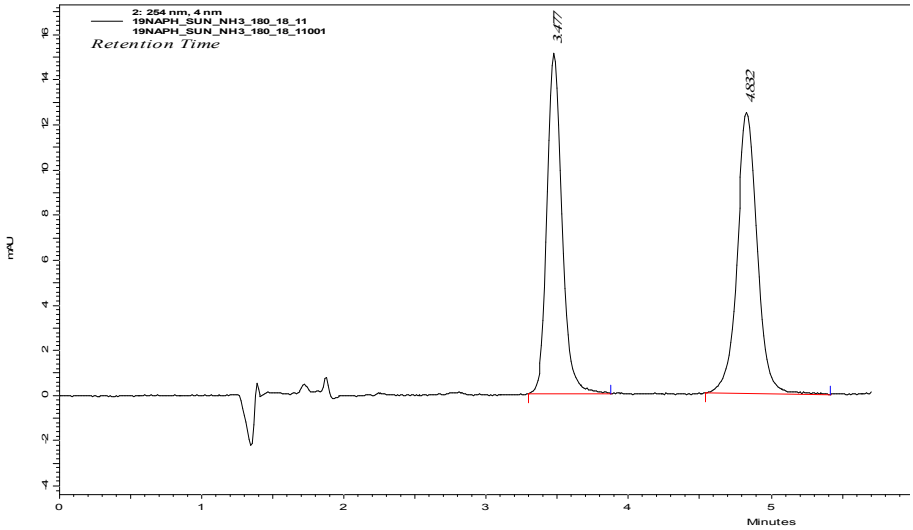


Fig 28: naphazoline hydrochloride 40µg/ml with NH_4OH (pH 10.5) in the sun test (t=180 min), before injection to HPLC the solution was acidified to pH 5.5
Rt 3.5-naphazoline; Rt 4.8-degradation product

After three hours of irradiation in the sun test, a degradation product with retention time about 4.8 min was detected. This retention time is not interfering with either compound under analysis.

4.3.2. Linearity

4.3.2.1. Naphazoline hydrochloride:

Stock solution:

0.05g were diluted in HPLC water and diluted to 100 ml volumetric flask with the same solvent. So a stock solution was made which contained 0.50 mg/ml of naphazoline hydrochloride. From this solution were prepared five concentrations - 50; 40; 35; 25; 20 µg/ml.

Concentrations:

50 µg/ml

1.00 ml of the stock solution was diluted to 10 ml volumetric flask with HPLC water.

40µg/ml

0.800 ml of the stock solution were diluted to 10 ml volumetric flask with HPLC water.

35µg/ml

0.700 ml of the stock solution were diluted to 10 ml volumetric flask with HPLC water.

25µg/ml

0.50 ml of the stock solution were diluted to 10 ml volumetric flask with HPLC water.

20µg/ml

0.800 ml of the stock solution were diluted to 20 ml volumetric flask with HPLC water.

Each solution was transferred to a vial and measured with HPLC.

Tab 7: Peak areas vs. concentration of naphazoline hydrochloride for linearity

peak area	**Concentration µg/ml**				
	50	**40**	**35**	**25**	**20**
area 1	306159	246982	214030	154856	122365
area 2	302950	245091	213734	151292	123416
area 3	302388	245812	213974	148909	122168
average	**303832.3**	**245961.67**	**213912.67**	**153074**	**122649.67**

Function: y = 6063.6x + 1724.5

Correlation coefficient: 0.9998

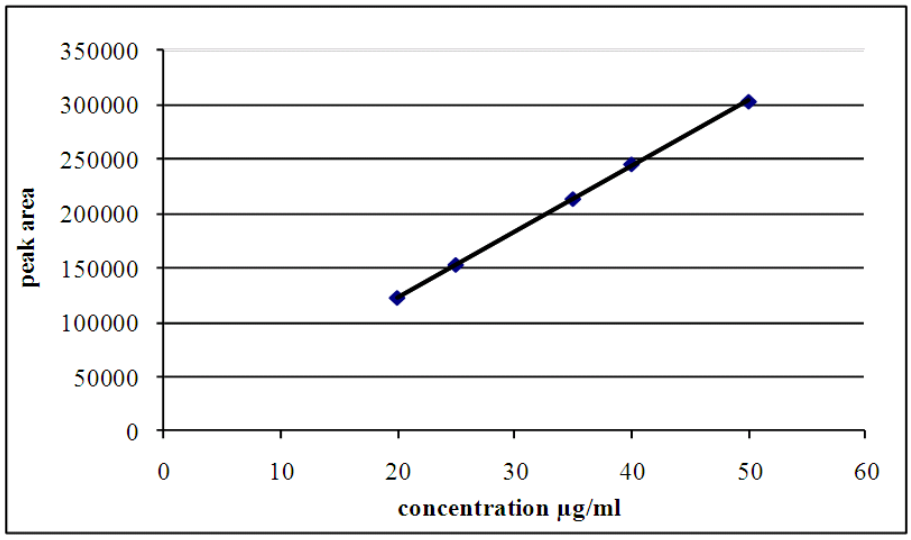


Fig 29: Naphazoline hydrochloride, calibration curve

4.3.2.2. Methylparaben:

Stock solution:

0.04g were diluted in HPLC water and diluted to 100 ml volumetric flask with the same solvent. So was made a stock solution which contained 0.40 mg/ml. From this solution were prepared five concentrations – 40; 34; 28; 20; 16µg/ml.

Concentrations:

40µg/ml

1.00 ml of the stock solution was diluted to 10 ml volumetric flask with HPLC water.

34µg/ml

0.850 ml of the stock solution were diluted to 10 ml volumetric flask with HPLC water.

28µg/ml

0.700 ml of the stock solution were diluted to 10 ml volumetric flask with HPLC water.

20 µg/ml

0.500 ml of the stock solution were diluted to 10 ml volumetric flask with HPLC water.

16µg/ml

0.800 ml of the stock solution were diluted to 20 ml volumetric flask with HPLC water.

Each solution was transferred to a vial and measured with HPLC.

Tab 8: Peak areas vs. concentration of methylparebene for linearity

peak area	Concentration µg/ml				
	40	**34**	**28**	**20**	**16**
area 1	2525088	2161194	1792360	1289126	1006435
area 2	2531571	2164335	1791588	1288995	1009587
area 3	2529727	2164371	1794982	1290784	1011652
average	**2528795.33**	**2163300**	**1792976.667**	**1289635**	**1009224.67**

Function: y = 63045x + 16736

Correlation coefficient: 0.9996

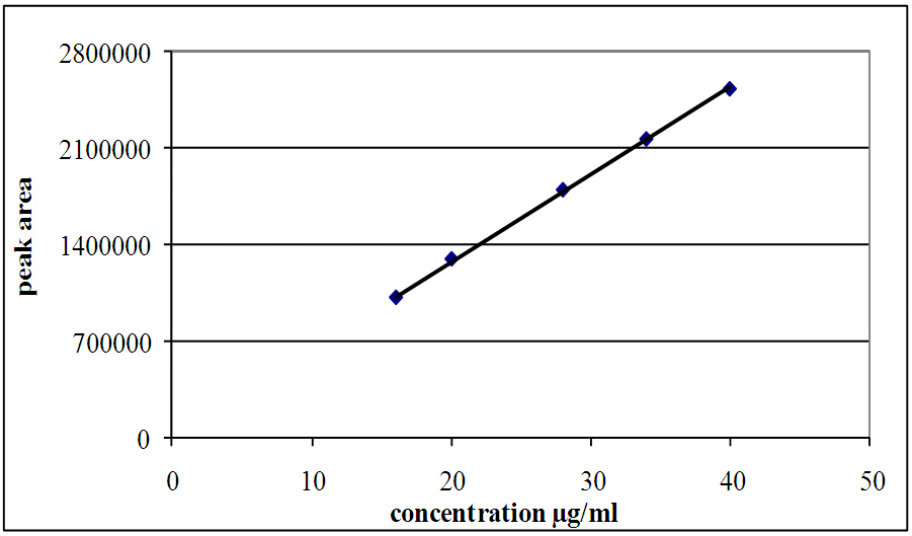


Fig 30: Methylparaben, calibration curve

4.3.3. Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantitation (LOQ) are based on determination of the signal-to-noise ratio. This ratio was performed by comparing measured signals from samples with noise that occurred on the base line and by establishing the concentration of naphazoline/methylparaben at which

naphazoline/methylparaben can be reliably detected. For the limit of detection, the signal-to-noise ratio in the chromatograms is 3:1. A typical signal-to-noise ratio for the limit of quantitation is 10:1.

For ratio detection solutions of the following concentrations were prepared and measured:

4.3.3.1. Naphazoline hydrochloride

0.800 ml of the stock solution (0.50 mg/ml) (4.3.2.1.) were diluted to 20 ml volumetric flask with HPLC water – 20µg/ml (solution 1)
2.50 ml of solution 1 were diluted to 10 ml volumetric flask with HPLC water – 5µg/ml (solution 2)
1 ml of solution 1 was diluted to 10 ml volumetric flask with HPLC water – 2µg/ml (solution 3)
0.50 ml of solution 1 were diluted to 10 ml volumetric flask with HPLC water – 1µg/ml
1 ml of solution 2 was diluted to 10 ml volumetric flask with HPLC water – 0.5 µg/ml
0.5 ml of solution 2 were diluted to 10 ml volumetric flask with HPLC water – 0.25µg/ml
1 ml of solution 3 was diluted to 10 ml volumetric flask with HPLC water – 0.2µg/ml
0.5 ml of solution 3 were diluted to 10 ml volumetric flask with HPLC water – 0.1µg/ml

The LOD for naphazoline is 0.2µg/ml and the LOQ is 1µg/ml.

