

Stability study of selected pharmaceutical formulations

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**SVEUČILIŠTE U ZAGREBU
FAKULTET KEMIJSKOG INŽENJERSTVA I TEHNOLOGIJE
SVEUČILIŠNI DIPLOMSKI STUDIJ KEMIJSKO INŽENJERSTVO**

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**STUDIJA STABILNOSTI IZABRANIH FARMACEUTSKIH
FORMULACIJA**

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LIST OF ABBREVIATIONS

ACN	acetonitrile
EMA	European medicines agency
EDQM	European Directorate for the Quality of Medicines
EtOH	ethanol
HETP	Height Equivalent to a Theoretical Plate
HPLC	high performance liquid chromatography
ICH	International Conference on Harmonisation
IPEC	International Pharmaceutical Excipients Council
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
PGE ₁	Prostaglandin E ₁
Ph. Eur.	European Pharmacopoeia
t ₀	starting time point related to stability study
USP	United States Pharmacopeia
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume

SAŽETAK RADA

Razvijena je i optimizirana HPLC metoda obrnute faze, za studiju stabilnosti farmaceutskih formulacija koje sadrže prostaglandin E₁ i papaverin HCl kao glavne komponente. Studija stabilnosti bit će izvedena kroz sedam vremenskih točaka: t₀ (početna vremenska točka), te nakon jedan, dva, tri, četiri, pet, i konačno, šest mjeseci. Kromatografska separacija postignuta je na Alltima C18 5 μ koloni (250 mm x 4,6 mm, 5 μ m) pri sobnoj temperaturi koristeći gradijentni protok pokretne faze. Pokretna faza A je mješavina 0,02 M fosfatnog pufera (pH 3.0) i acetonitrila (62:38 v/v). Pokretna faza B je mješavina 0,02 M fosfatnog pufera (pH 3.0) i acetonitrila (30:70 v/v). Protok pokretnih faza je 1,2 mL/min. UV detekcija izvedena je pri 205 nm. Metoda je validirana na specifičnost, linearnost, točnost, granice detekcije i kvantifikacije, te preciznost za dva glavna pika. Studija stabilnosti izvedena je za t₀ (početna vremenska točka).

Ključne riječi: farmaceutske formulacije, Prostaglandin E₁, Papaverin, HPLC, UV, studija stabilnosti, razvoj metode

ABSTRACT

An optimized reversed phase HPLC method, for the stability study of formulations containing prostaglandin E₁ and papaverine HCl as main components, was developed. Stability study will be performed at seven time points: t₀ (starting point), and then after one, two, three, four, five, and finally, six months. Chromatographic separations were achieved on an Alltima C18 5 μ column (250 mm x 4.6 mm, 5 μ m) and maintained at room temperature using gradient elution. Mobile phase A was a mixture of a 0.02 M phosphate buffer solution (pH 3.0) and acetonitrile (62: 38 v/v). Mobile phase B was a mixture of a 0.02 M phosphate buffer solution (pH 3.0) and acetonitrile (30: 70 v/v). Mobile phases were pumped at a flow rate of 1.2 mL/min. UV detection was performed at 205 nm. The method was validated for specificity, linearity, accuracy, limit of detection, limit of quantification, and precision of the two main peaks. Stability study was performed for t₀ (starting point).

Key words: pharmaceutical formulations, Prostaglandin E₁, Papaverine, HPLC, UV, stability study, method development

1. INTRODUCTION

1.1 Prostaglandin E₁

1.1.1 Structure

Prostaglandin E₁ is a type of prostaglandin. Prostaglandins are a group of physiologically active lipid compounds having diverse hormon-like effects in animals. They are found in most animal and human tissues and are derived enzymatically from fatty acids. Every prostaglandin contains 20 carbon atoms, including a 5-carbon ring. It was firstly isolated from seminal fluid in 1935 by the Swedish physiologist Ulf von Euler, and also independently by M.W. Goldblatt.¹

The synthetic variant of Prostaglandin E₁ (PGE₁) is known pharmaceutically as alprostadi². Its structure can be seen in Figure 1.

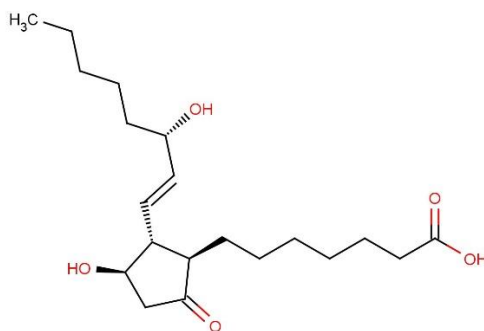


Figure 1 Structural formula of alprostadi

1.1.2 Synthesis

Alprostadi is biosynthesized from dihomo- γ -linolenic acid, which is an omega-6 fatty acid, in healthy humans without coronary artery disease or genetic disorders.³

Simple and selective synthesis of alprostadi using an asymmetric Michael addition was developed by Hayashi and Umemiya. It was based on the Michael addition of butanedial to nitroalkene. This synthesis was performed in only three steps, including three isolations and three chromatographic purifications. It includes one-pot deprotection with metal-based reagents employed in the synthesis, which contain only nontoxic metals.^{4 5}

1.1.3 Physicochemical properties

The molecular formula of alprostadil is $C_{20}H_{34}O_5$ ($M_r=354.5$ g/mol). IUPAC name is 7-[[$(1R, 2R, 3R)$ -3-Hydroxy-2-[[$(1E, 3S)$ -3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoic acid.⁶ It appears as a white to yellow crystalline solid. It is partly soluble in water, but soluble in organic solvents such as EtOH, MeOH or acetone. pK_a value is 4.85.⁷ Alprostadil is considered toxic and may impair fertility.⁸

1.1.4 Clinical use

Prostaglandin E_1 is used in the continuous treatment of erectile dysfunction because of its vasodilatory properties. Injectable forms are *Edex* and *Caverject*.⁹ Alprostadil (*Prostin VR*) is available as a generic and it usually comes in a formulation of two or three compounds. In this case it must be mixed by a compounding pharmacy, but is much less expensive. It should be kept refrigerated at 4 °C.

Alprostadil is also used for critical limb ischemia, an advanced stage of peripheral artery disease¹⁰ and in maintaining a patent ductus arteriosus in newborns.

Some of the adverse effects include accidental injury, cerebral or urethral bleeding, various kinds of pains, mainly in urethral area, fever, seizures and many more.

1.1.5 Pharmacokinetics

Alprostadil is considered today one of the drugs of choice, alone or in formulation with other drugs (vasoactive cocktails), for the diagnosis and treatment of erectile dysfunction. It is safe and easy to use.

About 80 % of the drug is absorbed within 10 minutes of injection. Regarding distribution, 81 % is bound to albumin and about 55 % to alpha-globulin. About 80 % is metabolized in one pass through the lungs. It is also rapidly metabolized locally by enzymatic oxidation. Metabolites are extracted by the kidneys, about 90 % through urine in the first 24 hours. Half-life is between 0.5 and 10 minutes.¹¹

1.1.6 Stability and dosage

Prostaglandin E₁ used in injections comes in as ethanol solution. It should be kept refrigerated at 2-8 °C, protected from light and excessive heat (>30°C). It should be stored in unopened foil pouches until use. In ethanol 10 mg/mL is stable for longer than 6 months at -20 °C. Prostaglandins are generally unstable in aqueous acid or alkaline solutions, and alprostadil has a maximum stability between pH 6-7. When aqueous solutions are frozen, the prostaglandin may precipitate, but will usually redissolve on shaking or with sonification.^{12 13}

Alprostadil has a high chance to undergo physical and chemical degradation in the formulation and one of the main degradation products is prostaglandin A₁ (PGA₁). Therefore, it is imperative to develop an analytical method that will determine the possible existence of this molecule as well, with all other formulation substances. The chemical structure of PGA₁ is shown in Figure 2.

Therapeutical alprostadil doses vary from 5 to 50 µg/mL.¹⁴ Prescribing limits for adults in the case of treatment for erectile dysfunction are generally 60-65 µg/mL.

