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Chemical Analysis of Ophthalmic Solutions

Pagagiotis Tavlarakis
Seton Hall University

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By

Panagiotis Tavlarakis

DISSERTATION

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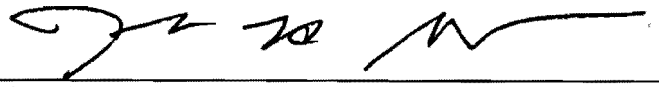
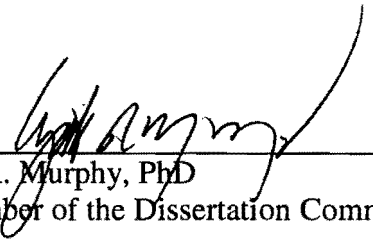
We certify that we have read this dissertation and that in our opinion it is adequate in scientific scope and quality as dissertation for the degree of Doctor of Philosophy.

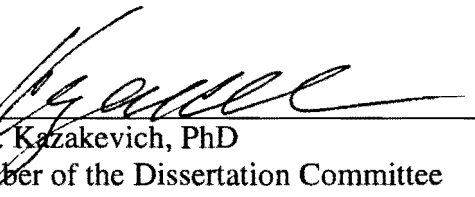
Approved



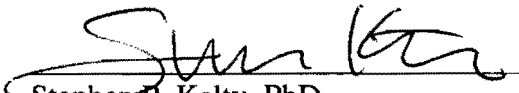
Nicholas H. Snow, PhD
Research Mentor



W. R. Murphy, PhD
Member of the Dissertation Committee



Y. V. Kazakevich, PhD
Member of the Dissertation Committee



Stephen P. Kelty, PhD
Chair of the Department of Chemistry and Biochemistry

Abstract

An array of over the counter ophthalmic solutions (eye drops) is available for the self treatment of minor ophthalmic disorders. They are used all over the world for the treatment of minor eye episodes including redness, dry eye and tear generation. Because components in ophthalmic solutions may cause adverse reactions, there is a need for modern, rugged chemical analysis techniques for these components using straightforward, inexpensive and readily available instrumentation and methods. This work focuses on the chemical analysis of ophthalmic solutions in three major areas; preservatives, lubricants, and active components. Each feature plays an integral part in the total effectiveness of the ophthalmic solution, so several components were studied extensively with different chromatographic techniques. The emergence of new complex matrices which target multi symptom relief in the eye makes development of straightforward new techniques increasingly challenging.

One of the three primary aspects of this work was the analysis of preservatives, which is important for inhibiting microbial growth and ensuring safety. Benzalkonium chloride (BAC) is widely used in ophthalmic solutions and is often difficult to analyze due to its multicomponent nature. Further, the several homologues do not posses identical bactericidal activity, with the C12 homologue most effective against yeast and fungi, the C14 homologue against gram-positive bacteria, and the C16 homologue against gram-negative bacteria. A fast, simple, isocratic, high performance liquid chromatography based in ultra violet (HPLC-UV) detection method was sufficient to fully separate the BAC homologues from each other and from other components in typical solutions.

Second, lubricants, or demulcents, are often present in ophthalmic solutions and

are used to control the solution viscosity. Additionally, they offer a medicinal benefit, relieving pain in inflamed or irritated mucous membranes. Three different demulcents were studied: polyvinylpyrrolidone (PVP), polyethylene glycol 400 (PEG400) and glycerin. As PVP and PEG400 are polymeric, size exclusion chromatography (SEC) is the technique of choice, however, most SEC methods focus on molecular weight characterization rather than quantitative analysis. In this work, total PVP in ophthalmic solutions was determined using the unusual combination of size exclusion chromatography, ultraviolet-visible detection and quantitation of an analyte peak that elutes before the void volume disturbance. A more conventional size exclusion chromatography method was used for the simultaneous determination of PEG400 and glycerin in ophthalmic solutions, using size exclusion chromatography, with refractive index detection.

Third, analysis of the active component, along with possible degradants and impurities is critical in any ophthalmic solution. The increasing attention that degradants and impurities are receiving from the regulatory authorities makes these methods important to the development of ophthalmic solutions. One challenge in ophthalmic solutions is the analysis of oxymetazoline hydrochloride (OXY) and its known degradation product (N-(2-aminoethyl)-2-[4-(1,1dimethylethyl)-3-hydroxy-2,6-dimethylphenyl]-acetamide) (OXY-DEG). Although a number of HPLC methods have been developed to determine OXY in a variety of products, none of these methods also determined OXY-DEG, which appears at much lower concentration. In this work, a straightforward, isocratic reversed-phase HPLC-UV method was sufficient to quantify both OXY and OXY-DEG in a single analysis, although their concentrations are orders of

magnitude different. Forced degradation studies performed during method validation revealed that OXY-DEG is likely a base hydrolysis product of OXY.

In the analysis of over the counter medications such as ophthalmic solutions, which are widely used at low cost, there is an increasing need for new analytical methods that are low cost, simple to operate and use straightforward instrumentation, while maintaining similar sensitivity and reproducibility requirements to techniques employing more sophisticated instrumentation. In this work, several straightforward techniques for the analysis of components in ophthalmic solutions, each of which represents an improvement over previous literature techniques, are demonstrated.

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Dedication

This work is dedicated to my Family

&

In loving memory of my father, Antonios Tavlarakis, who left early from this world but his valuable lessons in life had a profound impact on my life as well

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Chapter 1: Introduction

1.1 Historical Overview

The history of ophthalmic solutions coincides with the history of Ophthalmology, the branch of medicine that deals with the anatomy, physiology and diseases of the eye. The word has Greek roots from the word "ophthalmos" which means eye and the word "logos" which means speech, word, and communication of thought with words. Literally the word ophthalmology means the science of eyes [1].

Due to unrecorded uncertainties in the history of ophthalmology, it is impossible to say where exactly the treatment of the eyes began, but the earliest mention of any medical matter is found to go back as far as 2000 B.C. Any medical treatment at that time was totally at the hands of priests. The first written document about treatment of the eye came later, around 1650 B.C. in Egypt where a 110 page document (Papyrus Ebers) was found that described all the diseases and remedies that were known to Egyptians of that time. From the 110 pages, around 8 pages were dedicated to diseases of the eye and treatment for such diseases. Some of the remedies at the time used onions, castor oil, pomegranate, copper salts, hemlock and opium. Learning and performing medicine at that time was done at temples, as priests were doctors as well.

The separation of scientific medicine from temple practice was not completed until 460 B.C. during the golden age of Greece. Hippocrates considered the father of medicine is credited with accomplishing this separation by introducing the science of observation and reasoning into medicine.

In those days, ophthalmic diseases were treated with venesection, cupping and occasionally by drawing the humours away from the eye. Chronic diseases were treated mainly with local application of milk of women, gall of goats, and various preparations of copper, iron, and lead. For the next 800 years and from the time of Hippocrates to the end of the era of Roman Empire, Greek medicine dominates the field and was practiced not only in Greece but extended to Rome and Alexandria.