4.3.3.2. Methylparaben

0.800 ml of the stock solution (0.40 mg/ml) (4.3.2.2.) were diluted to 20 ml volumetric flask with HPLC water – 16 µg/ml – (solution 1)
2.50 ml of solution 1 were diluted to 10 ml volumetric flask with HPLC water – 4 µg/ml (solution 2)
1.00 ml of solution 1 was diluted to 10 ml volumetric flask with HPLC water – 1.6 µg/ml (solution 3)
0.50 ml of solution 1 were diluted to 10 ml volumetric flask with HPLC water – 0.8 µg/ml
1 ml of solution 2 was diluted to 10 ml volumetric flask with HPLC water – 0.4 µg/ml (solution 4)
0.5 ml of solution 2 were diluted to 10 ml volumetric flask with HPLC water – 0.2 µg/ml

1 ml of solution 3 was diluted to 10 ml volumetric flask with HPLC water – 0.16 µg/ml (solution 5)
0.5 ml of solution 3 were diluted to 10 ml volumetric flask with HPLC water – 0.08 µg/ml
1 ml of solution 4 was diluted to 10 ml volumetric flask with HPLC water – 0.04 µg/ml
0.5 ml of solution 4 were diluted to 10 ml volumetric flask with HPLC water – 0.02 µg/ml
1 ml of solution 5 was diluted to 10 ml volumetric flask with HPLC water – 0.016µg/ml

The LOD for methylparaben is 0.016µg/ml and the LOQ is 0.08 µg/ml

4.3.4. Precision

4.3.4.1. Naphazoline hydrochloride

To determine the precision, solutions with concentrations 50; 40; 35; 25; 20 µg/ml were prepared. Each concentration was injected three times in one day and again on the second and on the third day. Based on peak areas, were calculated average, standard deviation (s) and relative standard deviation (s %)

Tab 9: the precision of HPLC in a solution with concentration 50µg/ml of naphazoline hydrochloride:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	peak area
1	306159	303413	304200
2	302950	303495	303784
3	302388	304316	303795
average	303832.33	303741.33	303926.,33
s	2034.45185	499.361926	237.066095
s%	**0.67**	**0.16**	**0.08**

Tab 10: the precision of HPLC in a solution with concentration 40 μg/ml of naphazoline hydrochloride:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	peak area
1	246982	245147	244251
2	245091	244271	244759
3	245812	245485	244305
average	245961.67	244967.67	244505
s	954.34288	626.553536	279.014934
s%	**0.39**	**0.25**	**0.11**

Tab 11: the precision of HPLC in a solution with concentration 35 μg/ml of naphazoline hydrochloride:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	peak area
1	214030	213873	214276
2	213734	213516	212824
3	213974	214164	213486
average	213912.67	213851	213528.67
s	209.303607	324.559702	726.939704
s%	**0.01**	**0.15**	**0.34**

Tab 12: the precision of HPLC in a solution with concentration 25 μg/ml of naphazoline hydrochloride:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	peak area
1	154856	152161	152185
2	151292	151765	152505
3	148909	152154	151225
average	151685.67	152026.67	151971.67
s	2992.98051	226.637008	666.13312
s%	**1.97**	**0.15**	**0.44**

Tab 13: the precision of HPLC in a solution with concentration 20 µg/ml of naphazoline hydrochloride:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	peak area
1	122365	122579	121644
2	123416	122223	123372
3	122168	121718	125198
average	122649.67	122173.33	122411.5
s	670.933926	432.643425	1777.22518
s%	**0.54**	**0.35**	**1.45**

Tab 14: intermediate precision of the HPLC system (n=9):

	Concentration (µg/ml)				
	20	25	35	40	50
average	122411.5	151894.67	213764.11	245144.78	303833.33
s (n=9)	1112.70078	1245.4006	443.307017	891.007311	1057.14569
s%	**0.91**	**0.82**	**0.21**	**0.35**	**0.35**

4.3.4.2. Methylparaben

To determine the precision, solutions with concentrations 40; 34; 28; 20; 16 µg/ml were prepared. Each concentration was injected three times in one day and again on the second and the third day. From peak areas were calculated average, standard deviation (s) and relative standard deviation (s %)

Tab 15: the precision of HPLC in a solution with concentration 40 µg/ml of methylparaben:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	peak area
1	2525088	2531140	2520955
2	2531571	2532058	2525745
3	2529727	2531434	2525091
average	2528795.33	2531544	2523930.33
s	3340.40781	468.781399	2597.38048
s%	**0.13**	**0.02**	**0.10**

Tab 16: the precision of HPLC in a solution with concentration 34 µg/ml of methylparaben:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	peak area
1	2161194	2169086	2157985
2	2164335	2165898	2161258
3	2164371	2166938	2161711
average	2163300	2167307.33	2160318
s	1823.93832	1625.77407	2033.09346
s%	**0.08**	**0.08**	**0.09**

Tab 17: the precision of HPLC in a solution with concentration 28 µg/ml of methylparaben:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	paek area
1	1792360	1789707	1776673
2	1791588	1792475	1777748
3	1794982	1793714	1777787
average	1792976.67	1791965.33	1777402.67
s	1779.04956	2051.54389	632.210671
s%	**0.10**	**0.11**	**0.04**

Tab 18: the precision of HPLC in a solution with concentration 20 µg/ml of methylparaben:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	peak area
1	1289126	1288555	1262500
2	1288995	1292053	1262825
3	1290784	1290137	1260378
average	1289635	1290248.33	1261901
s	997.216626	1751.6556	1328.92927
s%	**0.08**	**0.14**	**0.11**

Tab 19: the precision of HPLC in a solution with concentration 16 µg/ml of methylparaben:

	day 1	**day 2**	**day 3**
injection	peak area	peak area	peak area
1	1006435	1011908	1005842
2	1009587	1013363	1005182
3	1011652	1009069	1005518
average	1009224.67	1011446.67	1005514
s	2627.30591	2183.85676	330.018181
s%	**0.26**	**0.22**	**0.03**

Tab 20: intermediate precision of the HPLC system (n=9):

	Concentration (µg/ml)				
	16	20	28	34	40
average	1008728.44	1290268.33	1787448.22	2163641.78	2528089.89
s (n=9)	3111.76442	14074.7106	7674.55708	3427.98315	3959.80184
s%	**0.31**	**1.1**	**0.43**	**0.16**	**0.16**

4.3.5. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and it provides an indication of its reliability during normal usage.

Method parameters:

Tab 21: HPLC conditions in the method

HPLC system	
temperature	30 °C
flow rate	1 ml/min
injection volume	10 µl
eluents	70B+30A(v/v)
pH	3

A-methanol; B-triethylamine 0.05M

Tab 22: naphazoline hydrochloride 40µg/ml measured by the proposed method

results			
Rt	peak area	conc µg/ml	content %
3.509	242291	39.67	99.18
3.509	241460	39.53	98.83
3.509	241779	39.59	98.98

Parameters of this method were changed to indicate the reliability of this method during routine application.

4.3.5.1. Changed flow rate:

Tab 23: changed flow rate from 1 to 1.1ml/min

changed flow rate from 1 to 1.1 ml/min			
Rt	peak area	conc µg/ml	content %
3.136	221706	36.28	90.70
3.125	221475	36.24	90.60
3.125	220230	36.04	90.10

Tab 24: changed flow rate from 1 to 1.2ml/min

changed flow rate from 1 to 1.2 ml/min			
Rt	peak area	conc µg/ml	content %
2.88	203208	33.23	83.08
2.88	202702	33.14	82.85
2.88	202967	33.19	82.98

Tab 25: changed flow rate from 1 to 0.9ml/min

changed flow rate from 1 to 0.9 ml/min			
Rt	peak area	conc µg/ml	content %
3.88	266926	43.08	107.70
3.89	266915	43.73	109.30
3.89	266697	43.70	109.25

4.3.5.2. Changed oven temperature:

Tab 26: changed oven temperature from 30 to 32°C

changed oven temperature from 30 to 32 °C			
Rt	peak area	conc µg/ml	content %
3.41	241420	39.53	98.83
3.41	242815	39.76	99.40
3.41	242701	39.74	99.35

Tab 27: changed oven temperature from 30 to 28°C

changed oven temperature from 30 to 28 °C			
Rt	peak area	conc µg/ml	content %
3.59	242571	39.72	99.30
3.59	242404	39.69	99.22
3.59	244018	39.96	99.90

4.3.5.3. Changed eluent:

Tab 28: changed eluent from 70 to 68%

changed eluent from 70 to 68% B			
Rt	peak area	conc µg/ml	content %
3.157	241859	39.60	99.00
3.168	243335	39.85	99.62
3.168	243169	39.82	99.55

Tab 29: changed eluent from 70 to 72%

changed eluent from 70 to 72% B			
Rt	peak area	conc µg/ml	content %
3.89	241806	39.59	98.97
3.88	242182	39.66	99.15
3.88	241757	39.59	98.97

4.3.5.4. Changed pH

Tab 30: changed pH from 3 to 2.8

changed pH from 3 to 2.8			
Rt	peak area	conc µg/ml	content %
3.509	242926	39.78	99.45
3.509	242551	39.72	99.30
3.509	242262	39.67	99.18

Tab 31: changed pH from 3 to 3.4

changed pH from 3 to 3.4			
Rt	peak area	conc µg/ml	content %
3.467	241748	39.58	98.95
3.467	241237	39.50	98.75
3.467	240889	39.44	98.60

The capacity of the proposed system to remain unaffected by small variations in method parameters was proved (except changes in flow rate).