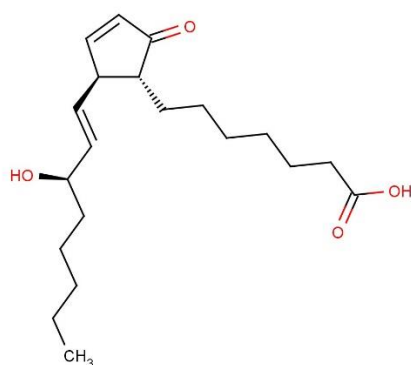


Figure 2 Chemical formula of PGA₁, degradation product of PGE₁

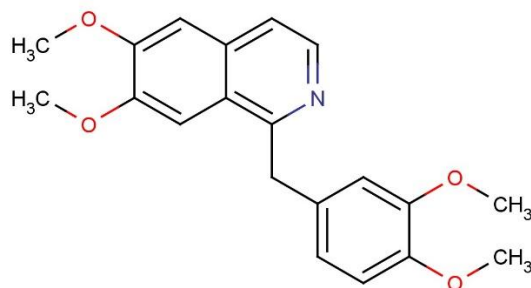
1.2 Papaverine

1.2.1 Structure

Papaverine is an opium alkaloid antispasmodic drug. While it is found in the opium poppy, papaverine differs in both structure and pharmacological action from opium alkaloids. It was discovered in 1848 by Georg Merck, a German chemist.¹⁵

Papaverine is often used as a conjugate with hydrochloride. This way it is available for intramuscular, intravenous, rectal and oral administration. The nitrogen atom is

protonated and forms a strong hydrogen bond with the chloride ion. Distance between these two atoms is 3.01 Å. The isoquinoline ring is slightly non-planar but the benzene ring is planar within experimental error. The molecular structure of papaverine is of interest in gaining a better understanding of its pharmacological action on smooth muscle.¹⁶



1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxyisoquinoline

Figure 3 Structural formula of papaverine with its IUPAC nomenclature

1.2.2 Synthesis

Since the papaverine structure is relatively simple, it was soon prepared by synthesis. The first synthesis was accomplished by Pictet and Gams.¹⁷ Since the original synthesis a number of improvements have been made.

1.2.3 Physicochemical properties

Molecular formula of papaverine is C₂₀H₂₁NO₄ (M_r=339.39 g/mol). IUPAC name is 1-(3,4-Dimethoxybenzyl)-6,7-dimethoxyisoquinoline. It is sparingly soluble in water and slightly soluble in alcohol. Papaverine occurs as a white crystalline powder. In this thesis papaverine hydrochloride (C₂₀H₂₁ClNO₄) is used. It is a colorless–lightly yellow liquid with relative molecular weight of 375.90 and pK_a of 6.4. It can be examined in UV light at 254 nm.^{18 19}

1.2.4 Clinical use

Papaverine is an inhibitor for the relief of cerebral and peripheral ischemia associated with arterial spasm and myocardial ischemia complicated by arrhythmias. The main actions of papaverine are exerted on cardiac and smooth muscle. It acts directly on the

heart muscle to depress conduction and prolong the refractory period. It relaxes various smooth muscles of the larger blood vessels. It increases cerebral blood flow because of its vasodilatory properties and therefore is used to treat problems resulting from poor blood circulation.²⁰ One of the most common problems papaverine is used to treat, is erectile dysfunction, where it is used alone or in formulations. It is also used as a preventive to migraine headaches.^{21 22}

Some of the frequent side effects that come from papaverine treatments are ventricular tachycardia, vertigo, increased transaminase levels and others.²³

1.2.5 Pharmacokinetics

Oral absorption of papaverine hydrochloride is found to be about 94 %. Volume of distribution is 0.99–1.52 L/kg and plasma protein binding is 87 %. Plasma half-life is 1.5 – 2.2 h. It is metabolized by the liver and excreted in the urine in an inactive form. After intracavernosal injection, the peak plasma concentration is several times lower than after extracavernosal injection.^{24 25}

1.2.6 Stability and dosage

Papaverine is stable under normal conditions, but it may discolor on exposure to light. It is not compatible with strong oxidizing agents and may be harmful if absorbed to skin.²⁶

Adult dosage is varying from 2.5 to 60 mg. A starting dose of 15 mg is recommended for most cases, although dosage adjustments should be made carefully.²⁷

1.3 Excipients

Excipients are natural or synthetic substances formulated alongside the active ingredient or ingredients in a pharmaceutical formulation. They are included for purpose of long-term stabilization, bulking up solid formulations or enhancement of the active ingredient. They can facilitate drug absorption, reduce viscosity, enhance solubility, or can also be useful in the manufacturing process. Conclusively, they are an important part of drug formulations and therefore its quality, safety and functional standards are regulated by IPEC.²⁸

1.3.1 Benzyl alcohol

Benzyl alcohol is popular as a preservative in the cosmetic and pharmaceutical industries. It is soluble in water up to a concentration of about 4 %, but is usually used in a concentration of 0.9 % as a bacteriostatic preservative in multiple-dose vials of solutions or drugs for parenteral therapy. Benzyl alcohol solutions are microbiocidal (i.e., they have an antimicrobial effect) but are not regarded as a sanitizing agent, which would require a faster reduction of microorganisms.

Benzyl alcohol is inexpensive, non-flammable, and can be disposed of relatively easily. Benzyl alcohol may be converted to benzaldehyde (and benzoic acid) by oxidation. Because benzyl alcohol is only partially soluble in water (4 g/100mL at room temperature) the reaction rate in water solutions is slow and no significant decrease in the benzyl alcohol concentration was observed during shelf life studies of the tested media. Benzyl alcohol will degrade some plastics, particularly at higher concentrations and temperatures.²⁹

1.3.2 Saline

Saline is a solution of sodium chloride (NaCl) in water, usually in concentration of 0.9 % w/v NaCl in water. In this case it is referred to as *normal saline*, and it is used for parenteral (intravenous) application. pH of this solution ranges from 4.5 to 7.0.³⁰

1.4 High Performance Liquid Chromatography

1.4.1 General

Chromatography in general comprises all separation techniques in which analytes partition between a stationary and a mobile phase. In liquid chromatography, the mobile phase is a liquid, while the stationary phase can be a solid or a liquid immobilized on a solid. High-performance liquid chromatography comprises all liquid chromatographic techniques that require the use of elevated pressures to force the liquid through the packed bed of the stationary phase. Therefore it is also called high-pressure liquid chromatography.

The traditional form of liquid chromatography employed a polar adsorbent such as silica or alumina, and a nonpolar mobile phase based on hydrocarbons. Today, this type is called normal-phase chromatography.

In reversed-phase chromatography, a nonpolar stationary phase is used in conjunction with polar, largely aqueous mobile phases. 70-80 % of HPLC applications utilize this technique. Most stationary phases are silica-based bonded phases. This kind of chromatography is popular nowadays thanks to its reproducibility and possibility of using a wide range of mobile phases.

An HPLC instrument typically includes a pump, an injector, a column, detector and a computer with specific software which will provide the analyst with the desired data. The pump delivers a known flow and composition of the mobile phase through the column. The injector brings the sample to the mobile phase stream which carries it to the column and the detector generates a signal proportional to the amount of sample component emerging from the column and allowing quantitative analysis. A schematic view of an HPLC system is presented in Figure 4.

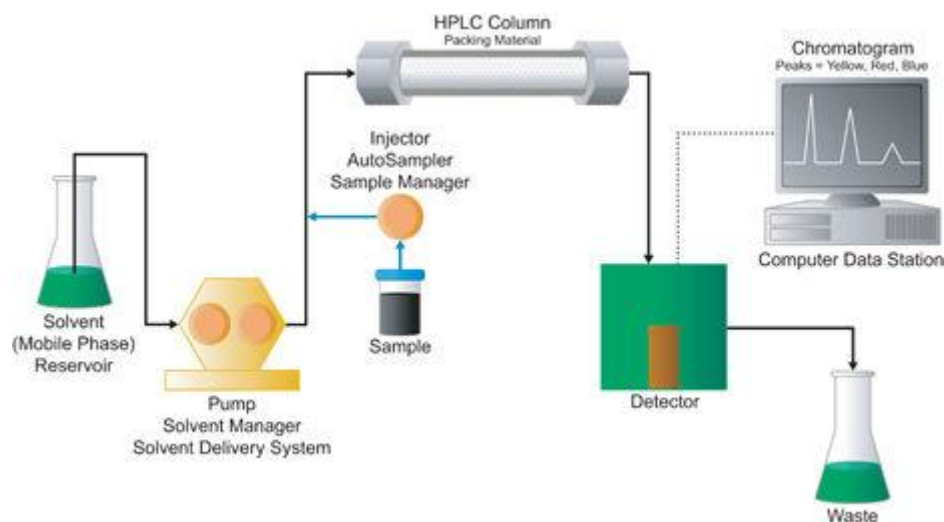


Figure 4 HPLC system scheme³¹

1.4.2 Isocratic and gradient

In isocratic chromatography, all conditions and settings of the separation are held constant. On the other hand, in gradient chromatography one or more parameters are varied. In nearly all practical cases, only one parameter is varied. The most typical gradient is variation of the mobile-phase composition from low elution to high elution strength.