The Alexandrian school introduced Collyria for treatment of eye diseases. It was a solid substance made up of several secret ingredients in a cake form with gum being the basis of the formulation. Prior to its application a fragment of the cake was dissolved in water, oil, milk of women, urine, bile, or saliva.

During this time the disease that was scourge to the eyes was trachoma, an infectious disease caused by bacteria which produce roughening of the inner surface of the eyelids, causing the eyelids to turn inward and scratch the cornea eventually creating a painful form of blindness.

The work of three men dominated medicine during this period: Celsus, Pliny, and Galen. Celsus wrote "De Medica or De Medicina" in A.D. 29, a compilation of detailed description of couching for cataract [2]. A humoural implication, collected in a space between the pupil and the lens, obstructing visual spirits. When this was fully developed it could be displaced away from the front of the eye by means of an operation. Pliny made the observation that the eyes of nocturnal animals were brilliant in the dark, in agreement with other Roman writers of this period that were interested in the problem of why the pupil is black. Pliny also credits the discovery of glass making to the Phoenicians. In these early days, glass was used as an art; later glass is used to correct

vision. Galen (A.D. 131-201) studied the anatomy of the eye and like Hippocrates, thought that the crystalline body (lens) was the most important part of the organ of vision. He believed that the retina was to perceive the alterations which occur in the crystalline body of the eye and to communicate them to the brain. He shared the same ideas for cataracts as Celsus.

During the period of A.D. 630-1375, medieval scientists combined theory and practice. They found it natural to study the eye with the practical applications of that knowledge. This period also marked the rise of Islam and conquest of the Eastern part of the Mediterranean. One unfortunate event it was the burning of the famous library of Alexandria, resulting in a big gap in the ancient writings that was never restored. The eastern knowledge of medicine began to filter into the west during the Crusades and within a decade the torch had been handed on to Western Europe.

During the 16^{th} and 17^{th} centuries, the facts of physiological optics began to slowly be accepted. Robert Hooke first measured the minimum visual angle which the basis of our present day test types. However, clinical progress was not made until the beginning of the 19^{th} century especially in the area of cataracts. First, Daviel of France published the operation of extracting the opaque lens from the eye through the interior chamber and later Brisseau convinced the Academie Royal des Sciences that a cataract is really an opaque lens.

The 19^{th} century was marked by many discoveries in ophthalmology, bacteriology, by the introduction of anesthesia, and antiseptic surgery just to mention a few. However, from the perspective of ophthalmic solutions, bacteriology and the introduction of antiseptic surgery highlight the importance of a bacteria free environment

and the tools used for eye surgery. In those days, ophthalmic solutions were kept in corked glass bottles, unpreserved, and were administered with a pipette. Current studies show that solutions stored under these conditions are 94% contaminated [3]. These results are not surprising since the pipette, the open bottle or the cork can come in contact with a pathogenic microorganism, even in a clean environment. As Pasteur famously demonstrated in 1864, contamination from microorganisms can be prevented with savvy packaging [4].

Ophthalmic solutions in twentieth, and twenty-first centuries are marked in improvements in packaging, preservation, and delivery systems that improve product lifetime and sustain release of the drug in the eye. Packaging moved from glass cork bottles to a sterile glass bottles with a screwed dropper, and in some instances to a plastic bottle with a tip for directly delivering the drop into the eye.

Preservation of ophthalmic solutions is very important and is required by the Food and Drug Administration for topical ophthalmic multidose bottles. More specifically, the United States Pharmacopeia specifically states that ophthalmic solutions in multidose containers should contain suitable substances to prevent or destroy micro-organisms when accidentally introduced when the container is opened during use. Despite concerns in recent years about the presence of benzalkonium chloride and the development of other preservative systems, benzalkonium chloride is by far the preservative system of choice in ophthalmic solutions.

Recent advances in ophthalmic solutions have focused on sustained release formulations for delivering the drug into the eye and formulations that target multiple symptoms at the same time. Because of the unique characteristics of the eye and the

multiple barriers that the formulation has to go through, the development of ophthalmic formulations is extremely challenging. In the next section, "formulation overview" these challenges are described in more detail.

1.2 Formulation Overview

Ophthalmic solutions are among of the most challenging and fascinating tasks facing product development researchers because the eye is well protected against the absorption of foreign materials, including therapeutic formulations. The easy accessibility of the eye makes this organ suitable for topical administration of a medication. However, any drug delivered through that route must go through several barriers in the precorneal area before the anatomical barriers of the cornea. Because of these barriers, only a small dose of the drug will reach the eye.

Upon topical administration of an ophthalmic solution in the eye, tear flow immediately increases and washes it away in a short period of time. Under normal conditions, the eye can accommodate a small volume before overflowing. Commercial eye drops typically deliver about 30 μL to 50 μL per drop and most of this amount will drain out of the eye with the first blink. Due to that loss, only a small amount (about 10 μL) will remain in the eye to penetrate the cornea and the inner tissue of the eye [5]. Consequently there is a very small time window, approximately 5 minutes, for any drug introduced topically to be absorbed by the eye and in many cases no more than 2% of the drug absorbed [5-7]. By contrast, a considerable amount of drug is absorbed by the nasolacrimal duct, with its greater surface area and higher permeability of the mucosal

membrane compared to that of the cornea [8].

Corneal permeability of the drug is low for ophthalmic solutions administered topically. The human cornea has 5 tissues with 3 of them, the epithelium, the endothelium, and the inner stroma being the main barriers to absorption. The epithelium is relatively lipophilic and has low porosity and high tortuosity, which makes it the main barrier for hydrophilic drugs. The middle stromal layer, which consists mainly of water interspersed with collagen fibrils and accounts for most of cornea's thickness, is the main barrier for lipophilic drugs [5, 9-11]. All these barriers result not only in low penetration of the drug but in systemic side effects of the ophthalmic drugs with topical administration [12-13].

An ideal ophthalmic solution will provide deep penetration beyond the initial layers of the eye. Consequently this can only be achieved by sustained drug release and the ability to remain in the vicinity a front of the eye for a long period of time.

The raw materials used in ophthalmic solutions must be of the highest quality available and each lot of the ingredients must be qualified against multiple pharmacopoeia specifications prior to its use, in order to be safe and to meet global requirements. Also, each ingredient has a specific function in the formulation. Some general characteristics of ingredients that must be studied are concentration, tonicity, viscosity and pH adjustment (buffer solutions), active ingredient stabilizers, and component solubility. Selection of all the formulation components is done based on the physical and chemical compatibility between them and biocompatibility with delicate ocular tissue.