4.4. Stability tests

In this part, photo- and thermo-stability of naphazoline were investigated. For this purpose, the sun test with naphazoline hydrochloride solution and with the Coldan + Okuzell mixture was performed. For research on the stability of naphazoline in high temperatures, the oven test with naphazoline hydrochloride was realised.

4.4.1. SUN TEST-Naphazoline

In this test, naphazoline hydrochloride first in a water solution, then in an alkalized solution and finally in an acid solution was given into the sun test. The test lasts 3 hours. Every ten minutes, samples were taken and analysed by HPLC. The initial concentration of these solutions was 40µg/ml.

For detection, if there is no degradation in the real samples of drops, these drops with real concentration and with real volume were prepared (5 ml mixture of Coldan and Okuzell Augentropfen, see 3.1.1.4.1.). A water solution of naphazoline in the same concentration (0.1mg/ml) and the same volume was prepared to compare if possible degradation depends on the composition of the drops or only on the content of naphazoline. The samples were taken at 0; 12; 24; 36; 48; 60 minutes.

The sun test has time factor: 15, which means 1 min in the sun test corresponds to 15 min of bright sunlight.

4.4.1.1. Naphazoline hydrochloride in water solution:

For the sun test, a solution with concentration 40µg/ml was prepared - 8.00 ml of the stock solution (0.50 mg/ml, 4.3.2.1.) were diluted with HPLC water to 100.0 ml volumetric flask. The flask was carefully closed and placed in the sun test machine. The samples were taken every ten minutes for three hours. These samples were immediately analysed by HPLC.

The concentration in µg/ml and the percentage decrease of naphazoline concentrations from the start time were calculated (see Tab 32). The relation between % from t=0 and time is demonstrated in figure 31.

Tab 32: naphazoline hydrochloride 40µg/ml in the sun test:

time (min)	peak area	conc µg/ml	% from t=0
0	241165	39.49	**100.0**
10	242180	39.66	**100.4**
20	241315	39.51	**100.1**
30	241801	39.59	**100.3**
40	243868	39.93	**101.1**
50	240908	39.44	**99.9**
60	242351	39.68	**100.5**
70	241138	39.48	**99.9**
80	240457	39.37	**99.7**
90	240262	39.33	**99.6**
100	241112	39.48	**99.9**
110	240604	39.36	**99.7**
120	241040	39.47	**99.9**
130	240737	39.42	**99.8**
140	239330	39.19	**99.2**
150	240282	39.34	**99.6**
160	239730	39.25	**99.4**
170	238211	39.00	**98.7**
180	239314	39.18	**99.2**

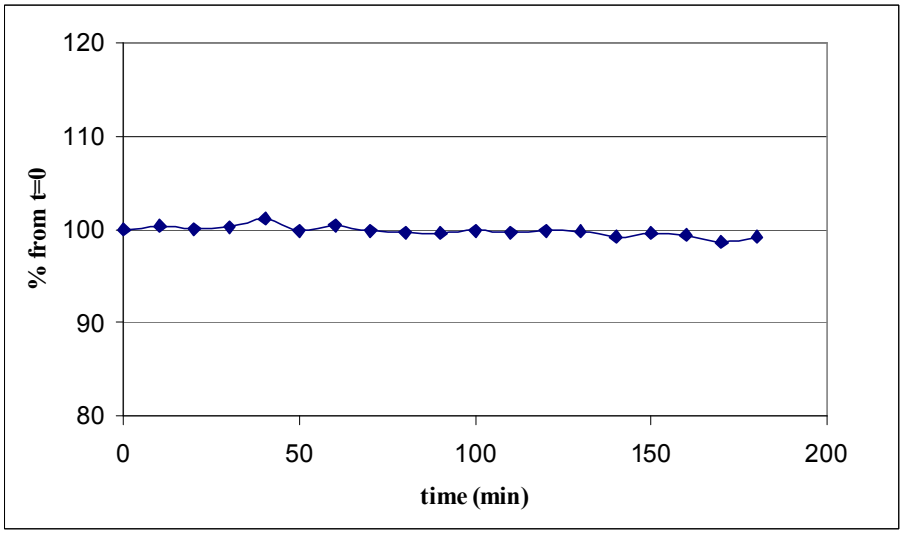


Fig 31: naphazoline hydrochloride 40 µg/ml in the sun test

4.4.1.2. Naphazoline hydrochloride in alkalized solution:

The solution of naphazoline hydrochloride was prepared: 8 ml of the stock solution (0.50 mg/ml, 4.3.2.1.) were diluted with HPLC water. The pH of this solution was changed to 10.52 with a solution of NH_4OH. This solution was diluted to 50 ml volumetric flask with HPLC water. This volumetric flask was carefully closed and placed in the sun test machine for three hours. The test lasted three hours. Every ten minutes, samples of 800 µl were taken and mixed with 800 µl buffer pH 3 (resulting in a sample pH of 5.5). The diluted samples were transferred to a vial and analysed by HPLC.
The concentration in µg/ml and the percentage decrease of naphazoline concentrations from the start time were calculated (see tab 33). The relation between % from t=0 and time is demonstrated in figure 32.

Tab 33: naphazoline hydrochloride 40µg/ml with NH_4OH in sun test:

time (min)	peak area	conc µg/ml	% from t=0
0	234995	38.47	**100.0**
10	233459	38.22	**99.3**
20	225103	36.84	**95.8**
30	213595	34.94	**90.8**
40	195027	31.88	**82.9**
50	173660	28.36	**73.7**
60	161728	26.39	**68.6**
70	140940	22.96	**59.7**
80	136280	22.19	**57.7**
90	133909	21.80	**56.7**
100	129048	20.99	**54.6**
110	123034	20.01	**52.0**
120	117717	19.13	**49.7**
130	116671	18.96	**49.3**
140	113197	18.38	**47.8**
150	111942	18.18	**47.3**
160	112646	18.29	**47.5**
170	106762	17.32	**45.0**
180	108357	17.62	**45.8**

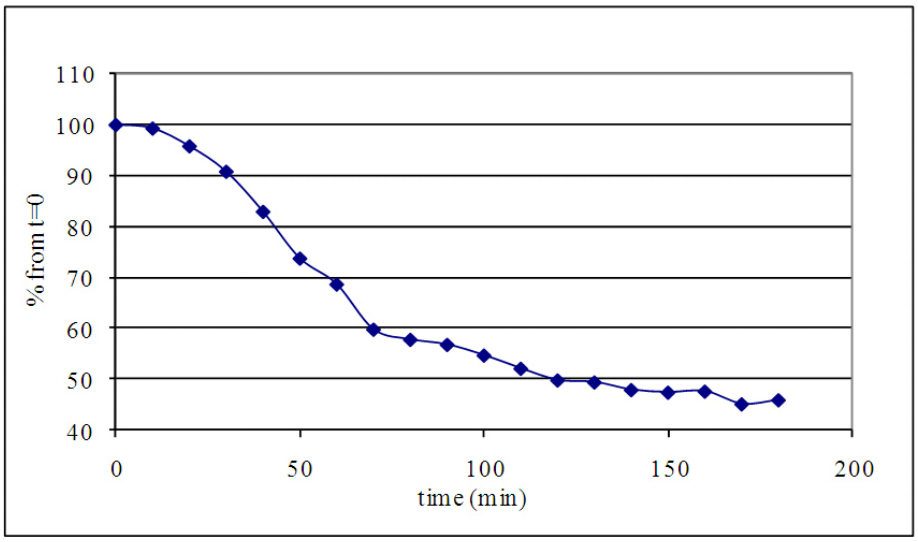


Fig 32: naphazoline hydrochloride 40µg/ml with NH_4OH in the sun test

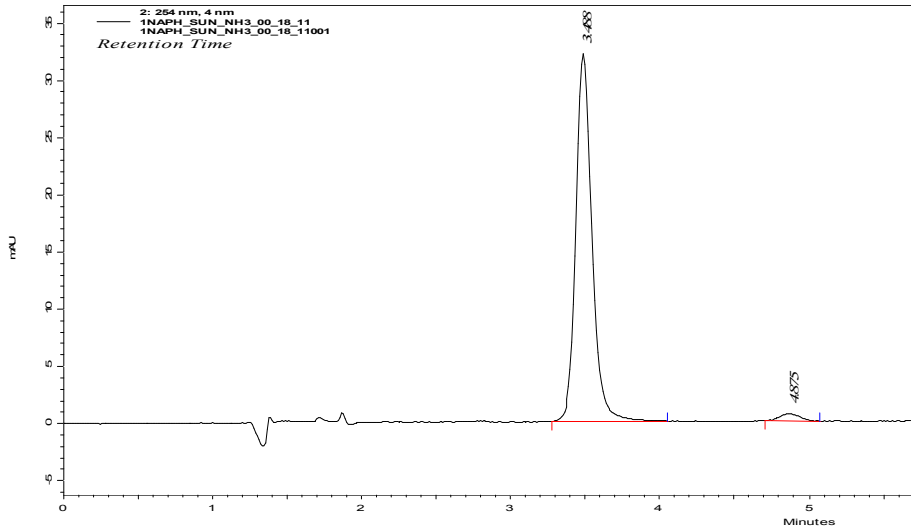


Fig 33: naphazoline hydrochloride 40µg/ml with NH_4OH in the sun test, t=0

4.4.1.3. Naphazoline hydrochloride in acid solution:

Naphazoline should be relatively stable in an acidic solution. To prove this fact, this solution was prepared: 8 ml of the stock solution (0.5mg/ml; 4.3.2.1.) were diluted to 100 ml volumetric flask, and the pH of this solution was acidified to pH 3 with the buffer solution (3.4.). This flask was closed and placed in the sun test machine. Samples were taken every ten minutes for three hours. These samples were immediately analysed by HPLC.