1.4.3 van Deemter equation

The van Deemter equation is a simple equation describing the dependence of the HETP on the linear velocity. It assumes that the HETP is composed of three different, independent contributions, which simply add up and therefore form the observed curvilinear relationship:

$$H = A + \frac{B}{\mu} + C\mu \quad [1]$$

In this equation, A is called the Eddy diffusion and is a function of the size and distribution of the interparticle channels and other non-uniformities in the packed bed. B is inversely proportional to the linear velocity; it describes the molecular diffusion in the axial direction. C is resistance to mass transfer and is directly proportional to the linear velocity. μ is the average mobile phase velocity. The mobile phase velocity giving the optimal efficiency can be derived at the minimal plate height as depicted in Figure 5.^{32 33}

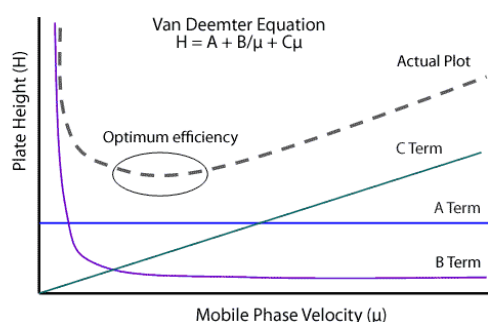


Figure 5 van Deemter equation parameters dependent on H

1.4.4 Reversed-phase HPLC

1.4.4.1 Stationary phase

The majority of reversed phase (RP) columns consist of alkyl derivatized silica particles which should not be used with aqueous bases as they will destroy the underlying silica particles. RP columns are usually used with an acidic mobile phase in the pH range 2-7. RP columns should be flushed with clean solvent after use to remove residual acids or buffers.

For RP chromatography, the most popular type is octadecyl carbon chains (C18)-bonded to silica with over 600 columns commercially available.³⁴

1.4.4.2 Mobile phase

Mixtures of water, buffers and organic solvents are used to elute analytes from a RP column.³⁵ The solvents have to be miscible with water and the most common organic solvents used are acetonitrile, methanol and tetrahydrofuran (THF). pH of the mobile phase can play a significant role on the retention of an analyte and can change the selectivity.

1.4.4.3 Mechanism

RP-HPLC operates on the principle of hydrophobic interactions, which play an important role in all processes in life science.³⁶

The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand on the stationary phase. The retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water.

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C and generally non-polar atomic bonds, such as S-S) is retained longer because it is poorly interacting with the water in the mobile phase. On the other hand, analytes with a higher polar surface area (with polar groups such as -OH, -NH₂, COO⁻, NH₃⁺ in their structure) are less retained as they are better integrated into water. Such interactions are subject to steric effects in that very large molecules may have only restricted access to the pores of the stationary phase, where the interactions with surface ligands (alkyl chains) take place. This typically results in less retention.

Retention time increases with the hydrophobic (non-polar) surface area of the analytes. Similarly, organic compounds with single C-C bonds elute later than those with a double or triple bond, as they are shorter than single bonds respectively.

1.5 Aim of the study

Intracavernous formulations for treatment of erectile dysfunction are used widely in a modern medicine. It was introduced during the 1980s and has since become a common

form of therapy.³⁷ Formulations studied in this thesis contain prostaglandin E₁ (alprostadil) at 20 and 10 µg/mL in association with papaverine HCl at 20 and 10 mg/mL respectively. While alprostadil was the main compound to be subjected to physical and chemical degradation, its degradation and possible degradation products are correlated to the stability of the formulation. The low specific absorbance of alprostadil in the ultraviolet region, and the presence of high levels of papaverine and benzylalcohol in the formulation were the main difficulties for simultaneous determination of alprostadil, papaverine and benzylalcohol which is used as preservative in the formulations.

The aim of this study is to develop a method that can be used to analyse formulations, with two different concentration profiles, overcoming the difficulties presented in the text above. The developed method has to be validated according to the ICH Harmonised Tripartite Guidelines.

Different compositions of mobile phase and different values of pH were studied to find the best chromatographic conditions for the analysis of these formulations. A sensitive, selective, linear, precise and accurate reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed.

Finally, stability of the formulation will be studied at seven different time points.

2. MATERIALS AND METHODS

2.1 Instrumentation

Most analyses were performed on a Dionex HPLC system (Sunnyvale, CA, USA) that consisted of a LPG-3400A HPLC pump, an ASI-100 autosampler equipped with a 100 μ L syringe and a LINEAR UVIS 200 UV detector which was operated at 205 nm. The dwell volume of the system was 0.4 mL. Part of the analyses was performed on a Merk-Hitachi (Darmstadt, Germany) that consisted of a L-6200 Intelligent pump, an Elite LaChrom L-2200 autosampler equipped with a 100 μ L syringe and an Elite LaChrom L-2400 UV detector which was operated also at 205 nm. The dwell volume of that system was 4.0 mL. For data processing and acquisition, Chromeleon software version 6.8 from Dionex (Sunnyvale, CA, USA) was used. The pH measurements were performed on a 691 Metrohm pH meter (Herisau, Switzerland). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to further purify demineralized water. Chromatographic separations were achieved on an Alltima C18 5 μ column (250 mm x 4.6 mm, 5 μ m) from Alltech Associates, Inc. (Nicholasville, Kentucky, USA) All prepared mobile phases were degassed by purging with helium. The Dionex instrumentation is shown in Figure 6. The Alltima column is shown in Figure 7.



Figure 6 Dionex pump and autosampler

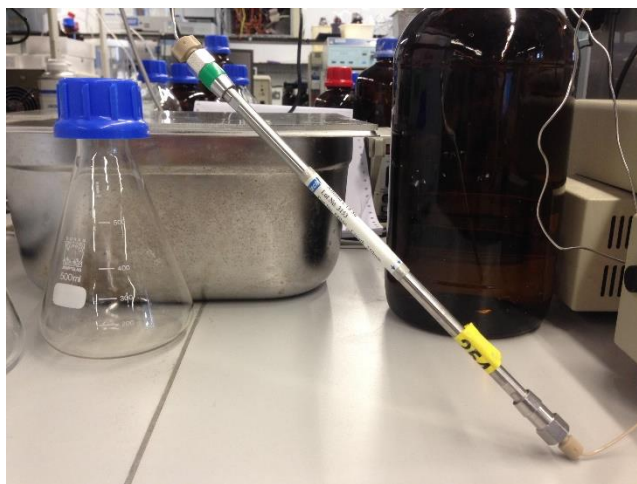


Figure 7 Alltima C18 column

2.2 Chemicals and reagents

HPLC gradient grade acetonitrile was from Fisher Scientific (Leicestershire, UK). Potassium dihydrogen phosphate and phosphoric acid were from Merck (Darmstadt, Germany).

2.3 Samples and reference substances

Commercial samples of alprostadil were obtained from Pfizer as the commercial drug Prostin® VR, 0.5 mg of alprostadil per mL of ethanol as an injectable solution. Papaverine HCl ampules were obtained in a concentration of 100 mg/3 mL as in injectable solution. Benzyl alcohol came as a 2 mL ampule and a 0.9 % sodium chloride solution Viaflo bag was from Baxter.

2.4 Experimental part

2.4.1 Sample preparation

Stability study and method validation presented in this thesis were developed for two formulations, both consisting of alprostadil, papaverine HCl, benzyl alcohol and saline solution. Concentrations of main components in the formulations were different for each of the two formulations as can be seen in Table 1.