The pH and buffering of ophthalmic solutions is very important to product

stability and product quality in general. Ideally an ophthalmic solution would be buffered at pH 7.4, which is the pH of tear fluid. In addition to formulation stability, pH adjustment enhances comfort, safety, and activity of the product. The pH of ophthalmic solutions is usually buffered within a range to provide maximum product stability and shelf life of about two years. The pH value expected to be maintained within the range during the entire shelf life of the product. An optimal pH range for ophthalmic formulations is between 7.0-7.4 and usually phosphate buffer is selected as a starting point.

Well buffered solutions prevent unwanted changes in pH due to hydroxyl ion release from the glass bottles in which solutions are stored. Unwanted pH changes can create discomfort to the patient especially for changes outside the tolerable pH range (6.6-8.5) of the cornea. Larger pH changes, outside the pH range of 4 to 10 will create permeability changes.

Precipitation and deterioration of the drug can occur after administration for formulations with pH that is not close to physiological pH of tears. The introduction of any formulation into the eye that causes discomfort due to precipitation is likely to stimulate tear production and increase the rate of drug removal from the eye making such formulations unsuitable for their intended use.

Sterility is of high importance and every ophthalmic solution must be manufactured under conditions to render it sterile in its final package and for the shelf life of its product. Each lot of product should be tested and released with appropriate pharmacopoeia methods to verify product sterility. There are different methods (steam, dry-heat, gas, ionizing radiation, filtration and aseptic processing) of sterilization of

ophthalmic solutions and selection of the method is done based on the compatibility of active components with other ingredients in the formulation. To reduce the biggest source of microbial contamination, only sterile purified water should be used preparing ophthalmic solutions.

Clarity is one of the ophthalmic solution properties, as by definition solutions are expected to be free of foreign mater and undissolved material. It is a critical parameter, so samples are tested to verify that they are free of foreign matter. Clarity is enhanced by filtration and it is essential that this is performed by equipment that is clean and does not contribute to contamination of the formulation. The overall process can be done in a combined step with sterilization and must take place in a clean environment. Solutions that fail the clarity test should not be used for instillation to the eye due to product instability and possible discomfort to the eye which will stimulate tear production and drug removal.

Tonicity is a measure of the osmotic pressure of two solutions separated by semipermeable membrane [14]. It is important to the eye that ophthalmic solutions should be adjusted for correct tonicity. When the concentration of the solute is higher outside the membrane (cell membrane) the solution is hypertonic, when the concentration of the solute is lower outside the membrane the solution is hypotonic and when the concentration is the same in both sides of the membrane the solution is isotonic [15]

Depending on the drug and its intended use, solutions can be prepared with different tonicity. The external part of the eye is more tolerant to tonicity variations. An isotonic solution is more important for intraocular use. However, in some cases of dry eye, tear fluid is reported to be hypertonic and a hypotonic artificial product is used to

balance this condition. Common ingredients used for the tonicity adjustment of the ophthalmic solutions are sodium chloride, potassium chloride, manitol, dextrose, propylene glycol, and glycerine.

There are three additional areas very important to ophthalmic formulations: preservation, demulcents, and active components. These are discussed extensively in the next chapters. In these chapters the importance of preservatives in ophthalmic solutions was discussed and one of the most widely used preservative systems (benzalkonium chloride) in over the counter ophthalmic solutions was studied. Two chapters will focus on the study of demulcents or lubricants in ophthalmic solutions. Demulcents in over the counter ophthalmic solutions have a dual role, to control viscosity and offer pain relief in irritated mucous membranes. Polyvinylpyrrolidone, PEG400 and glycerin are the three demulcents which were studied in the following chapters. Vasoconstrictors are one of the major areas of active components in ophthalmic solutions. They are used primarily for the treatment of redness and minor irritations in the eye. One of the vasoconstrictors used in ophthalmic solutions is oxymetazoline and it is member of other vasoconstrictors that have similar chemical structure and exhibit the same degradation profile. Oxymetazoline and its known degradant were studied in the next chapters.

Chapter 2: Preservatives

2.1 Introduction

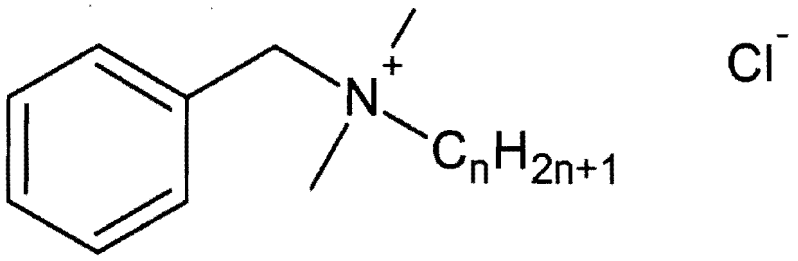
Preservatives are chemical substances that are used in ophthalmic solutions to preserve the integrity of the solution by killing microbes or inhibiting microbial growth, thus extending the product shelf life. This is extremely important given the environment in which these solutions are kept and in that how they are often mishandled. Solutions kept in medicine cabinets, purses, and pockets, exposed in extreme conditions of heat, humidity, provide a good environment for microbial growth. Often, users mishandle multidose bottles by touching the tip of the dropper with their fingers or with their eyes, introducing contamination. There are several types of preservatives within two major classifications, but only a few are applicable in ophthalmic solutions. Preservatives are classified in two major categories, antioxidants and antimicrobial. Antioxidants are inhibit oxidation reactions that disrupt the cell metabolism. Antimicrobial preservatives kill or inhibit the growth of microbes introduced during manufacturing or usage. The most commonly used preservative in ophthalmic solutions is benzalkonium chloride, which kills microorganisms by disrupting cell membranes.

Surfactants are also a major area of preservatives within the antimicrobial category. They are classified as cationic, anionic, nonanionic and amphoteric based on their net charge in solution and chemical structure. When dissolved in water surfactants reduce the surface tension between the liquid/vapor surface or at the water/oil interface. Cationic surfactants ionize in aqueous solution to produce positively charged organic ions

that are responsible for surface activity. Cationic surfactants are widely used in ophthalmic formulations because of their low toxicity, high surface activity, aqueous solubility, and their high preservative efficacy that often reduces or eliminate the need for additional preservatives. Benzalkonium chloride preservative efficacy is well established and it is widely used in ophthalmic solutions. Its physical attributes of high water solubility, and lack of color or odor in solution makes the BAC quaternary ammonium salt suitable as a preservative in ophthalmic solutions.

Benzalkonium chloride (BAC) is a mixture of alkylbenzyl dimethylammonium chlorides of various alkyl chain lengths with a general formula: $[C_6H_5CH_2N(CH_3)_2R]Cl$, in which R represents a mixture of alkyls with n-$C_{12}H_{25}$, n-$C_{14}H_{29}$, and n-$C_{16}H_{33}$ comprising the major portion of the BAC. The chemical structure of benzalkonium chloride is presented in Figure 1. It is commonly used as an antiseptic with the greatest activity associated with the C_{12}-C_{14} alkyl derivatives.