The concentration in µg/ml and the percentage decrease of naphazoline concentrations from the start time were calculated (see tab 34). The relation between % from t=0 and time is demonstrated in figure 34.

Tab 34: naphazoline hydrochloride 40µg/ml pH 3 in the sun test

time	peak area	conc µg/ml	% from t=0
0	239572	39.22	**100.0**
10	239856	39.27	**100.1**
20	238947	39.12	**99.7**
30	239477	39.20	**99.9**
40	239534	39.22	**100.0**
50	239302	39.18	**99.9**
60	240004	39.29	**100.2**
70	240199	39.32	**100.2**
80	240808	39.43	**100.5**
90	239968	39.29	**100.2**
100	239933	39.28	**100.1**
110	238109	38.98	**99.4**
120	238979	39.13	**99.8**
130	240654	39.04	**99.5**
140	240223	39.33	**100.3**
150	239136	39.15	**99.8**
160	239319	39.18	**99.8**
170	239020	39.14	**99.8**
180	239958	39.29	**100.2**

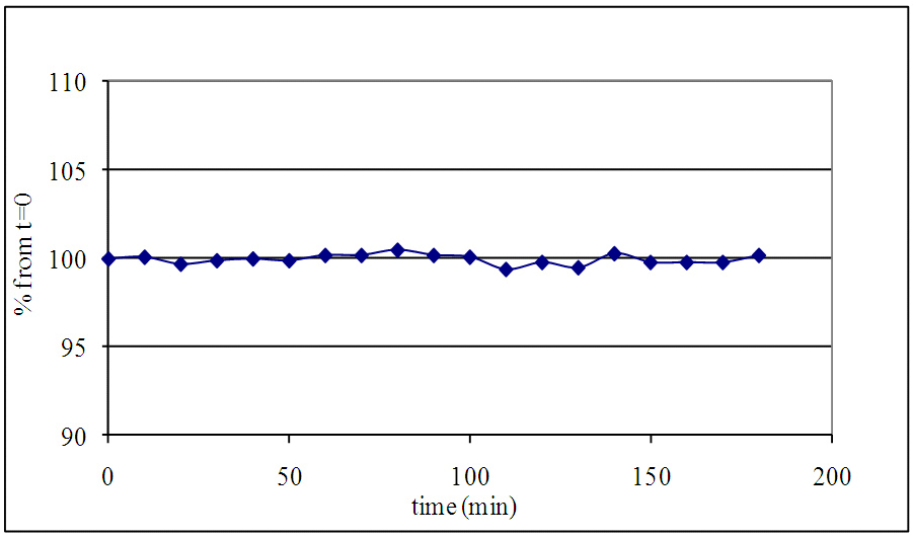


Fig 34: naphazoline hydrochloride pH 3 in the sun test

4.4.2. SUN TEST-Drops

4.4.2.1. Mixture of Coldan and Okuzell Augentropfen 0.5mg/5ml:

The real in-house preparation contains 0.5mg/5ml (0.1mg/ml) of naphazoline hydrochloride. Therefore, the same concentration was prepared: 0.5 ml of Coldan Augentropfen were diluted with Okuzell Augentropfen to 5 ml volumetric flask. This volumetric flask was carefully closed and placed in the sun test for one hour. Every 12 minutes, samples were taken: 400 µl of the solution from the flask were diluted with 600 µl of HPLC water (concentration in a vial 40µg/ml). These samples were analysed by HPLC.

The concentration in µg/ml and the percentage decrease of naphazoline concentrations from the start time were calculated (see tab 35). The relation between % from t=0 and time is demonstrated in figure 35.

Tab 35: mixture of Coldan Augentropfen and Okuzell Augentropfen, 40µg/ml in the sun test

time min	peak area	conc µg/ml	% from t=0
0	246093	40.30	100.0
12	241058	39.47	97.9
24	245106	40.14	99.6
36	242308	39.68	98.5
48	243302	39.84	98.8
60	244687	40.07	99.4

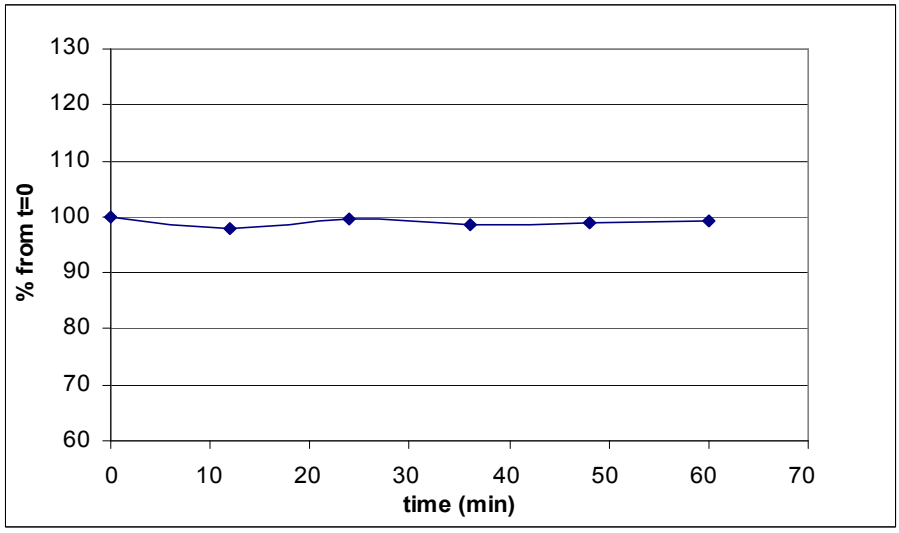


Fig 35: mixture of Coldan Augentropfen and Okuzell Augentropfen, 40µg/ml in the sun test

4.4.2.2. Naphazoline hydrochloride solution:

The same concentration (0.5mg/5ml) of naphazoline hydrochloride was prepared: 1.0 ml of the stock solution (0.50 mg/ml, see 4.3.2.1.) was diluted with HPLC water to 5.0 ml volumetric flask. This volumetric flask was carefully closed and placed in the sun test for one hour. Every 12 minutes, samples were taken: 400 µl of the solution in the flask were diluted with 600 µl of HPLC water (concentration in a vial 40µg/ml). This sample was analysed by HPLC.

The concentration in µg/ml and the percentage decrease of naphazoline concentrations from the start time were calculated (see tab 36). The relation between % from t=0 and time is demonstrated in figure 36.

Tab 36: solution of naphazoline hydrochloride 40µg/ml in the sun test

time min	peak area	conc µg/ml	% from t=0
0	245772	40.25	100.0
12	245349	40.18	99.8
24	245099	40.14	99.7
36	245776	40.25	100.0
48	245555	40.20	99.2
60	245187	40.15	99.7

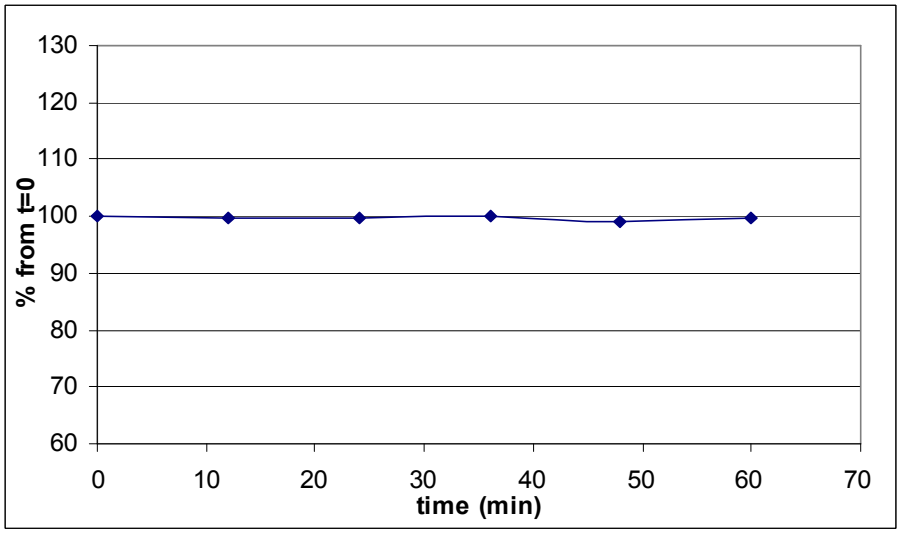


Fig 36: solution of naphazoline hydrochloride 40µg/ml in the sun test

4.4.3. Oven test

The alkaline solution of naphazoline hydrochloride as described in 4.4.1.2 was prepared for this purpose. This test lasted 3 hours. Every ten minutes, samples were taken and analysed by HPLC. The initial concentration of these solutions was 40µg/ml.

Naphazoline hydrochloride in alkalized solution

For the oven test, a solution was prepared: 8.00 ml of the stock solution (0.50 mg/ml; 4.3.2.1.) were diluted with water. The pH of the solution was changed to pH 10.5 with a solution of NH_4OH. This alkalized solution was diluted with HPLC water to 50.0 ml volumetric flask. The flask was carefully closed and placed in the oven, which was preheated to 50 °C. The test was lasted three hours. Every ten minutes, samples of 800 µl were taken and mixed with 800 µl buffer pH 3 (resulting in a sample pH of 5.5). The diluted samples were transferred to a vial and analysed by HPLC.
The concentration in µg/ml and the percentage decrease of naphazoline concentrations from the start time were calculated (see tab 37). The relation between % from t=0 and time is demonstrated in figure 37.