Table 1 Formulations used for stability study and method development

<u>Formulation 1</u>	Prostin VR	20 µg
	Papaverine HCl	20 mg
	Benzyl alcohol	9 mg
	Saline	to 1 mL
<u>Formulation 2</u>	Prostin VR	10 µg
	Papaverine HCl	10 mg
	Benzyl alcohol	9 mg
	Saline	to 1 mL

As a reference for formulation 1, Prostin VR was diluted with MilliQ water or saline solution to obtain an alprostadil concentration of 20 µg/mL which was used in the analytical method. For the determination of alprostadil, formulation 1 was injected as such. Papaverine ampules were diluted with MilliQ water in order to get a drug formulation concentration of 20 mg/mL. However, due to a very large peak area, this concentration had to be 1000 times diluted with MilliQ water to obtain a test concentration that was used to perform analysis. For the determination of papaverine, formulation 1 was also diluted 1000 times. The same way of sample and reference preparation can be applied to formulation 2.

Injection volume used in the method is 50 µL.³⁸ However, in few cases, 100 µL was used. Reason for that will be explained further in the thesis.

2.4.2 Current European Pharmacopoeia method for individual drugs

2.4.2.1 Prostaglandin E₁

With similar column properties as used in this thesis, the Pharmacopoeia method is the following: it consists of two mobile phases, A and B. Mobile phase A is prepared by dissolving 3.9 g of KH₂PO₄ in water and diluting it to 1000.0 mL with the same solvent. It needs to be adjusted to pH 2.5 with a 2.9 g/L solution of phosphoric acid (around 600 mL). 740 mL of the buffer solution and 260 mL of acetonitrile are required for mobile phase A. Mobile phase B is prepared the same way, but solvents are added in a different ratio. 200 mL of the buffer solution and 800 mL of acetonitrile are required for mobile phase B. Flow rate is 1 mL/min with 20 µL loop injector and detection wavelength 200 nm. Retention time of alprostadil with this system is about 63 min.³⁹

2.4.2.2 Papaverine

For papaverine similar column properties as mentioned above for alprostadil are used. Mobile phase A is 3.4 g/L solution of KH_2PO_4 adjusted to pH 3.0 with dilute phosphoric acid. Mobile phase B is acetonitrile, and C is methanol. Gradient method for papaverine included in the Pharmacopoeia is shown in Table 2.

Table 2 Gradient method for papaverin HPLC analysis

Time (min)	Mobile phase A (per cent V/V/V)	Mobile phase B (per cent V/V/V)	Mobile phase C (per cent V/V/V)
0 - 5	85	5	10
5 - 12	85 → 60	5	10 → 35
12 - 20	60	5	35
20 - 24	60 → 40	5 → 20	35 → 40
24 - 27	40	20	40
27 - 32	40 → 85	20 → 5	40 → 10

With this composition of mobile phases the flow rate used is 1 mL/min, detection wavelength is 238 nm and injection volume is 10 μL .⁴⁰

2.4.3 Chromatographic conditions

The separation was performed on an Alltima C18 column (250×4.6mm; 5 μm particles) with a gradient method and a mixture of phosphate buffer (KH_2PO_4 0.02 M, pH 3) and acetonitrile as a mobile phase. The mobile phase ratio was variable: 0-11 min and 19-29 min with a (62:38, v/v) mixture of phosphate buffer and ACN, and from 12-18 with a (30:70, v/v) mixture. The mobile phases were manually mixed. The injection volume was 50 μL . Samples were held at a temperature of 4 °C in the autosampler with analysis performed at room temperature. Mobile phase flow rate was 1.2 mL/min for the duration of analysis. Wavelength used was 205 nm.

2.4.4 Procedures for method validation

Validation was performed for the main peaks, alprostadil and papaverine, for specificity, linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), precision, repeatability and solution stability.

2.4.4.1 Specificity

Specificity was shown by examining potential interferences between formulation components like benzyl alcohol, alprostadil and papaverine and their degradation products.

2.4.4.2 Degradation studies

Forced degradation studies were performed on alprostadil and papaverine in order to demonstrate the selectivity and stability indicating capability of the proposed method. Analytes were exposed to a temperature of 80 °C for 24 hours. Samples were then analyzed by the proposed method.

2.4.4.3 Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) for both alprostadil and papaverine were determined at a signal-to-noise ratio of 3 and 10, respectively.

2.4.4.4 Linearity

Linearity responses of the detector for alprostadil and papaverine were examined around the test concentrations (20 µg/mL for both analytes). Percentages were 120 %, 100 %, 80 %, 60 % and 50 % of the test concentration corresponding to concentrations of 24, 20, 16, 12, 10 µg/mL respectively.

2.4.4.5 Accuracy

The accuracy was calculated by comparing numerical values of peak areas given by the main peaks in spiked blanks and their reference solution peaks with an acceptance criterion between 98.0 and 102.0 %.

2.4.4.6 Precision

Repeatability was determined by injecting the two formulation solutions six times. Alprostadil concentrations in the formulations were 20 µg/mL and 10 µg/mL respectively while concentrations of papaverine were 20 mg/mL and 10 mg/mL. Test

solutions for alprostadil were injected as such. Test formulations for papaverine were diluted 1000 times with MilliQ water, giving a test concentrations of 20 and 10 $\mu\text{g}/\text{mL}$. The percentage RSD was calculated for the two main peaks in both formulations.

3. RESULTS AND DISCUSSION

3.1 Development and optimisation of the analytical method

The main difficulty in the development of the method for determination of alprostadil and papaverine in the formulation simultaneously is the low specific absorbance in the UV zone and low concentration of alprostadil. That, together with the high concentrations of papaverine and benzyl alcohol, made the proper separation of the two main peaks challenging, but in the end, achievable.

UV detection was chosen instead of other detection modes cited in the literature because of its robustness and precision.

Levels of papaverine and benzyl alcohol were high compared to alprostadil, and therefore saturating the detector when no dilution was performed. The separation of alprostadil depended largely on the wideness of the papaverin and benzyl alcohol peaks. Optimization of chromatographic conditions was made and good resolution with symmetrical peak shapes was achieved. Analysis time is relatively short.

3.1.1 Wavelength selection

The UV detector could only select one wavelength at a time, and it was set to 205 nm, which is found to be the optimal wavelength for alprostadil determination based on the signal to noise ratio. Low specific absorbance of alprostadil was preventing the usage of higher wavelength settings.

3.1.2 Mobile phase

Composition of the mobile phase and its pH value are both important factors in the development of the analytical method. Chosen organic solvent was acetonitrile (ACN). Its cut-off value is at 190 nm. So it is compatible with detection at 205 nm. Different values of pH and proportions of ACN and phosphate buffer have been tested. pH 3.0 was selected as it gave good resolution between the main peaks. pH 3.5 and 4.0 were tested as well, but with pH 3.0 the separation was optimal. Proportions of ACN under 40 % allowed a good resolution between the peaks of papaverine, benzyl alcohol, and alprostadil. Proportions above that value were not suited for a good separation and lead

to coelution of papaverine and benzyl alcohol. Proportions of ACN under 30 % were not suited because they affected elution times, and therefore would make the analysis longlasting. The proportion of 38 % ACN and 62 % phosphate buffer (0.02 M) lead to a good separation and short retention times for the main peaks with the alprostadil peak eluting around 11 min.

However, with degradation studies, analysis time was prolonged to 29 minutes. A gradient method by changing the mobile phase composition was used to improve the analysis. Mobile phase A is as described above (38 % ACN and 62 % phosphate buffer 0.02 M). Composition of mobile phase B was 70 % of ACN and 30% of phosphate buffer 0.02M after testing several different compositions. Due to possible precipitation of the buffer, compositions above 70 % of ACN were discarded. Compositions under 70 % ACN were suitable for the method, but in the end 70 % of ACN was chosen because of an optimal analysis time. In table 3, the gradient used in this method is shown.

Table 3 Method - mobile phase gradient

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 11	100	0
11 - 12	100 → 0	0 → 100
12 - 18	0	100
18 - 19	0 → 100	100 → 0
19 - 29	100	0

3.1.3 Flow rate

In order to shorten the analysis time, but keep the flow rate in the optimal range, a flow of 1.2 mL/min was used.