Benzalkonium chloride has been in clinical use since 1935 and is an active ingredient in a wide variety of prescription and over-the-counter products. Is generally found in topical solutions for cleaning, minor wound care and disinfecting in 1:750 dilution with water, or about 0.133%. For major wound care, mucous membrane and ophthalmic applications, concentrations are usually 10-50 times lower. BAC is one of the typical preservative systems used in ophthalmic solutions [16-17]. Is more active against bacteria but is weak against mold and is more active above a pH of 6 [18]. The homologues do not posses identical bactericidal activity. In general, the C_{12} homologue is most effective against yeast and fungi, the C_{14} homologue against gram-positive bacteria, and the C_{16} homologue against gram-negative bacteria [19-20].



n = 8, 10, 12, 14, 16, 18

Figure 1: Chemical structure of benzalkonium chloride (BAC), with "n" representing different alkyl chain lengths. Major portion of the BAC comprising by a mixture of alkyl chains with length of n-C_{12}, n-C_{14}, and C_{16}.

Recently, benzalkonium chloride was proven to enhance the antibacterial efficacy of antibiotics such as gatifloxacin [21]. Many analytical methods (HPLC and HPCE) were developed to determine BAC in a variety of products [16, 19-20, 22-26]. However, the described methods did not examine the fast separation of BAC homologues and their applicability might limited to a specific ophthalmic solutions. In some instances, due to the low concentrations of BAC, a salting-out technique was employed with the sum of all the homologues that might be present [16].

In this work we describe a new method for the analysis of the benzalkonium chloride in ophthalmic solutions. This represents an improvement over the current USP method for total BAC, which involves a titration [27]. The new method is a stability indicating method, its applicability was tested in different ophthalmic solutions and successfully validated based on International Conference on Harmonization guidelines [28].

2.2 Experimental

2.2.1 Reagents and Chemicals

The raw material for BAC (Century Pharmaceuticals, Indianapolis, USA) was provided by the product development group. ACS reagent sodium acetate trihydrate and reagent grade glacial acetic acid were purchased from Sigma Aldrich (Saint Louis, Missouri, USA). HPLC grade methanol was purchased by J.T. Baker (Phillipsburg, NJ, USA).

2.2.2 Equipment

The Waters Alliance HPLC system (Waters Corporation, Milford, Massachusetts, USA) was used for the method development and method validation. The Alliance liquid chromatography system was equipped with 2695 separation module, 2487 UV detector and 996 photodiode array detector. Data collection and processing was done using the Empower chromatographic data acquisition system.

2.2.3 Chromatographic Conditions

A simple isocratic high performance liquid chromatography (HPLC) method was developed for the determination of benzalkonium chloride (BAC) in ophthalmic solutions. The chromatographic column used was a YMC, CN, 5 μm, 150 mm x 4.6 mm. The flow was kept at 1.0 mL/min during the length of the run and the column temperature was 40°C. The wavelength was 262 nm and the injection volume was 100 μL. The mobile phase utilized was 35:65 of 0.075 M sodium acetate trihydrate buffer with pH value adjusted to 5.0 with acetic acid : methanol.

2.2.4 Testing Parameters

Testing of the chromatographic method was performed in accordance to International Conference on Harmonisation (ICH) guidelines [28] and typical operating procedures for pharmaceutical analysis. The test parameters will be discussed in the same order as they were investigated during the method validation.

2.2.4.1 Specificity

The specificity is the ability of the method to measure accurately and specifically the analytes in the presence of components that may be present in the sample matrix. In order to measure the degree of interference, ophthalmic samples, placebos, and standards of BAC were exposed to stress conditions of acid, base, peroxide, light and heat. Subsequently, the solutions were analyzed according to the chromatographic parameters presented in this paper to ensure no extraneous peak coeluted with the multicomponent BAC peaks. A UV diode array detector was used to check a 3-dimensional spectrum.

2.2.4.2 Accuracy

In order to measure the exactness of the analytical method between the true value and an accepted reference value, we spiked an ophthalmic solution without benzalkonium chloride (BAC) with BAC standard at three working levels 70%, 100%, and 130% of the theoretical concentration. Six preparations were performed at each level and assayed as per method conditions. The average result from each individual level was compared to its respective theoretical concentration value to check for any potential bias. All three average values were not significantly lower or higher than the theoretical value e.g. ±1.5%.

2.2.4.3 Linearity

In order to show that there is a direct proportional relationship between the analyte response and its concentration, five concentration level solutions of BAC, corresponding from 50-150% of theoretical concentration were prepared and injected. In

addition, the ability of the system to retain and carryover the analyte into subsequent injections was evaluated by injecting a blank solution (diluent only) in duplicate immediately after the 150% linearity level.

2.2.4.4 System Performance

In order to ensure performance of the system before and during the analysis, system performance parameters, as defined in USP/NF, [29] were established as a direct result of ruggedness and robustness experiments.

2.2.4.5 Precision

2.2.4.5.1 System Precision: We determined the system precision using six replicate measurements of a 100% theoretical standard solution containing benzalkonium chloride. The error contributed by the system, independent of the sample preparation, should be less than the acceptance criteria of 2.0 %.

2.2.4.5.2 Repeatability: The repeatability, which is the error contributed by sample preparation, was determined by six identical sample preparations of the same sample.

2.2.4.5.3 Intermediate: In order to evaluate the degree of agreement among test results obtained from multiple samplings of the same lot of samples on a different day using different instrument, column and analyst, six identical sample preparations of the same sample were used.

2.2.4.6 Robustness

2.2.4.6.1 Chromatographic parameters: Robustness of a method is a measure of its capacity to remain unaffected by small but deliberate variations in chromatographic parameters. The parameters under test were wavelength, flow, column temperature, mobile phase ratio, and pH of the buffer in the mobile phase. These parameters were changed one at a time. System suitability and samples run were conducted with unchanged method parameters and modified parameters.

2.2.4.6.2 Solution Stability: Standard and sample solutions were stored at room temperature and tested at initial, 48 hrs and 144 hrs. The solutions were tested against a freshly prepared standard at each time point.

2.3 Results and Discussion

2.3.1 Developmental Work

There are many factors to consider when developing methods. One of the factors is the choice of the proper detection scheme which depends on the analyte's properties. UV detection was selected since benzalkonium chloride has an intense chromophore absorbing in the UV. The selection of the wavelength in our method (λ=262 nm) was made based on a second maximum absorbance of BAC. More specific benzalkonium chloride has two UV maxima at about 210 nm and 262 nm. The second maximum is weaker but serves well with the selected mobile phase of methanol : sodium acetate trihydrate (65:35) since baseline noise was observed at wavelength around 210 nm. In

addition, the 262 nm wavelength was selected because methanol has a cutoff wavelength of 205 nm.