Tab 37: naphazoline hydrochloride 40µg/ml with NH_4OH in oven test:

time (min)	peak area	conc µg/ml	% from t=0
0	233578	38.24	**100.0**
10	229409	37.55	**97.7**
20	227489	37.23	**97.4**
30	228390	37.38	**97.8**
40	213878	34.99	**91.5**
50	212433	34.75	**90.9**
60	200461	32.78	**85.7**
70	193687	31.66	**82.8**
80	186898	30.54	**79.9**
90	177937	29.06	**75.9**
100	174797	28.54	**74.6**
110	168224	27.46	**71.8**
120	160547	26.19	**68.5**
130	157140	25.63	**67.0**
140	133014	21.65	**56.6**
150	141108	22.99	**60.1**
160	138180	22.50	**58.8**
170	125478	20.41	**53.4**
180	121877	19.82	**51.8**

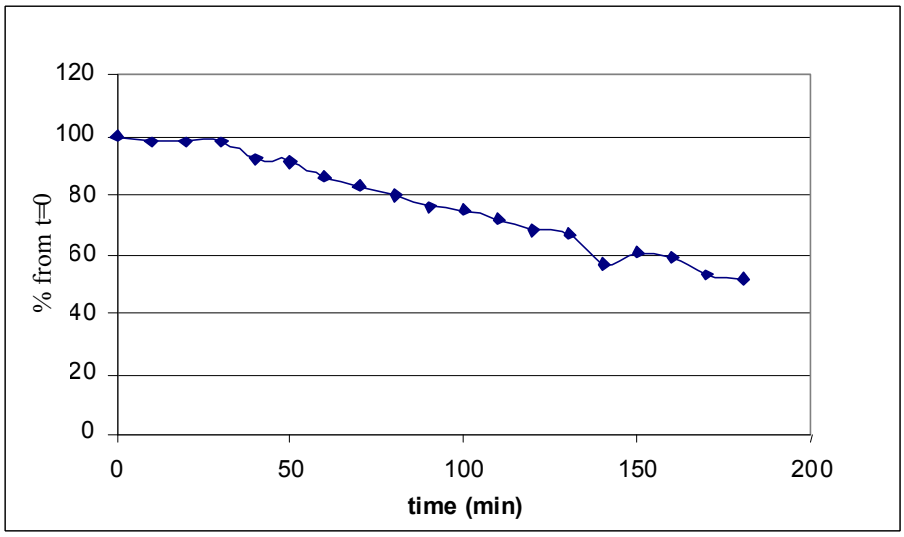


Fig 37: naphazoline hydrochloride 40µg/ml with NH_4OH in oven test, 50°C.

4.4.4. Discussion:

One of the aims of our study was to investigate the photo- and thermo- stability of different naphazoline solutions. For this purpose aqueous, alkalized and acid solution were prepared and placed in the sun test machine.

After the sun test with aqueous naphazoline solution, the concentration of naphazoline was almost the same (4.4.1.1.). Was proved, that aqueous solutions are relatively stable. The fact that alkaline solutions are prone to hydrolysis [2] was verified. For this purouse the alkalized naphazoline solution (4.4.1.2.) was tested. In this case, a small signal of a degradation product appeared already at the beginning of the test (Fig: 33), which means that the degradation was induced by alkaline media and the sun light accelerated this hydrolysis. The concentration of naphazoline was decreasing depending on time (Fig 32). The sun test with naphazoline acid solution, demonstrated no changes in naphazoline concentration, this solution was relatively stabile (4.4.1.3.).

To recognize if degradation and its acceleration were due to the sunlight or only due to the elevated temperature, the oven test was performed. The same solution (concentration 4mg/50 ml) was tested in the oven with a temperature of 50 °C (4.4.3.). A degradation product was also detected at the beginning of the test , but the decline of naphazoline concentration was slower and linear (Fig 37).

The results of the sun and oven test were compared. Was determined that a degradation after sun light exposure is quicker than after heat exposure.

The sun test with a mixture of Coldan and Okuzell Augentropfen simulates 15 hours of the sample's exposure to bright sunlight. After this exposure, no degradation products have been detected in the drop mixture (4.4.1.2.).

4.5. Analysis of ophthalmic preparations

4.5.1. Naphazolinhydrochlorid 0.01%, Augentropfen 5 ml

We had three charges of in-house preparation Naphazolinhydrochlorid 0.01%, Augentropfen:

- 12.05.2010
- 01.10.2010
- 05.01.2011

4.5.1.1. Analysis of Naphazolinhydrochlorid 0.01%, Augentropfen, 12.05.2010:

400 µl of Naphazolinhydrochlorid Augentropfen were diluted with 600 µl of HPLC water and analysed by HPLC under proposed conditions. Concentration in a vial 40µg/ml.

For the analysis, we had two bottles of Naphazolinhydrochlorid, Augentropfen from 12.05.2010, and three vials with the same concentration were prepared from each bottle. . From each vial, three injections were measured. . The percentage content was calculated as well as the average, standard deviation (s) and relative standard deviation (s %) of concentrations and contents.

Tab 38: the first sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared on 12.05.2010

	12.5.2010					
	sample 1					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	38.84	97.10	38.01	95.03	38.44	96.10
2	38.99	97.47	38.39	95.98	38.50	96.25
3	39.03	97.57	38.32	95.80	38.34	95.85
average	**38.95**	**97.38**	**38.24**	**95.60**	**38.43**	**96.07**
s	0.10016653	0.24758837	0.20223748	0.50461206	0.08082904	0.20207259
s%	0.26	0.25	0.52	0.53	0.21	0.21

Tab 39: the second sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared on 12.05.2010

	12.5.2010					
	sample 2					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	38.22	95.55	38.37	95.93	38.43	96.08
2	38.00	95.00	38.62	96.55	38.14	95.35
3	38.35	95.88	38.35	95.88	38.10	95.25
average	**38.19**	**95.48**	**38.45**	**96.12**	**38.22**	**95.56**
s	0.17691806	0.44455971	0.15044379	0.37322915	0.18009257	0.45310043
s%	0.46	0.46	0.39	0.39	0.47	0.47

4.5.1.2. Analysis of Naphazolinhydrochlorid 0.01%, Augentropfen, 01.10.2010

400 µl of Naphazolinhydrochlorid Augentropfen were diluted with 600 µl of HPLC water and analysed by HPLC under proposed conditions. Concentration in a vial 40µg/ml.

For the analysis, we had three bottles of Naphazolinhydrochlorid, Augentropfen from 01.10.2010, and three vials with the same concentration were prepared from each bottle. From each vial, three injections were measured. The percentage content was calculated as well as the average, standard deviation (s) and relative standard deviation (s %) of concentrations and contents.

Tab 40: the first sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared on 01.10.2010

	1.10.2010					
	sample 1					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	40.65	101.62	39.81	99.53	38.81	97.03
2	40.66	101.65	39.67	99.18	38.57	96.43
3	40.78	101.95	39.79	99.48	38.84	97.10
average	**40.70**	**101.74**	**39.76**	**99.40**	**38.74**	**96.85**
s	0.07234178	0.18248288	0.07571878	0.18929694	0.14798649	0.36828431
s%	0.18	0.18	0.19	0.19	0.39	0.38

Tab 41: the second sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared on 01.10.2010

	1.10.2010					
	sample 2					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	39.90	99.75	38.84	97.10	38.98	97.45
2	40.01	100.02	38.33	95.83	38.76	96.90
3	39.82	99.55	38.99	97.48	39.11	97.78
average	**39.91**	**99.77**	**38.72**	**96.80**	**38.95**	**97.38**
s	0.09539392	0.23586719	0.34597688	0.86407947	0.17691806	0.44455971
s%	0.24	0.24	0.89	0.89	0.45	0.45

Tab 42: the third sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared on 01.10.2010

	1.10.2010					
	sample 3					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	39.98	99.95	39.41	98.53	38.89	97.23
2	39.97	99.93	39.75	99.38	38.65	96.63
3	39.89	99.72	39.35	98¨.75	38.85	97.13
average	**39.95**	**99.87**	**39.50**	**98.89**	**38.80**	**97.00**
s	0.04932883	0.1274101	0.21571586	0.44117268	0.12858201	0.32145503
s%	0.12	0.13	0.55	0.45	0.32	0.32

4.5.1.3. Analysis of Naphazolinhydrochlorid 0.01%, Augentropfen, 05.01.2011

400 µl of Naphazolinhydrochlorid Augentropfen were diluted with 600 µl of HPLC water and analysed by HPLC under proposed conditions. Concentration in a vial 40µg/ml.

For the analysis, we had six bottles of Naphazolinhydrochlorid, Augentropfen from 05.01.2011. Three bottles were prepared by volume. Three bottles were prepared by weighing. From each bottle, three vials with the same concentration were prepared. From each vial, three injections were measured. The percentage content was calculated as well as the average, standard deviation (s) and relative standard deviation (s %) of concentrations and contents.