3.2 Method validation

3.2.1 Specificity

The method is specific enough to have a good separation between alprostadil peak (around 11th minute) and partly co-eluted papaverine and benzyl alcohol peaks (between 3rd and 6th minute) which can be seen in Figure 8. It is also specific enough for a good separation between papaverine and benzyl alcohol peaks if diluted 1000 times

with MilliQ water. This can be seen in Figure 9. Detection of alprostadil in this case is not possible due to its very small concentration after dilution.

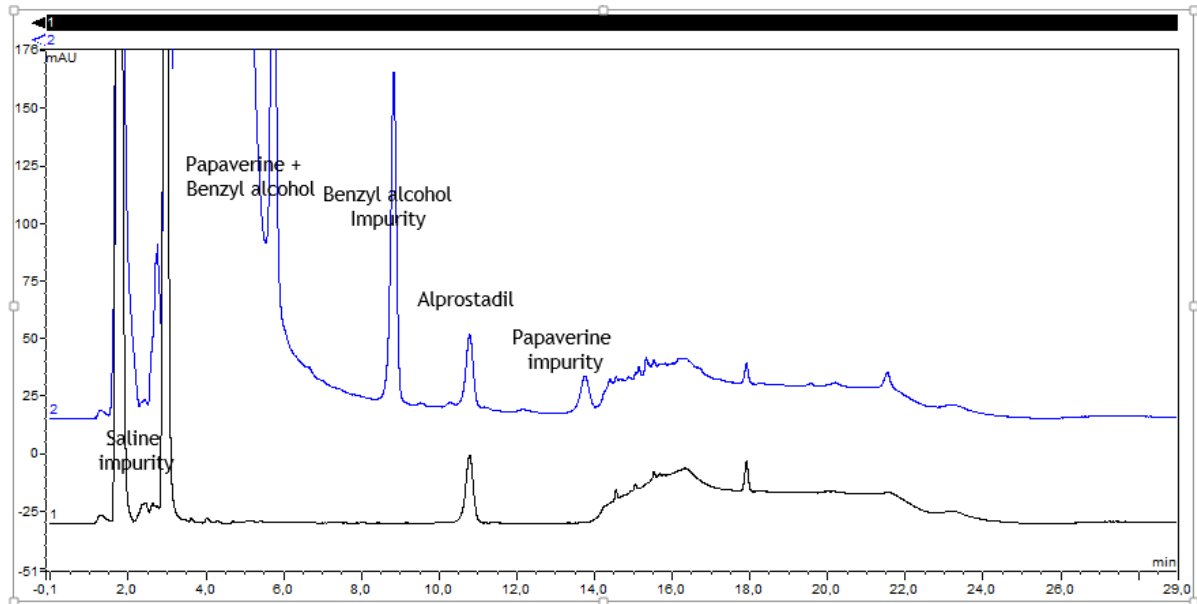


Figure 8 Chromatogram of alprostadil reference (1) and the formulation injected as such (2)

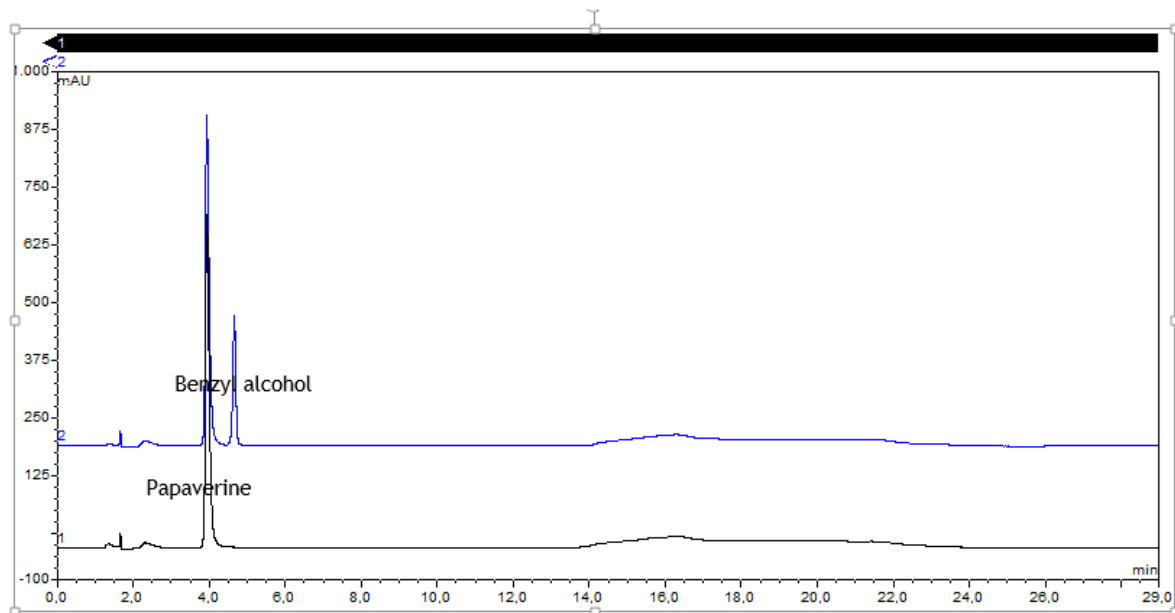


Figure 9 Chromatogram of papaverine reference (1) and the formulation [1:1000 diluted] (2)

3.2.2 Degradation studies

Chromatogram obtained under stress conditions for alprostadil is shown in Figure 10. Both alprostadil and papaverine samples were exposed to a temperature of 80 °C for 24

hours. Alprostadil sample chromatograms after exposure reveal new peaks that are presumed to be degradation products. After 7 hours of exposure, the alprostadil peak area was considerably decreased and after 24 hours the alprostadil peak was completely gone. The papaverine sample chromatogram after degradation study did not reveal new peaks related to degradation products. It remained stable, although a small peak area decrease was noted. From chromatograms 2 and 3, shown in Figure 10, it is clear why a gradient program was introduced. This adjustment was necessary because degradation peaks appeared around 26 and 30 min if no gradient was applied. With gradient, they appeared around 19 minutes (chromatogram 3). After their elution, the mobile phase composition returns to its starting conditions. That gives 29 minutes as total analysis time for this method.

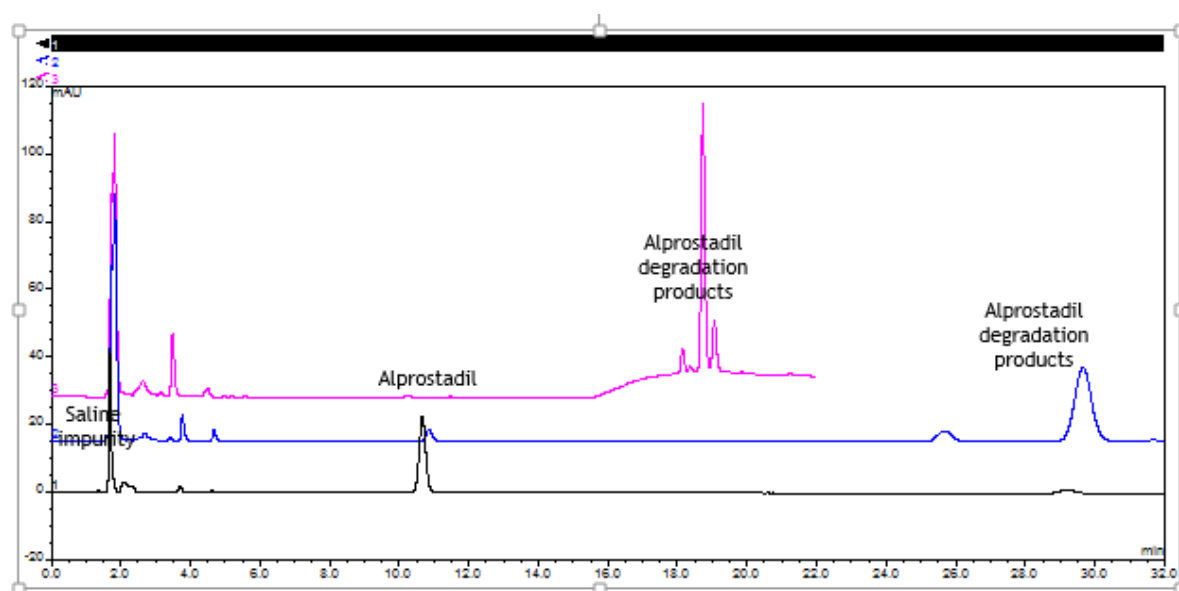


Figure 10 Chromatograms of alprostadil degradation studies: alprostadil sample before exposure to 80 °C, isocratic (1); alprostadil sample after 7h of exposure to 80 °C, isocratic (2); alprostadil sample after 24 hours of exposure to 80 °C, gradient (3)

3.2.3 Sensitivity

Limit of detection and limit of quantification were determined. For alprostadil, LOQ calculated was 240 ng/mL, with an injection volume of 50 μ L corresponding to a signal-to-noise ratio of 10. LOD in this case was 100 ng/mL, with an injection volume of 50 μ L corresponding to a signal-to-noise ratio of 3.