Ideally for method development of new compounds with reversed phase HPLC several columns should be screened based on the pH tolerance. However, BAC is a mixture of well defined molecules and for that reason only one column was used during the method development. Different HPLC methods were reported in the literature for the analysis of benzalkonium chloride using reversed phase chromatography [30-34]. Due to its multicomponent nature and its low concentration in ophthalmic solutions (50-100 ppm), BAC is a difficult material to analyze. The method should be quantitative and qualitative in order to identify and distinguish the homologue components from each other and from other excipients. As a result, due to the low concentration of BAC and the fact that different formulations contain different sources of raw material which have different homologues made the method development very challenging. Several formulations with different homologues and different amounts of BAC were obtained and analyzed with the current method. The method appears to be suitable for these types of formulations. Figure 2 shows an overlay chromatogram of several different ophthalmic formulations containing the C_{12}, C_{14}, C_{16} and small amount of C_{18} BAC homologues.

In the isocratic method, constant eluent composition in the column implies that equilibrium was established in the column and components moving through the column with constant velocity. Many attempts were made to elute all BAC homologues with gradient mobile phases but poor resolution between the BAC homologues and poor applicability between different formulations made the method impractical.

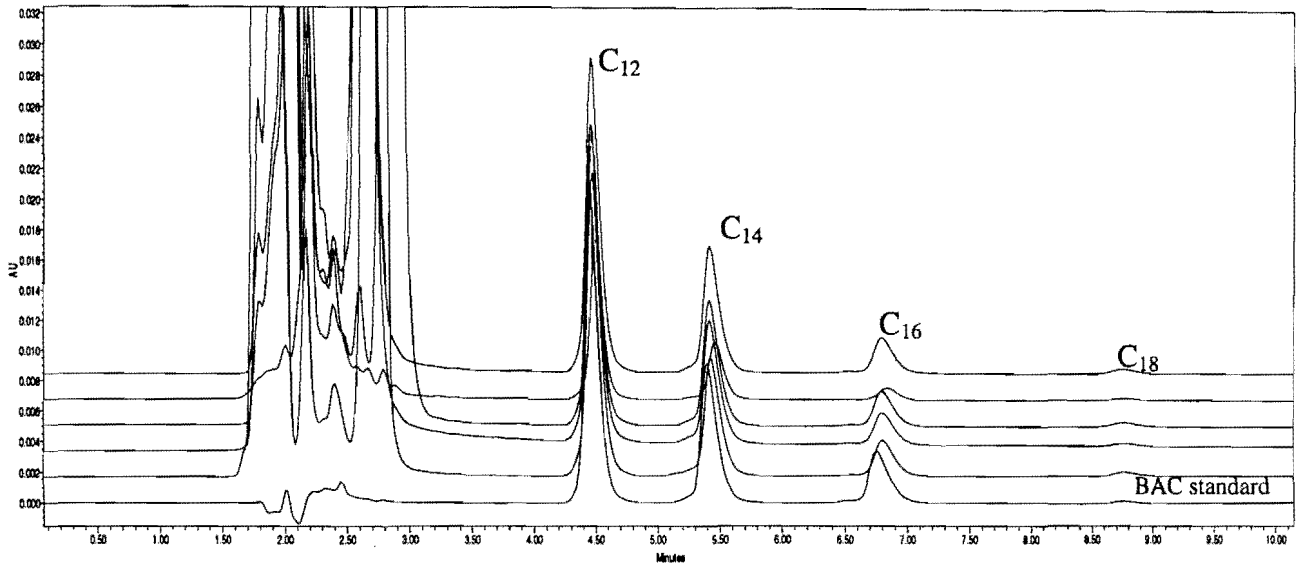


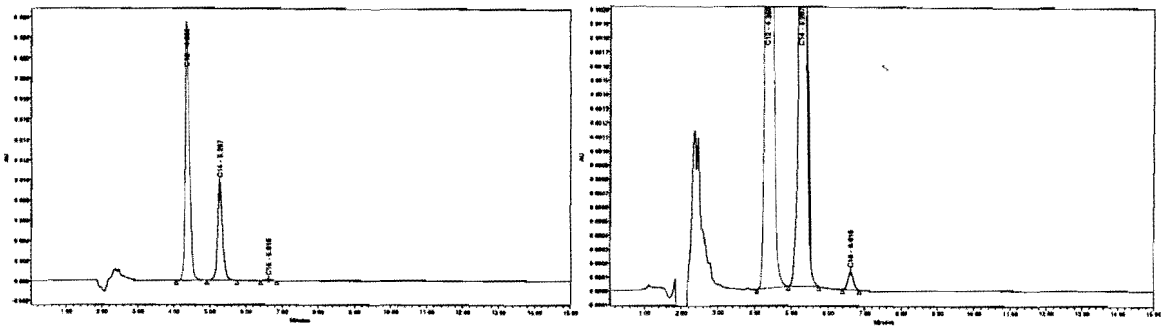
Figure 2: Overlay chromatogram of several different ophthalmic formulations that contain C_{16} and C_{18} of benzalkonium chloride (BAC) homologues. BAC comprises primarily by a mixture of alkyl chains with length of C_{12}, C_{14}, and C_{16}.

Since benzalkonium chloride is known for its adsorption in membrane material [17], a filter membrane (PTFE) was tested from two different vendors to evaluate the need of filtering solutions during the sample preparation. PTFE filters with dimensions of 25 mm and 0.45 μm porosity were tested. Results were compared against a non filtered sample. No significant change was observed between the vendors and filtered vs. unfiltered samples.

System performance parameters were selected to provide confidence that the method is capable of determining BAC in ophthalmic solutions. A typical chromatogram for the benzalkonium chloride system performance standard solution is shown in Figure 3. The selected parameters include injection precision, resolution between C_{12}, C_{14} and C_{14}, C_{16} homologues (when applicable), and a tailing factor of BAC homologues (C_{12}, C_{14}, C_{16}). In addition, check standard conformity and bracketing standard conformity were proved during each chromatographic run.

2.3.2 Experimental Work

The specificity of the method was tested and no interference was observed for the BAC peaks from forced degradation samples and ophthalmic solutions without BAC. During these studies equivalent amounts as per method of ophthalmic solution samples with and without BAC, BAC standard were stressed for the following conditions: (1) heat at 75°C for 1 hr; (2) 5 mL of 1 N hydrochloric acid at 75°C for 1 hr; (3) 5 mL of 1 N sodium hydroxide at 75°C for 1 hr; (4) 5 mL of 30% hydrogen peroxide at 75°C for 1 hr; and (5) light exposure for 24 hrs as per ICH option 1.



(a) (b)

Figure 3: System performance chromatogram (a): Full scale chromatogram (b): Expanded chromatogram. Performance parameters such as injection precision, resolution between the homologues, and tailing factor are evaluated.

Target Concentration Level	Concentration (*µg/mL*)	Response (*Area*)
20%	9.95	58175.46
50%	24.88	149170.44
100%	49.77	296918.96
120%	59.72	359065.31
160%	79.62	480266.94
Slope	6047756.17	
Y Intercept (%)	-2147.48	
Correlation	0.99995	

Table 1: Linearity parameters of benzalkonium chloride. Benzalkonium chloride found to have a linear response over a range of 20%-160% of theoretical concentration 0.05 mg/mL.