4.5.1.3.1. Drops prepared by volume

Tab 43: the first sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared by volume, 05.01.2011

	5.1.2011					
	sample 1					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	40.31	100.8	39.54	98.85	40.23	100.58
2	40.50	101.2	39.39	98.48	40.14	100.35
3	40.23	100.6	39.38	98.45	40.08	100.20
average	**40.35**	**100.87**	**39.44**	**98.59**	**40.15**	**100.38**
s	0.1386843	0.305505	0.089629	0.222785	0.0754983	0.191398
s %	0.34	0.3	0.23	0.23	0.19	0.19

Tab 44: the second sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared by volume, 05.01.2011

	5.1.2011					
	sample 2					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	39.84	99.60	39.27	98.18	39.83	99.58
2	39.79	99.48	39.36	98.40	39.71	99.28
3	39.97	99.92	39.53	98.83	39.70	99.25
average	**39.87**	**99.67**	**39.39**	**98.47**	**39.75**	**99.37**
s	0.0929157	0.22745	0.132035	0.330606	0.0723418	0.182483
s %	0.23	0.23	0.33	0.34	0.18	0.18

Tab 45: the third sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared by volume, 05.01.2011

	5.1.2011					
	sample 3					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	38.94	97.35	39.16	97.90	38.79	96.98
2	39.02	97.55	39.13	97.83	38.74	96.85
3	39.05	97.63	39.21	98.03	38.89	97.23
average	**39.00**	**97.51**	**39.17**	**97.92**	**38.81**	**97.02**
s	0.0568624	0.144222	0.040415	0.101489	0.0763763	0.193132
s %	0.15	0.15	0.1	0.1	0.2	0.2

4.5.1.3.2. Drops prepared by weighing

Tab 46: the first sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared by weighing, 05.01.2011

	5.1.2011					
	sample 1					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	40.25	100.6	40.06	100.15	40.11	100.30
2	39.92	99.80	39.99	99.98	40.06	100.15
3	39.99	99.98	39.98	99.95	40.12	100.30
average	**40.05**	**100.13**	**40.01**	**100.03**	**40.10**	**100.25**
s	0.1738774	0.419682	0.043589	0.107858	0.0321455	0.086603
s %	0.43	0.42	0.11	0.11	0.08	0.09

Tab 47: the second sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared by weighing, 05.01.2011

	5.1.2011					
	sample 2					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	39.41	98.53	40.03	100.08	39.60	99.00
2	39.50	98.75	39.83	99.58	39.70	99.25
3	39.54	98.85	39.98	99.95	39.58	98.95
average	**39.48**	**98.71**	**39.95**	**99.87**	**39.63**	**99.07**
s	0.0665833	0.163707	0.104083	0.259422	0.064291	0.160728
s %	0.17	0.17	0.26	0.26	0.16	0.16

Tab 48: the third sample of Naphazolinhydrochlorid, Augentropfen, 40µg/ml, prepared by weighing, 05.01.2011

	5.1.2011					
	sample 3					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	40.04	100.10	39.87	99.68	39.82	99.55
2	39.92	99.80	40.05	100.13	39.96	99.90
3	39.95	99.88	40.18	100.45	39.88	99.70
average	**39.97**	**99.93**	**40.03**	**100.09**	**39.89**	**99.72**
s	0.06245	0.155349	0.155671	0.386825	0.0702377	0.175594
s %	0.16	0.16	0.39	0.39	0.18	0.18

Tab 49: intermediate percentage content of naphazoline hydrochloride in Naphazolinhydrochlorid, Augentropfen, 40µg/ml

	1.10.2010	**12.5.2010**	**5.1.2011 V**	**5.1.2011 W**
AV content %	98.63	96.03	98.87	99.75
s	1.13867254	0.7442415	1.2640267	0.53960099
s%	1.15	0.77	1.28	0.54

AV-average; V-prepared by pipetting; W-prepared by weighing

4.5.1.4. Discussion:

The content of naphazoline in the samples from 12.05.2010 was about 96.03%. Because these samples were a half-year old, it can be assumed that the lower value was caused by hydrolysis of the compound.

The content of naphazoline in the samples from 01.10.2010 and from 05.01.2011 was almost the same (98.63% the first and 98.88 second). Both samples were prepared by volume.

The content of naphazoline in the samples from 05.01.2011 (prepared by weighing) was 99.75%. This result was the most exact. The results of this investigation were consulted with representatives of the hospital pharmacy.

4.5.2. Bor-Naphazolinhydrochlorid 0.025%, Augentropfen 10ml

We had four charges of in-house preparation Bor-Naphazolinhydrochlorid Augentropfen:

- 16.09.2010
- 22.11.2010
- 05.01.2011
- 19.01.2011

4.5.2.1. Analysis of Bor-Naphazolinhydrochlorid Augentropfen 0.025%, 16.09.2010

100 µl of Bor-Naphazolinhydrochlorid Augentropfen were diluted with 900 µl of HPLC water and analysed by HPLC. Concentration in a vial 25µg/ml.

For the analysis, we had three bottles of Bor-Naphazolinhydrochlorid Augentropfen from 16.09.2010. From each bottle, one sample was prepared. Each sample was injected three times. The percentage content was calculated as well as the average, standard deviation (s) and relative standard deviation (s %) of concentrations and contents.

Tab 50: samples of Bor-Naphazolinhydrochlorid, Augentropfen, 25µg/ml, prepared on 16.9.2010

	16.9.2010					
	sample 1		**sample 2**		**sample 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.29	97.16	24.44	97.76	24.12	96.48
2	24.12	96.48	24.41	97.64	24.12	96.48
3	24.10	96.40	24.22	96.88	23.96	95.84
average	**24.17**	**96.68**	**24.36**	**97.43**	**24.07**	**96.27**
s	0.10440307	0.41761226	0.1193035	0.4772141	0.09237604	0.36950417
s%	0.43	0.43	0.49	0.50	0.38	0.38

4.5.2.2. Analysis of Bor-Naphazolinhydrochlorid Augentropfen 0.025%, 22.11.2010

100 µl of Bor-Naphazolinhydrochlorid Augentropfen were diluted with 900 µl of HPLC water and analysed by HPLC. Concentration in a vial 25µg/ml.

For the analysis, we had three bottles of Bor-Naphazolinhydrochlorid Augentropfen from 16.09.2010. From each bottle, one sample was prepared. Each sample was injected three times. The percentage content was calculated as well as the average, standard deviation (s) and relative standard deviation (s %) of concentrations and contents.

Tab 51: samples of Bor-Naphazolinhydrochlorid, Augentropfen, and 25µg/ml, prepared on 22.11.2010

	22.11.2010					
	sample 1		**sample 2**		**sample 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.13	96.52	23.41	93.64	23.44	93.76
2	24.39	97.56	23.40	93.60	23.35	93.40
3	24.22	96.88	23.31	93.24	23.23	92.92
average	**24.25**	**96.99**	**23.37**	**93.49**	**23.34**	**93.36**
s	0.13203535	0.5281414	0.0550757	0.2203028	0.10535654	0.42142615
s%	0.54	0.54	0.24	0.24	0.45	0.45

4.5.2.3.. Analysis of Bor-Naphazolinhydrochlorid Augentropfen 0.025%, 05.01.2011

100 µl of Bor-Naphazolinhydrochlorid Augentropfen were diluted with 900 µl of HPLC water and analysed by HPLC. Concentration in a vial 25µg/ml. These samples were prepared in a lower amount than usual.

For the analysis, we had three bottles of Bor-Naphazolinhydrochlorid Augentropfen from 05.01.2011. From each bottle, three vials with the same concentration were prepared. Each vial was injected three times. The percentage content was calculated as well as the average, standard deviation (s) and relative standard deviation (s %) of concentrations and contents.

Tab 52: first sample of Bor-Naphazolinhydrochlorid, Augentropfen, 25µg/ml, prepared on 05.01.2011

	5.1.2011					
	sample 1					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.61	98.44	25.16	100.64	24.66	98.64
2	24.70	98.80	25.35	101.40	24.60	98.40
3	24.67	98.68	25.34	101.36	24.68	98.72
average	**24.66**	**98.64**	**25.28**	**101.13**	**24.65**	**98.59**
s	0.0458258	0.183303	0.106927	0.427707	0.0416333	0.166533
s %	0.18	0.18	0.42	0.42	0.17	0.17

Tab 53: second sample of Bor-Naphazolinhydrochlorid, Augentropfen, 25µg/ml, prepared on 05.01.2011

	5.1.2011					
	sample 2					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.46	97.84	24.70	98.80	24.55	98.20
2	24.54	98.16	24.62	98.48	24.62	98.48
3	24.47	97.88	24.70	98.80	24.56	98.24
average	**24.49**	**97.96**	**24.67**	**98.69**	**24.58**	**98.31**
s	0.043589	0.174356	0.046188	0.184752	0.0378594	0.151438
s %	0.18	0.18	0.19	0.19	0.15	0.15

Tab 54: third sample of Bor-Naphazolinhydrochlorid, Augentropfen, 25µg/ml, prepared on 05.01.2011

	5.1.2011					
	sample 3					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.93	99.72	24.95	99.80	25.07	100.28
2	24.93	99.72	24.98	99.92	24.97	99.88
3	24.97	99.88	25.03	100.12	24.93	99.72
average	**24.94**	**99.77**	**24.99**	**99.95**	**24.99**	**99.96**
s	0.023094	0.092376	0.040415	0.161658	0.072111	0.288444
s %	0.09	0.09	0.16	0.16	0.29	0.29

Tab 55: intermediate content of naphazoline in Bor-Naphazolinhydrochlorid, Augentropfen 25µg/ml

	22.11.2010	**16.9.2010**	**5.1.2011**
AV content %	94.61	96.79	99.21
s	1.81603965	0.62762338	1.0064612
s%	1.92	0.65	1.01

AV-average

The samples from 22.11.2010 and 16.9.2010 are prepared by routine technique, but the content of naphazoline in these samples is less than declaration. The samples of Bor-Naphazolinhydrochlorid, Augentropfen from 5.1.2011 were prepared in a lower amount than usual. Compared to the results of the charges prepared by routine technique, problems with dissolving and homogenization can be assumed.

4.5.2.4. Analysis of Bor-Naphazolinhydrochlorid Augentropfen 0.025%, 19.01.2011

This charge was prepared in a large volume. Every twentieth sample was taken and provided for HPLC analysis.

100 µl of Bor-Naphazolinhydrochlorid Augentropfen were diluted with 900 µl of HPLC water and analysed by HPLC. Concentration in a vial 25µg/ml.