For papaverine the LOQ calculated was 40 ng/mL, with an injection volume of 50 μ L corresponding to a signal-to-noise ratio of 10. LOD in this case was 12 ng/mL, with an injection volume of 50 μ L corresponding to a signal-to-noise ratio of 3.

As mentioned before, two different instrumentations, on which analyses were performed, were used in this thesis. Firstly, method development was performed on a Merck-Hitachi system (Darmstadt, Germany). Problem occurred when LOQ and LOD for papaverine were in the process of validation. It was found that there was a system peak co-eluting with the papaverine peak. Therefore LOQ and LOD for papaverine could not be determined. Many attempts were taken in order to separate the co-eluted peaks, but in the end they were all unsuccessful. Solution was to change the system and from that point, all analyses and validation procedures were performed on a Dionex HPLC system (Sunnyvale, CA, USA). Validation procedures already validated on the Merck-Hitachi system were validated again on the Dionex system.

3.2.4 Linearity

Linear calibration curves between peak area and concentration were obtained for both alprostadil and papaverine. Alprostadil regression data analysis shows that R^2 value is 0.9999 (Figure 11). The 95 % confidence interval of the intercept does not contain zero, which is considered unreliable in statistical terms. However, with the standard error being extremely small, the interval is also small, and the fact that it does not contain zero is acceptable. When the intercept is expressed versus the peak area of 100 % concentration, it amounts to 1.7 % which is lower than the generally accepted limit of 5 %. The residual plot shows no trend (Figure 12). Therefore, linear correlation can be considered good and satisfying.

Papaverine regression data analysis shows R^2 value of 0.9998 (Figure 13). The 95 % confidence interval in this case contains zero which shows good linear correlation. The residual plot obtained from data analysis is shown in Figure 14.

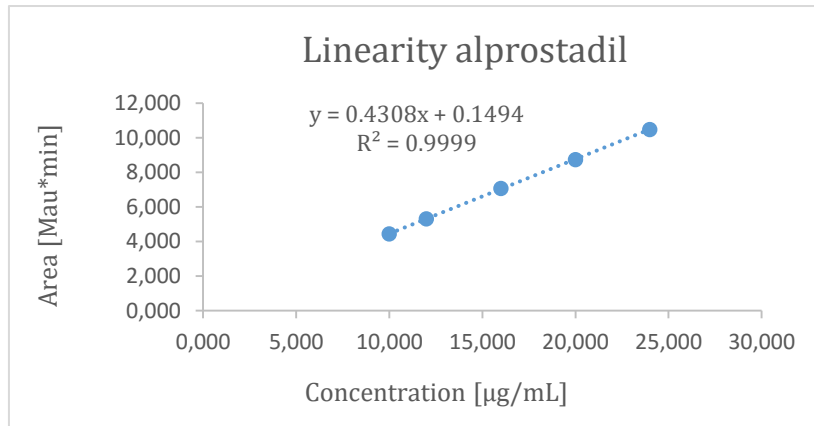


Figure 11 Calibration plot showing linearity of alprostadil

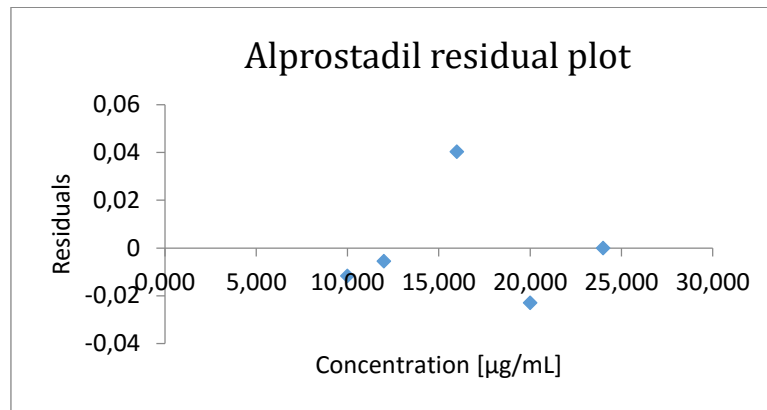


Figure 12 Residual plot for alprostadil

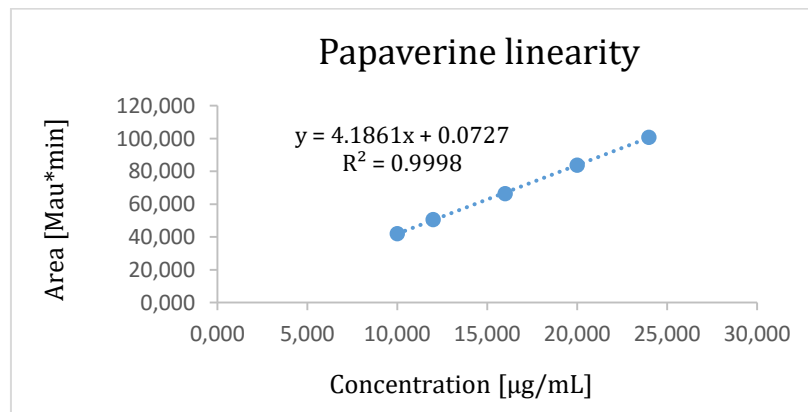


Figure 13 Calibration plot showing linearity of papaverine

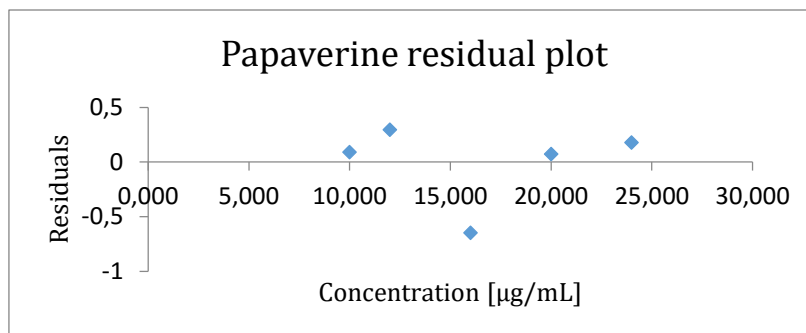


Figure 14 Residual plot for Papaverine

3.2.5 Accuracy

Percentages of recovery were determined by comparing spiked analyte peak area mean gained from three injections, and reference peak area mean, also from three injections. Acceptance criteria were expressed in percentages from 98.0-102.0 %. Two formulations have been tested, with different concentrations of main components. Alprostadil and papaverine recovery for both formulations can be seen in Tables 4 and 5.

Table 4 Recovery of alprostadil

Alprostadil	Sample mean [Mau*min]	Reference mean [Mau*min]	Recovery (%)
Formulation 1 Inj. Vol (50µL)	8.962	9.014	99.4
Formulation 2 Inj. Vol (100 µL)	7.066	7.016	100.7

Table 5 Recovery of papaverine

Papaverine	Sample mean [Mau*min]	Reference mean [Mau*min]	Recovery (%)
Formulation 1	84.815	85.532	99.2
Formulation 2	87.692	86.283	101.9

Injection volume used for this method is, as stated before, 50 µL. However, 100 µL injection volume was used for accuracy validation of alprostadil in formulation 2. Formulation 2 contains 10 µg/mL of alprostadil, which is two times less than what

formulation 1 contains. This way similar peak areas should be obtained. As mentioned before, peak areas and concentrations of alprostadil in both formulations are very small. Therefore, accuracy test for alprostadil in formulation 2 was performed in a way to get the same peak area as in formulation 1. This way, for both formulations, peak areas already validated in linearity test were obtained.