The repeatability of the method was evaluated by six identical sample preparations of a homogeneous batch and the results were found to be within the specifications. The percent relative standard deviation of the six preparations for BAC was found to be 0.7%.

In order to further validate our results, the experiment was conducted again on different day by a different analyst, using different instruments, and different columns. The experimental mean agreement between the two experiments was found to be 0.4, both experimental results were within the acceptance criteria. Results are displayed on Table 2b.

The accuracy of the method was established by assaying three different BAC concentration levels 70%, 100%, and 130% of the theoretical concentration (0.05mg/mL). Six preparations of ophthalmic solutions without benzalkonium chloride, at each level, were spiked with standard BAC and injected into the HPLC system. Results are reported in Table 2c with BAC mean recovery values varied from 99.2 to 99.5 % LC. No bias was observed for BAC, since the results for the mean recovery from all three accuracy levels were not significantly lower or higher (±1.5%) than the theoretical value. The range in which the method is shown to be linear and accurate for benzalkonium chloride is between 70-130% of theoretical concentration (0.05 mg/mL).

The method remained unaffected by small, deliberate variations in chromatographic parameters and mobile phase preparation. The parameters tested were wavelength, flow, temperature, mobile phase ratio, and pH of the buffer. Table 3 shows the results of the small deliberately variations of the method conditions.

Replicate #	Peak Response (*Area*)
1	302895.88
2	296933.32
3	298289.29
4	298556.27
5	298255.89
6	298355.70
Mean	**298881.06**
% RSD	**0.7**

(a)

Replicate	% Label Claim (%LC)	
	Benzalkonium Chloride	
	Experiment 1	Experiment 2
1	99.5	100.4
2	101.0	101.3
3	101.2	100.3
4	99.5	101.6
5	100.8	100.8
6	101.5	101.8
Mean	**100.6**	**101.0**
%RSD	**0.8**	**0.6**
Mean Agreement	**0.4**	

(b)

Preparation #	BAC		
	Mean Recovery Value %LC		
	70% Level	100% Level	130% Level
1	99.4	99.4	98.9
2	99.2	99.1	99.3
3	99.2	99.4	99.6
4	99.3	99.4	99.6
5	99.1	99.7	99.7
6	98.9	100.1	99.4
Mean	**99.2**	**99.5**	**99.4**
%RSD	**0.2**	**0.3**	**0.3**

(c)

Table 2: Experimental data of (a) System precision results, (b) Intermediate method precision results, (c) Accuracy results (ophthalmic solution spiked with BAC at three levels, 70, 100, 130% of theoretical)

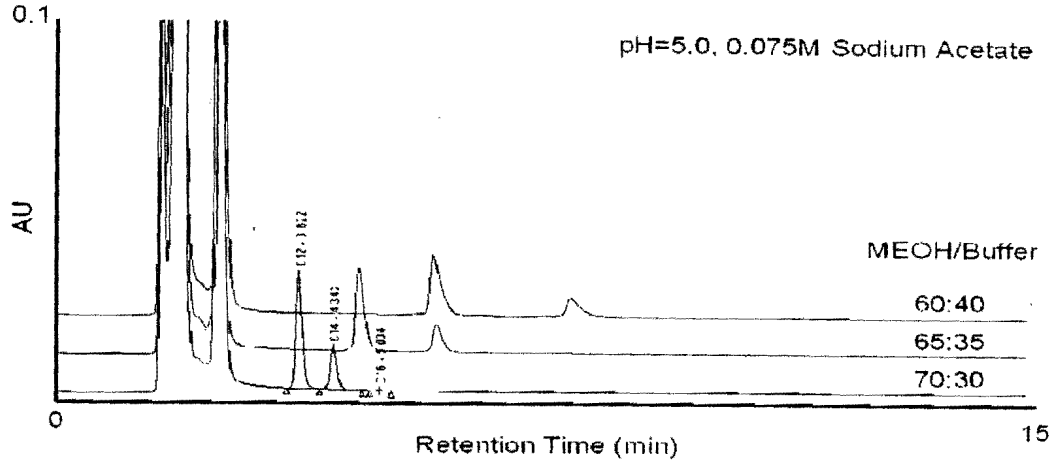
Parameter Changed	Percent Difference	Comment (%LC)
No Change (65:35), pH-5.0	N/A	98.5
Wavelength 260 nm	0.5	99.0
Wavelength 264 nm	0.4	98.9
Column Temperature 38 °C	0.3	98.8
Column Temperature 42 °C	0.7	99.2
Flow 0.9 mL/min	1.1	99.6
Flow 1.1 mL/min	1.3	99.8
Mobile phase ratio (60:40), pH-5.0	1.4	99.9
Mobile phase ratio (70:30), pH-5.0	0.7	99.2
Mobile phase pH=4.8, (65:35)	0.7	99.2
Mobile phase pH=5.2, (65:35)	0.4	98.9

Table 3: Robustness results. Parameters which evaluated were wavelength, flow, column, mobile phase ratio and mobile phase pH.

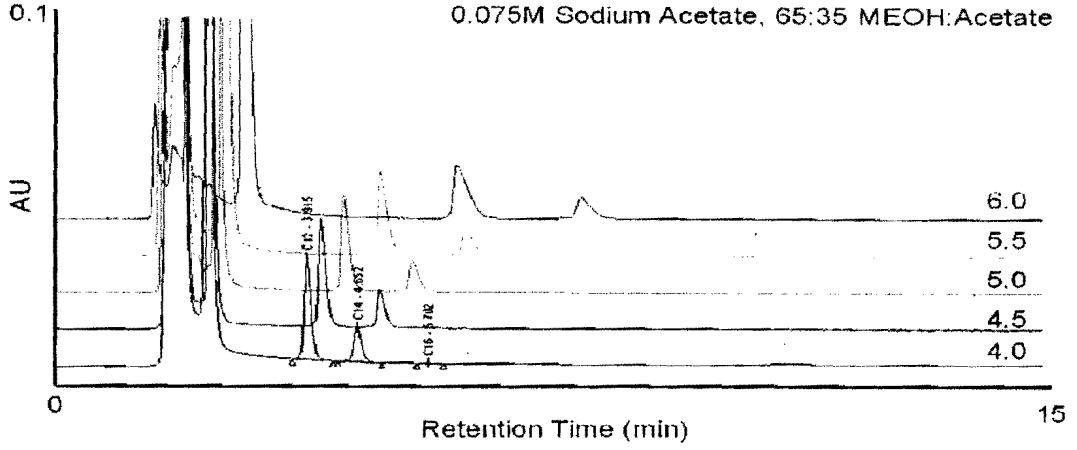
The robustness results were within acceptance criteria of ±3.0 %LC, however some parameters are more critical than others. More specific the mobile phase ratio at 60:40 is critical to the shape of the BAC homologues and tailing factor (T_f) did not meet suitability criteria as per the method. The results variations that were observed between the normal method conditions and the changed parameters were from 0.3% to 1.4%.