For the analysis, we had seven bottles of Bor-Naphazolinhydrochlorid Augentropfen from 19.01.2011. From each bottle, three vials with the same concentration were prepared. Each vial was injected three times. The percentage content was calculated as well as the average, standard deviation (s) and relative standard deviation (s %) of concentrations and contents.

Tab 56: the first sample of Bor-Naphazolinhydrochlorid, Augentropfen (1st sample from production), 25µg/ml, prepared on 19.01.2011

	19.1.2011					
	sample 1					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.14	96.56	24.11	96.44	24.34	97.36
2	24.41	97.54	24.36	97.44	24.39	97.56
3	24.34	97.36	24.42	97.68	24.51	98.04
average	**24.30**	**97.15**	**24.30**	**97.19**	**24.41**	**97.65**
s	0.140119	0.521664	0.164418	0.657673	0.0873689	0.349476
s %	0.58	0.54	0.68	0.67	0.36	0.36

Tab 57: the second sample of Bor-Naphazolinhydrochlorid, Augentropfen (20th sample from production), 25µg/ml, prepared on 19.01.2011

	19.1.2011					
	sample 2					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.05	96.20	24.79	99.16	24.87	99.48
2	24.27	97.08	24.70	98.80	24.70	98.80
3	24.22	97.88	24.48	97.92	24.67	98.68
average	**24.18**	**97.05**	**24.66**	**98.63**	**24.75**	**98.99**
s	0.1153256	0.840317	0.159478	0.637913	0.1078579	0.431432
s %	0.47	0.86	0.65	0.65	0.43	0.44

Tab 58: the third sample of Bor-Naphazolinhydrochlorid, Augentropfen (40th sample from production), 25µg/ml, prepared on 19.01.2011

	19.1.2011					
	sample 3					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.50	98.00	24.93	99.72	24.66	98.64
2	24.38	97.52	24.97	99.88	24.61	98.44
3	24.51	98.04	24.79	99.16	24.49	97.96
average	**24.46**	**97.85**	**24.90**	**99.59**	**24.59**	**98.35**
s	0.0723418	0.289367	0.094516	0.378065	0.0873689	0.349476
s %	0.30	0.30	0.38	0.38	0.35	0.35

Tab 59: the fourth sample of Bor-Naphazolinhydrochlorid, Augentropfen (60th sample from production), 25µg/ml, prepared on 19.01.2011

	19.1.2011					
	sample 4					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.93	99.72	24.67	98.68	23.83	95.32
2	24.92	99.68	24.68	98.72	23.98	95.92
3	24.95	99.80	24.59	98.36	23.85	95.40
average	**24.93**	**99.73**	**24.65**	**98.59**	**23.89**	**95.55**
s	0.0152753	0.061101	0.049329	0.197315	0.0814453	0.325781
s %	0.06	0.06	0.20	0.20	0.34	0.34

Tab 60: the fifth sample of Bor-Naphazolinhydrochlorid, Augentropfen (80th sample from production), 25µg/ml, prepared on 19.01.2011

	19.1.2011					
	sample 5					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.74	98.96	24.68	98.72	24.26	97.04
2	24.57	98.28	24.67	98.68	24.13	96.52
3	24.64	98.56	24.83	99.32	24.19	96.76
average	**24.65**	**98.60**	**24.73**	**98.91**	**24.19**	**96.77**
s	0.08544	0.34176	0.089629	0.358515	0.0650641	0.260256
s %	0.35	0.35	0.36	0.36	0.27	0.27

Tab 61: the sixth sample of Bor-Naphazolinhydrochlorid, Augentropfen (100th sample from production), 25µg/ml, prepared on 19.01.2011

	19.1.2011					
	sample 6					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.51	98.04	24.62	98.48	24.29	97.16
2	24.7	98.80	24.99	99.96	24.28	97.12
3	24.71	98.84	24.61	98.44	24.26	97.04
average	**24.64**	**98.56**	**24.74**	**98.96**	**24.28**	**97.11**
s	0.1126943	0.450777	0.216564	0.866256	0.0152753	0.061101
s %	0.46	0.46	0.88	0.88	0.06	0.06

Tab 62: the seventh sample of Bor-Naphazolinhydrochlorid, Augentropfen (120th sample from production), 25µg/ml, prepared on 19.01.2011

	19.1.2011					
	sample 7					
	vial 1		**vial 2**		**vial 3**	
injection	conc µg/ml	content %	conc µg/ml	content %	conc µg/ml	content %
1	24.59	98.36	24.52	98.08	23.94	95.76
2	24.30	97.20	24.66	98.64	24.01	96.04
3	24.48	97.92	24.69	98.76	23.85	95.40
average	**24.46**	**97.83**	**24.62**	**98.49**	**23.93**	**95.73**
s	0.1464013	0.585605	0.090738	0.362951	0.0802081	0.320832
s %	0.60	0.60	0.37	0.37	0.33	0.33

Tab 63: intermediate content of naphazoline hydrochloride in Bor-Naphazolinhydrochlorid, Augentropfen prepared on 19.01.2011

	sample 1	**sample 2**	**sample 3**	**sample 4**	**sample 5**	**sample 6**	**sample 7**
AV-cont %	97.33	98.22	98.59	97.95	98.09	98.21	97.35
s	0.5159836	1.057187	0.8278755	1.8835546	1.0373042	0.9760692	1.3038063
s %	0.53	1.08	0.84	1.92	1.06	0.99	1.33

AV-cont - average content %

The charge from 19.01.2011 was made in a large volume. This time, special emphasis was placed on thorough homogenization of the production lot before filling to the dosage containers. In table 63, we can see that the percentage content of every twentieth sample practically corresponds to the first sample. But the concentration of naphazoline is nevertheless slightly lower than the declared amount. These results were consulted with representatives of hospital pharmacy.

5. CONCLUSION

The project included development and validation of an HPLC method for quantitation of naphazoline. Photo- and thermo stability of these preparations were investigated as well.

For selective and stability indicating determination of naphazoline a new HPLC method was developed. Mobile phase composed of methanol : triethylamine 0.05M/pH 3 set up using phosphoric acid (30:70 v/v), and a BDS HYPERSIL C_{18} column (150x4(mm), particle size 5µm) were used for the chromatography. Flow rate was 1 ml/min, detection was performed at 254 nm and 280 nm.

The HPLC method was validated. The specificity of this method and linear response of DAD detector were demonstrated. The correlation coefficient for naphazoline was 0.9998. LOD was 0.2µg/ml and LOQ 1µg/ml. The precision was also determinated.

A stability assessment for the formulations was carried out with a special emphasis on the photodegradation upon light exposure. The sun test with aqueous, alkalized and acid naphazoline solution was performed. It was proven, that aqueous solutions are relatively stable. In alkalized solution, a small signal of a degradation product appeared already at the beginning of the test, which means that the degradation was induced by alkaline media and the sun light accelerated this hydrolysis. The concentration of naphazoline was decreasing depending on time. The acid solution was stabile.

The oven test with the alkalized solution was performed. A degradation product was also detected at the beginning of the test, but the decline of naphazoline concentration was slower and linear.

The sun test with a mixture of Coldan and Okuzell Augentropfen simulates 15 hours of the sample's exposure to bright sunlight. This mixture was stabile.

The content of naphazoline hydrochloride in different production lots of both in-house eye drops were analysed by the proposed HPLC method.

The content of naphazoline in the Naphazolinhydrochlorid 0.01%, Augentropfen samples from 12.05.2010 was about 96.03%. It can be assumed that the lower value was caused by hydrolysis of the compound. This sample was a half-year old.

The content of naphazoline in the samples from 01.10.2010 and from 05.01.2011 was almost the same (98.63% the first and 98.88 second). Both samples were prepared by volume.

The content of naphazoline in the samples from 05.01.2011 (prepared by weighing) was 99.75%. This result was the most exact.

The samples from 22.11.2010 and 16.9.2010 are prepared by routine technique, but the content of naphazoline in these samples is less than declaration- 94.61% and 96.79%. The samples of Bor-Naphazolinhydrochlorid, Augentropfen from 5.1.2011 were prepared in a lower amount than usual, the content of naphazoline was 99,21%. Compared to the results of the charges prepared by routine technique, problems with dissolving and homogenization can be assumed.

The charge from 19.01.2011 was made in a large volume. The percentage content of every twentieth sample practically corresponds to the first sample. But the concentration of naphazoline is nevertheless slightly lower than the declared amount. These results were consulted with representatives of hospital pharmacy.

Based on the results of the HPLC analysis, the production processes were evaluated and optimized, thus contributing to medication safety for in-house preparations.

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7. SHRNUTÍ

Naphazolin je derivátem 2-imidazolinu, je to sympatomimetická látka s agonistickým působením na alfa receptory, které se vyskytují hlavně v krevních cévách a bronších. Naphazolin má vasokonstrikční a dekongescenční vlastnosti. Je používán při terapii rinitidy, sinusitidy nebo alergické konjunktivitidy. Pro oční a nosní podání je používán ve formě vodných přípravků.

Naphazolin je dostupný v celé řadě komerčně vyráběných přípravků. Jedním z nich je Coldan® Augentropfen (oční kapky). V nemocničních lékárnách se z ekonomických a terapeutických důvodů dává přednost individuálně připravovaným přípravkům s naphazolinem. V naší práci jsme analyzovali dva odlišné přípravky obsahující naphazolin. První byl směs dvou komerčně dostupných preparátů- Coldan® Augentropfen a Okuzell® Augentropfen, tyto kapky jsou připravovány v poměru 1:9 (3.1.1.4.1.). Druhý přípravek byl Bor-Naphazolin Augentropfen. Tento je kompletně připravovaný v nemocniční lékárně (3.1.1.4.2.). Cílem naší práce bylo analyzovat tyto dva přípravky a prokázet jejich kvalitu za účelem zvýšení bezpečnosti pro pacienta.