All accuracy tests follow acceptance criterion and are in the range between 98.0 and 102.0 %. However, with 100 µL injections used for accuracy validation of alprostadil in formulation 2 a decrease in peak area, compared to formulation 1, for both sample and reference is noticed. Since the device on which analysis were performed was validated, it is concluded that the reason for such a deviation lies in different amounts of excipients present in each of the formulations, specifically saline. Main components are held in a different environment and this could affect the difference in peak areas when it concerns alprostadil since this component is sensitive.

To confirm conclusions mentioned above alprostadil reference was tested in both water and saline as dissolution media. Difference in peak areas was noted and results can be seen in Table 6.

Table 6 Recovery of alprostadil in different dissolution media

	Area [Mau*min]	Recovery (%)
Reference Saline	8.365	97.82
Reference Water	8.551	

3.2.6 Precision

The % RSD values for precision were <2 %, which confirms that the method is sufficiently precise. Results can be seen in Tables 7 and 8. For alprostadil presented in formulation 2, the same precision validation principle was used as mentioned in 4.2.5 for accuracy.

Table 7 Precision validation of alprostadil

Alprostadil	Sample mean [Mau*min]	SD	RSD (%)
Formulation 1 Inj. Vol. (50 µL)	8.927	0.056	0.62
Formulation 2 Inj. Vol. (100 µL)	7.055	0.034	0.48

Table 8 Precision validation of papaverine

Papaverine	Sample mean [Mau*min]	SD	RSD (%)
Formulation 1	86.442	0.452	0.52
Formulation 2	88.329	0.329	0.37

3.3 Stability study

Stability study of formulation 1 presented in Table 9 will be tested in the time span of several months, at seven different time points. Formulation was prepared as one solution. Part of the solution was filled in syringes (1 mL) and part in vials (5 mL). Stability study was conducted on both vial and syringe solutions.

Stability study samples and references preparation will be carried out the same way as it was during method validation. Percentages of recovery will be determined by comparing spiked analyte peak area mean gained from three injections, and reference peak area mean, also from three injections. It will be conducted for both of the main compounds in the formulation.

Table 9 Formulation for stability study

Formulation 1		
	Prostin VR	20 µg
	Papaverine HCl	20 mg
	Benzyl alcohol	9 mg
	Saline	to 1 mL

3.3.1 Starting point (t_0)

Results obtained for starting point (t_0) of stability study can be seen in Tables 10 and 11. Results indicate that developed analytical method is suitable for stability testing at this time point and that method is comparable between syringe and vial solutions. Recovery criterion was expressed in percentages which should be in range from 95.0 to 105.0 %.

Relevant chromatograms are shown in Figures 15 and 16.

Table 10 Recovery of alprostadil (t_0)

Alprostadil	Sample mean [Mau*min]	Reference mean [Mau*min]	Recovery (%)
Vial Inj. Vol (50 μ L)	8.409	8.365	100.53
Alprostadil	Sample mean [Mau*min]	Reference mean [Mau*min]	Recovery (%)
Syringe Inj. Vol (50 μ L)	8.448	8.365	101.00

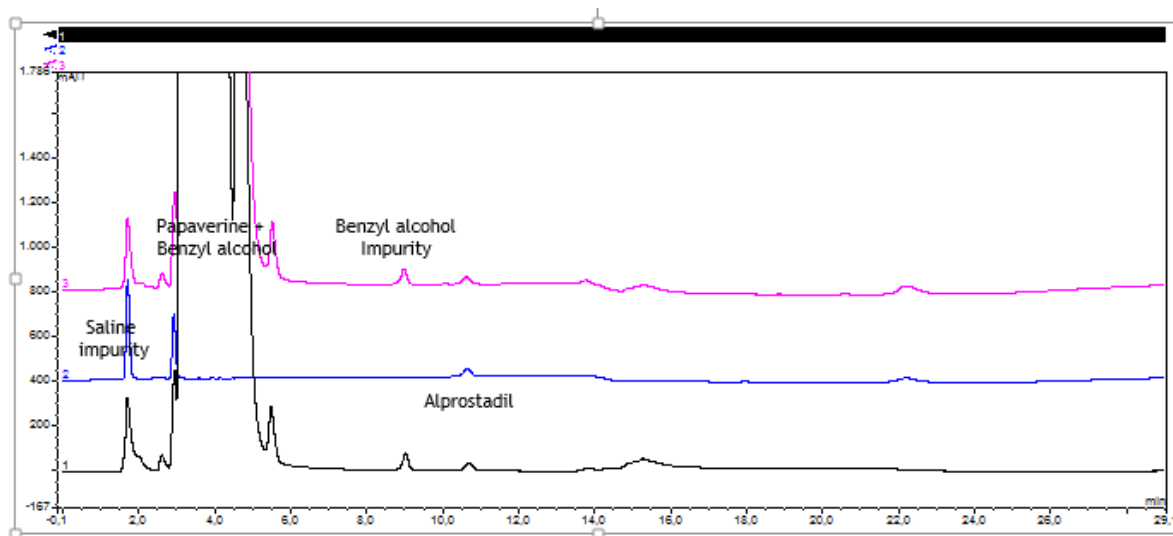


Figure 15 Stability study t_0 chromatogram: vial sample (1); alprostadil reference (2); syringe sample (3)

Table 11 Recovery of papaverine (t_0)

Papaverine	Sample mean [Mau*min]	Reference mean [Mau*min]	Recovery (%)
Vial Inj. Vol (50 μ L)	86.381	85.817	100.66
Papaverine	Sample mean [Mau*min]	Reference mean [Mau*min]	Recovery (%)
Syringe Inj. Vol (50 μ L)	81.124	83.208	97.50

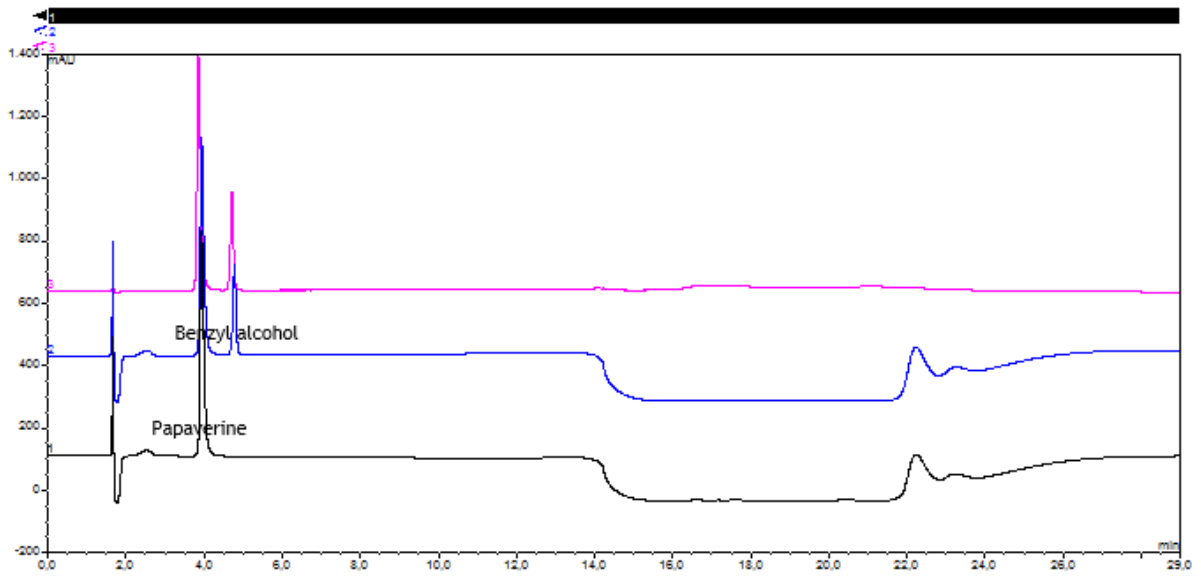


Figure 16 Stability study t_0 chromatogram: papaverine reference (1); vial sample (2); syringe sample (3)

4. CONCLUSION

An optimized reversed phase HPLC method, for the stability study of formulations containing prostaglandin E₁ and papaverine HCl as main components, has been developed. The method is specific, linear, accurate, sensitive, and precise when it comes to the two main components. The method will be used to check the stability of alprostadil – papaverine formulation 1 over several months. Starting time point (t₀) has been tested for stability and results obtained can indicate that developed analytical method is suitable for such a test at starting time point. Following stability study tests will show whether the method will continue to provide a good base for stability study of given formulation.