Critical parameters of the method such as mobile phase ratio, pH, and buffer concentration were evaluated and the results followed normal trends for reversed phase chromatography for cationic compounds. Figure 6 shows overlay chromatograms of mobile phase ratio, pH and buffer concentration.

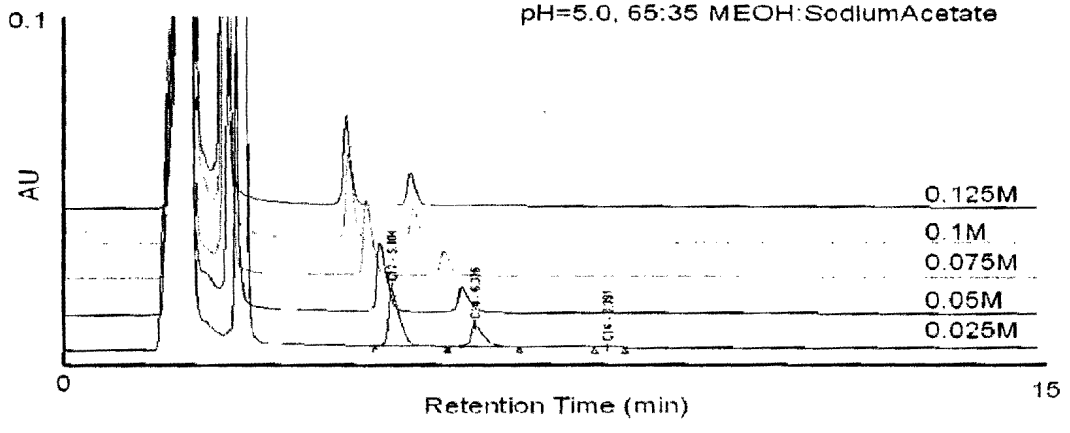
The stability of the standard and sample solutions for BAC was evaluated. The results are displayed on Table 4 and show no significant decrease over a period of 240 hrs for BAC in standard and 72 hrs for BAC in sample.



(a)



(b)



(c)

Figure 6: Robustness overlay chromatograms of critical method parameters: (a): organic:buffer ratio (b): pH and (C): buffer concentration

Time Point	Benzalkonium Chloride in Standard	
	Response	**% Difference from Initial**
Initial	100.0	N/A
24 Hours	100.7	0.7
72 Hours	101.9	1.9
144 Hours	102.0	2.0
Time Point	**Benzalkonium Chloride in Sample**	
	Response	**% Difference from Initial**
Initial	96.8	N/A
24 Hours	97.6	0.8
48 Hours	98.6	1.8

Table 4: Solution stability results for BAC in standard and sample solutions. Standard solution was stable over a period of six days and sample solution was stable over a period of two days.

2.4 Conclusion

The importance of preservatives in over the counter ophthalmic solutions is well known given the environment in which these solutions are kept. Often, users store the multidose bottles in medicine cabinets, purses, and pockets, exposed to extreme conditions of heat and humidity, all of which provide a good environment for microbial growth. Benzalkonium chloride is by far the preservative system of choice in ophthalmic solutions. In this chapter a new method for the determination of total benzalkonium chloride in ophthalmic solutions is described. The method separates the BAC homologues from each other and from other formulation components. The homologue separation is dependent to mobile phase ratio, pH and buffer strength. The testing for this method was performed according to ICH guidelines and met all acceptance criteria. Our experimental results indicated that the method is precise, accurate, and linear at concentration ranges of 0.035 mg/mL to 0.065 mg/mL for total BAC. The analytical procedure is simple, fast, isocratic based on reversed phase chromatography (RPC) and applicable to a variety of ophthalmic solutions with BAC from different vendors.

Chapter 3: Demulcents (Polyvinylpyrrolidone)

3.1 Introduction

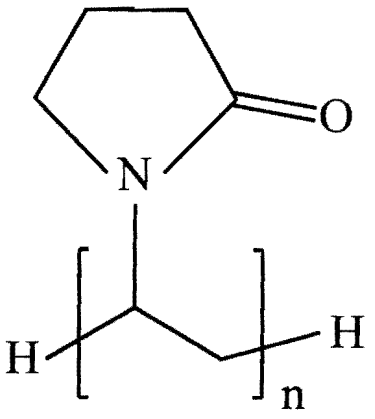
Lubricants, or demulcents, are often present in ophthalmic solutions and are used to control the solution viscosity. Additionally, they offer a medicinal benefit, relieving pain in inflamed or irritated mucous membranes. There are several demulcents in use in ophthalmic solutions, with selection done based on excipient compatibility, viscosity and the intent of medicinal benefit. In this study, several demulcents were studied: polyvinylpyrrolidone (PVP), polyethylene glycol-400 (PEG-400) and glycerin. Since PVP and PEG400 are polymeric, size exclusion chromatography (SEC) is the technique of choice, however, most SEC methods focus on molecular weight characterization rather than quantitative analysis. In this work, total PVP in ophthalmic solutions was determined using the unusual combination of size exclusion chromatography, ultraviolet-visible detection and quantitation of an analyte peak that elutes before the void volume disturbance [35]. A more conventional size exclusion chromatography method was used for the simultaneous determination of PEG400 and glycerin in ophthalmic solutions, using size exclusion chromatography, with refractive index detection [36], described in Chapter 4.

Povidone (Polyvinylpyrrolidone, PVP) is a chain polymer of 1-vinyl-2-pyrrolidone, developed in the late 1930's [37]. PVP is obtained by a multistep synthesis that concludes by polymerization of vinylpyrrolidone in aqueous solution in the presence of hydrogen peroxide [38]. A wide range of molecular weights, from a few thousand to a few million Daltons can be obtained by controlling the degree of polymerization. PVP is

a white hygroscopic powder and unlike many synthetic polymers is soluble in a variety of traditional solvents such as water, chlorinated hydrocarbons, alcohols, amides, and amines [39]. In our studies, PVP with a molecular weight of about 50,000 with a K-value of 30, typical in ophthalmic solutions, were used [40]. Figure 7 shows the structure of polyvinylpyrrolidone.

PVP originally was used as a plasma substitute and in a variety of applications. Its hygroscopic properties, film formation, and adhesion to different materials have made PVP widely used in pharmaceuticals, cosmetics and industrial production. The interactions between the carbonyl groups in PVP and the hydroxyl group in polyphenols are well known and have been reported in the literature. Due to these interactions PVP is used to isolate polyphenols and as a colloidal stabilizer in beers by selective removal of tannoid polyphenols [41-42]. PVP formulations have been used to produce desired solution viscosity, allowing the deposition of a uniform coating thickness of a photoresist in the manufacture of high resolution display screens [43]. In ophthalmic solutions, PVP is used as a demulcent or moisturizer and is generally present at approximately 1% concentration in an aqueous matrix also containing other excipients and active formulation components. It has been shown in combination with polyethylene glycol 400 and dextran 70 to be effective for the temporary relief of minor irritations, for protection of the eye against further irritation from the wind or sun and relief from eye dryness [44].