Tento projekt zahrnoval vývoj a validaci HPLC metody pro kvantifikaci naphazolinu ve zmíněných přípravcích. V rámci práce byla také testována foto- a termo stabilita těchto přípravků.

V prvním kroku byla provedena rešerše dostupných literárních zdrojů (3.1.1.5.). Žádná z publikovaných HPLC metod nebyla shodná s námi navrženou metodou.

Bylo potřeba dosáhnout separace všech komponent očních přípraků a stejně tak i jejich rozkladných produktů. Pro nalezení optimálních podmínek pro separaci byl nejprve naphazolin analyzován s různými mobilními fázemi s rozdílnou hodnotou pH. Byla použita EcoCART® 125-3 HPLC Cartridge LiChrospher® 100 RP-18 endcapped kolona s velikostí částic 5µm. První pokusy byly prováděny na HPLC přístroji pro předběžné testování (SHIMADZU LC 10AS, 4.1.3.1.). Jako mobilní fáze byly připraveny 3 roztoky pufrů- 2% kyselina octová pH 2; směs kyseliny octové a 5mM kyseliny heptansulfonové pH 2,47; 0,05M triethylamin pH 3 (4.1.4.) - jednotlivé pufry byly kombinovány s methanolem v poměru 50:50 (v/v). Takto vzniklé mobilní fáze byly použity pro analýzu

naphazolinu hydrochloridu (100µg/ml). Nejlepšího tvaru píku bylo dosaženo použitím mobilní methanol : triethylamin 0,05M; pH 3. Složení této faze bylo dále optimalizováno (Tab: 4). Za nejvhodnější byl zvolen poměr methanol : triethylamin 30:70 (v/v). S využitím této mobilní fáze byly analyzovány i další sloučeniny obsažené v kapkách (Fig. 8;9) a také rozkladné produkty naphazolinu (4.2.1.2.). Tato mobilní fáze byla vybrána jako optimální pro separaci všech jednotlivých složek vzorků. Tato metoda byla přenesena na druhý HPLC přístoj (SHIMADZU LC-20AD, 4.1.4.).

Pro analýzy na přístroji SHIMADZU LC-20AD byla použita BDS C18 kolona, 150x4 (mm), s velikostí částic 5µm, mobilní fáze methanol : 0,05M triethylamin pH 3 v poměru 30:70 (v/v). Znovu byly analyzovány všechny roztoky sloučenin obsažené v kapkách – naphazolin (Fig: 14), methylparaben (Fig. 15), kyselina boritá (Fig: 16) benzalkonium (Fig: 17) a směs rozkladného produktu a methylparabenu (Fig: 18). Všechny složky byly separovány a navržená metoda byla validována.

V rámci validace byla nejprve prokázána specificita navržené metody. Dále byla testována linearita. Pomocí pěti roztoků naphazolinu - koncentrace 50; 40; 35; 25; 20µg/ml-byla sestavena kalibrační křivka (Fig: 29). Její rovnice byla y = 6063,6x + 1724,5 a korelační koeficient 0,9998. Pro methylparaben byla rovnice kalibrační křivky y = 63045x + 16736 a korelační koeficient 0,9996. Pro obě sloučeniny byl stanoven detekčí (LOD) a kvantitativní limit (LOQ) (4.3.3.). Pro naphazolin byl LOD 0,2µg/ml a LOQ 1µg/ml, pro methylparaben LOD 0,016µg/ml a LOQ 0,08 µg/ml. Byla prokázána přesnost metody pro naphazolin (4.3.4.1.) a methylparaben (4.3.4.1.) – v případě obou sloučenin bylo měřeno 3 krát pět koncetrací ve 3 dnech. Robustnost metody byla testována tak, že jednotlivé parametry navržené metody – rychlost průtoku, teplota, složení mobilní fáze a pH (4.3.5.1.- 4.3.5.4.) byly měněny v určitém rozmezí.

V rámci stabilitních testů byla sledována foto- a termo-stabilita roztoků naphazolinu. Pro tento účel byly připraveny 3 roztoky naphazolinu – ve vodném, alkalickém a kyselém prostředí. Tyto byly umístěny do Suntestu - přístroje pro simulaci jasného slunečního světla (4.1.3.3..). Faktor přístroje je 15, což znamená, že 1 min expozice v tomto přistroji odpovídá 15 min přímého slunečného světla. Test trval tři hodiny. Každých deset minut byly jednotlivé vzorky odebírány a analyzovány. Byla vypočítána koncentrace v µg/ml a procentuální snižování obsahu naphazolinu v čase.

Tento test u vodného roztoku naphazolinu (4.4.1.1.) nezpůsobil výrazný pokles koncentrace naphazolinu. Roztok zůstal po působení jasného světla stabilní (Fig: 31). V roztoku, který byl alkalizován pomocí roztoku NH_4OH (4.4.1.2.), došlo ještě před umístěním vzorku do testovacího přístroje ke vzniku rozkladného produktu (Fig: 33), což znamená, že rozkladná reakce je navozena alkalickým prostředím a sluneční světlo dále tuto rozkladnou reakci urychluje. Koncentrace naphazolinu klesala v závislosti na čase (Fig: 32). U roztoku, který byl okyselen na pH 3 (4.4.1.3.), nedošlo k rozkladné reakci a množství naphazolinu bylo stejné, jako před začátkem testu (Fig: 34). Kyselé prostředí nemělo vliv na stabilitu naphazolinu.

V dalším kroku byl proveden test v sušárně předehřáté na 50°C (4.4.3.) s cílem zjistit, zda k rozkladu naphazolinu dochází působením slunečního světla nebo pouze vlivem zvýšené teploty. Tento test trval tři hodiny, vzorky byly odebírány po deseti minutách. Stejnému testu byl podroben alkalickýroztok naphazolinu připravený obdobně jako v případě 4.4.1.2.. Stejně jako při testu na sluneční světlo vznikal rozkladný produkt hned od změny pH roztoku. Pokles koncentrace naphazolinu byl ale pomalejší než během slunečního testu a probíhal lineárně (Fig: 37). Degradace byla urychlena světlem více než jen působením teploty.

Dále byla testována stabilita naphazolinu v reálných vzorcích - očních kapkách. Byla připravena směs Coldan a Okuzell Augentropfen ve stejné koncentraci a objemu (0,5mg/5ml) jako jsou v nemocnici připravované vzorky kapek (3.1.1.4.1.). Vzorky byly umístěny do Suntestu. Tento test simuloval 15 hodin působení přímého slunečního světla. Během této doby nedošlo ke snížení množství naphazolinu ve vzorku (Fig: 35).

Pro analýzu vzorků kapek byly k dispozici různé šarže připravovaných přípravků. U přípravku Naphazolinhydrochlorid 0,01%, Augentropfen 5 ml, to byly šarže z 12.05.2010; 01.10.2010 a z 05.01.2011. Z každé lahvičky každé jednotlivé šarže byly odebrány 3 vzorky a byly 3 krát měřeny. Byla vypočítána koncentrace a procentuální obsah naphazolinu, průměr, směrodatná odchylka a relativní směrodatná odchylka. Výsledky byly zaznamenány a porovnány (Tab: 49). Obsah naphazolinu ve vzorcích z 12.05.2010 byl 96%. Protože tento vzorek byl půl roku starý, lze předpokládat, že v průběhu skladování došlo k hydrolýze sloučeniny. Výsledky vzorků kapek připravovaných rutinním

postupem ukázaly nižší obsah naphazolinu než je deklarované množství. Koncentrace nejvíce odpovídající deklarované hodnotě byla zjištěna u vzorků připravovaných alternativním postupem (šarže z 05.01.2011).

Při analyze vzorků Bor-Naphazolinhydrochlorid 0,025%, Augentropfen 10ml byly k dispozici čtyři šarže- z 16.09.2010; 22.11.2010; 05.01.2011 a z 19.01.2011. Každý vzorek byl třikrát analyzován, byla vypočítána koncentrace, procentuální obsah naphazolinu, průměr, směrodatná odchylka a relativní směrodatná odchylka. Obsah naphazolinu u vzorků z 16.9.2010 a 22.11.2010 byl 94,6 a 96,7%, což je nižší než deklarované množství (Tab: 55), tyto vzorky byly připraveny rutinním postupem. Vzorky z 05.01.2011 byly připraveny v menším množství než při obvyklém postupu. Obsah naphazolinu byl 99,2%. V porovnání s výsledky rutinně připravovaných vzorků je tento obsah vyšší. U rutinních vzorků lze předpokládat problémy s rozpouštěním nebo homogenizací při přípravě. Šarže 19.01.2011 byla připravena ve velkém objemu (4.5.2.4.). Během přípravy byl kladen důraz na pečlivou homogenizaci roztoku před přeplněním do jednotlivých lahviček. Každý dvacátý vzorek byl odebrán a hodnocen pomocí HPLC. Z každé lahvičky byly odebrány tři vzorky, každý z těchto vzorků byl 3 krát analyzován. Výsledky byly zaznamenány a porovnány (Tab: 63). Obsah naphazolinu je ve všech vzorcích této šarže přibližně stejný, koncentrace naphazolinu je přesto lehce nižší než požadované množství.

Všechny výsledky analýzy očních přípravků byly konzultovány se zástupcem nemocniční lékárny za účelem optimalizace procesu přípravy a zvýšení bezpečnosti přípravků pro pacienta.

8. CURRICULUM VITAE

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1986	born 16 November in Pelhřimov
1993 to 2001	primary school in Hnojník
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From 2005	Charles University, Prague, Faculty of Pharmacy, Hradec Králové
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