5. ŽIVOTOPIS

Rođen sam u Zagrebu 28.09.1991. Nakon osnovne škole Šestine upisujem Sedmu gimnaziju u Zagrebu. Uz matično obrazovanje, završavam osnovnu i srednju glazbenu školu Vatroslava Lisinskog u Zagrebu na Odjelu za glasovir i orgulje. Fakultet kemijskog inženjerstva i tehnologije upisujem 2010. godine te stječem titulu sveučilišnog prvostupnika inženjera kemijskog inženjerstva 2014. godine kada upisujem i diplomski studij na istom fakultetu.

Kroz preddiplomski studij s prof. Aleksandrom Sander i prof. Markom Rogošićem koautor sam znanstvenog rada "Ravnoteža kapljevina-kapljevina za sustav ugljikovodik - tiofen (piridin) – ionska kapljevina". S radom sam sudjelovao na dvije međunarodne znanstvene konferencije te iščekujem publikaciju.

Od 2013. godine do danas član sam studentske udruge eSTUDENT gdje sam godinu dana obnašao funkciju voditelja tima za odnose s javnošću. Preko udruge sam sudjelovao u ljetnoj školi u Dubrovniku na temu intelektualnog kapitala 2014. godine. Iste godine timski sam se prijavio na Case Study Competition te ponudio rješenje za Inin marketinški slučaj.

Zadnji semestar studija provodim u gradu Leuvenu u Belgiji, na Fakultetu farmaceutskih znanosti Katoličkog sveučilišta Leuven gdje izrađujem diplomski rad.

6. REFERENCES

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- ¹ R. J. Nelson (2005) „*An introduction to behavioral endocrinology* (3rd ed.)“ Sunderland, Mass: Sinauer Associates. p. 100
- ² W. Cawello, A. Leonhardt, H. Schweer, H. W. Seyberth, R. Bonn, A. L. Lomeli (September 1995) „*Dose proportional pharmacokinetics of alprostadil (prostaglandin E₁) in healthy volunteers following intravenous infusion*“. *British Journal of Clinical Pharmacology* 40 (3): 273–6
- ³ S. M. Meller (BA), E. Stilp (MD), C. N. Walker (MD), C. Mena-Hurtado (MD) (June 2013) „*The Link Between Vasculogenic Erectile Dysfunction, Coronary Artery Disease, and Peripheral Artery Disease: Role of Metabolic Factors and Endovascular Therapy*“. *Journal of Invasive Cardiology*. 25 (6): 313–319
- ⁴ J. C. Jung and O. S. Park „*Efficient Asymmetric Synthesis of Prostaglandin E₁*“ *Z. Naturforsch.* 2007, 62b, 556 – 560; received July 31, 2006
- ⁵ M. North „*Sustainable Catalysis: Without Metals or Other Endangered Elements Part 1*“, pg 171, 2015, Royal Society of Chemistry
- ⁶ www.chemspider.com [April 2016]
- ⁷ European Pharmacopoeia 8.0 Volume II 01/2014 pg 1350, EDQM, Strasbourg, France
- ⁸ www.sigmaaldrich.com Material Safety Data Sheet (MSDS) [April 2016]
- ⁹ www.drugs.com [April 2016]
- ¹⁰ V. N. Varu, M.E. Hogg, M.R. Kibbe, (January 2010). „*Critical limb ischemia.*“. *Journal of vascular surgery* 51 (1): 230–41.
- ¹¹ www.drugs.com [April 2016]
- ¹² V. Vieillard, N. Ghorbel, C. Deffaux, A. Astier, R. Yiou and M. Paul „*Development and Validation of a Stability- Indicating High Pressure Liquid Chromatography Method for Determination of Prostaglandin E₁ and its Degradation Products in an Intracavernous Formulation*“; Vieillard et al., *Pharmaceutica Analytica Acta* 2013, 4:4
- ¹³ www.sigmaaldrich.com [April 2016]
- ¹⁴ Product information sheet; Data for Biochemical Research, 3rd ed. (Oxford Press, 1986), 346-347
- ¹⁵ M. Georg (1848) „*Vorläufige Notiz über eine neue organische Base im Opium*“ [Preliminary notice of a new organic base in opium]. *Annalen der Chemie und Pharmacie*
- ¹⁶ www.link.springer.com [April 2016]
- ¹⁷ M. S. Hifnawy, F. J. Muhtadi „*Analytical Profiles of Drug Substances*“; Volume 17, 1988, pg 367–447
- ¹⁸ www.druginfosys.com [April 82016]
- ¹⁹ European Pharmacopoeia 8.0 Volume II 01/2014 pg 2666. EDQM, Strasbourg, France
- ²⁰ www.chemicaland21.com [April 2016]
- ²¹ P. Desvaux (2005) „*An overview of the management of erectile disorders*“ *Presse medicale* (Paris, France : 1983) 34 (13 Suppl): pg 5–7.
- ²² A. J. Bella, G. B. Brock (2004) „*Intracavernous Pharmacotherapy for Erectile Dysfunction*“. *Endocrine* 23 (2–3): pg 149–155

-
- ²³ B. L. Clyde, A. D. Firlik, A. M. Kaufmann, M. P. Spearman, H. Yonas (April 1996) "*Paradoxical aggravation of vasospasm with papaverine infusion following aneurysmal subarachnoid hemorrhage. Case report*". J. Neurosurg. 84 (4): 690–5
- ²⁴ www.druginfosys.com [May 2016]
- ²⁵ www.medsafe.govt.nz [May 2016]
- ²⁶ www.chemicaland21.com [May 2016]
- ²⁷ www.druginfosys.com [May 2016]
- ²⁸ L. Bhattacharyya, S. Schuber, C. Sheehan, R. William (2006) "*Excipients: Background/Introduction*". In Katdare, Ashok; Chaubal, Mahesh. Excipient Development for Pharmaceutical, Biotechnology, and Drug Delivery Systems, Informa Health Care USA Inc., New York, pg 1-2
- ²⁹ „*Use of benzyl alcohol as a shipping and storage solution for chromatography media*“; Process chromatography Application note 28-9899-01 AC GE; 2014; Healthcare Bio-Sciences AB, Uppsala, Sweden
- ³⁰ www.baxter.ca [May 2016]
- ³¹ www.waters.com [May 2016]
- ³² U. D. Neue; "*HPLC columns : theory, technology, and practice*", 1997, New York, Wiley-VCH
- ³³ www.chromatographytoday.com [May 2016]
- ³⁴ USP Chromatographic Reagents 2007-2008: Used in USP-NF and Pharmacopeial Forum. United States Pharmacopeia. 2007.
- ³⁵ A. Mehta (2012) "*Principle of Reversed-Phase Chromatography HPLC/UPLC (with Animation)*". PharmaXChange
- ³⁶ F. Gerber, M. Krummen, H. Potgeter, A. Roth, C. Siffrin, C. Spoendlin (2004) "*Practical aspects of fast reversed-phase high-performance liquid chromatography using 3µm particle packed columns and monolithic columns in pharmaceutical development and production working under current good manufacturing practice*". Journal of Chromatography A, 1036 (2); pg 127-133
- ³⁷ R. Virag *et al*: "*Intracavernous self-injection of vasoactive drugs in the treatment of impotence: 8-year experience with 615 cases.*" The Journal of Urology, 1991; 145: pg 287-293
- ³⁸ R. A. Uebel, C. A. Wium and A. C. Schmidt: „*Stability evaluation of a prostaglandin E1 saline solution packed in insulin syringes*“; International Journal of Impotence Research (2001) 13, pg 16-17
- ³⁹ European Pharmacopoeia 8.0 Volume II 01/2014 pg 1513. EDQM, Strasbourg, France
- ⁴⁰ European Pharmacopoeia 8.0 Volume II 01/2014 pg 2963. EDQM, Strasbourg, France