Many chromatographic methods have been reported in the literature for the determination of PVP, either qualitative determination of the molecular weight range of the polymer or quantitative determination in formulations and products, with most



IUPAC name: Polyvinylpyrrolidone
Molecular formula: $(C_6H_9NO)_n$
Formula weight: 35000-51000

Figure 7: Structure of polyvinylpyrrolidone

focusing on the qualitative aspects such as the form of polyvinylpyrrolidone present and whether materials with same K-value are structurally the same [45-46].

A variety of capillary electrophoresis (CE) methods for the characterization and determination of povidone have been reported, including capillary zone electrophoresis (CZE) and capillary gel electrophoresis (CGE) [47-50]. Polyvinylpyrrolidone has been determined in several pharmaceutical matrices with solid phase microextraction and GC. The fibers were polypyrrole (PPy) and desorption was performed at the inlet of gas chromatograph equipped with a nitrogen phosphorous detector [51]. PVP has also been used as stationary phase materials or extractant [52-55]. SEC determinations of polyvinylpyrrolidone have focused on molecular weight characterization of the polyvinylpyrrolidone itself or the use of polyvinylpyrrolidone as a molecular weight calibrator for other determinations [56-64].

SEC is not usually used in combination with UV detection, however it is clearly applicable if the analytes and other compounds of interest in the analytical samples contain a chromophore. Some pharmaceutical applications of SEC with UV detection include a recent study of the mass balance in the oxidative degradation of rapamycin and the analysis of various proteins and biological polymers in formulations [65-67]. Determination of total PVP present in the matrix of a pharmaceutical formulation using SEC and UV detection has not been previously reported. In this work we describe a fast, straightforward new HPLC method for the analysis of total PVP in ophthalmic solutions. The new method is stability indicating and was successfully validated based on the International Conference on Harmonization guidelines for pharmaceutical quality assurance [68].

3.2 Experimental

3.2.1 Reagents and Chemicals

The raw material for PVP was purchased from BASF AG (Ludwigshafen, Germany). HPLC grade methanol was obtained from Fisher Scientific Inc. (Fairlawn, New Jersey, USA). ACS reagent grade sodium acetate was purchased from Sigma Aldrich (Saint Louis, Missouri, USA). Water was obtained using a Milli-Q (Millipore, Milford, MA) purification system located in our laboratory.

Laboratory formulations of typical opthalmic solutions were prepared in water at 0.5 mg/mL concentration of povidone. There were diluted by adding 10 mL of formulation to a 50 mL volumetric flask and diluting to the mark with water prior to HPLC analysis. The final working concentration of the sample and standard solutions was 0.1 mg/mL.

3.2.2 Instrumental Conditions

An Alliance HPLC system equipped with a 2695 separation module with 2487 UV and 996 photodiode array detectors was used for all experiments. Data collection and processing was performed using an Empower chromatographic data acquisition system. (Waters Corporation, Milford, Massachusetts, USA) The chromatographic column was a TSKgel G1000PW, 7.5 mm ID x 30 cm, 12 μm column (TOSOH Bioscience, Tokyo, Japan). The flow was kept at 1.0 mL/min during the length of the run

and the column temperature was 50°C. The UV detector wavelength was 220 nm and the injection volume was 25 µL. The mobile phase was premixed 800 mL 0.1 M sodium acetate and 200 mL methanol generating a mobile phase pH of about 10.

3.2.3 Testing Parameters

Testing of the chromatographic method was performed in accordance with ICH guidelines and typical operating procedures for pharmaceutical analysis. The test parameters are presented in the same order as they were investigated during the method validation. Specificity was determined by exposing ophthalmic solution samples with and without polyvinylpyrrolidone, and polyvinylpyrrolidone standards to stress conditions of acid, base, hydrogen peroxide, light and heat and subsequently analyzing them according to the method. A photodiode array detector was used during validation to ensure that no interfering compounds co-eluted with PVP but is not necessary for the final method. Accuracy was measured by spiking with PVP ophthalmic solutions without PVP at three working levels 0.35, 0.50 and 0.80 mg/mL (70%, 100%, and 130% of the standard concentration). Six preparations were performed at each level and assayed as per method conditions. The average result from each individual level was compared to its respective theoretical concentration value to check for any potential bias.

To ensure that the method is linear in the working concentration range, five concentration level solutions of polyvinylpyrrolidone, corresponding from 0.25-0.75 mg/mL (50-150% of the expected analyte concentration) were prepared and injected. In addition, the ability of the system to retain and carryover the analyte into subsequent

injections were evaluated by injecting a blank solution (diluent only) in duplicate immediately after the 150% linearity level. To ensure performance of the system before and during the analysis, system performance parameters, as defined in the USP/NF, were established as a direct result of ruggedness and robustness experiments [69].

System precision was determined using six replicate measurements of a 100% theoretical concentration standard solution (0.5 mg/mL PVP concentration) containing polyvinylpyrrolidone, with an acceptance criterion of the RSD being less than 2.0%. Repeatability was determined by six identical sample preparations of the same lot. To determine agreement among test results obtained from multiple samplings of the same lot of samples on different days using different instruments, columns and analysts, six identical samples from the same lot were prepared and analyzed.

Robustness was determined by examining small variations in: wavelength (±4 nm), flow rate (±0.1 mL/min), column temperature (±5°C), and mobile phase preparation (±10%). These parameters were changed one at a time. System performance and sample runs were both conducted with unchanged method parameters and modified parameters. In addition a quantitative comparison study was performed between the raw material that was used to prepare the batch and other raw materials of polyvinylpyrrolidone including a USP reference standard. One sample preparation was run and quantitated with five different standards as per the method. Further, to assess sample stability, standard and sample solutions were stored at room temperature and tested at initial, 24 hrs, 96 hrs, and 192 hrs. The solutions were tested against a freshly prepared standard at each time point.

3.3 Results and Discussion

3.3.1 Developmental Work

The physical and chemical properties of polyvinylpyrrolidone have been well established, since its discovery in 1930 [37-40]. UV detection was selected for this work since PVP has a chromophore in the ultraviolet range, with a maximum at 213.5 nm. The UV spectrum of PVP is shown in Figure 8. In the final method, 220 nm was selected for the UV detector wavelength. During method development, excessive noise, possibly from the solvent (methanol has a UV cutoff of 205 nm) or impurities in the solvents precluded the use of 213.5 nm. No deleterious quantitative effects from detecting PVP on the slope of the UV spectrum rather than the maximum were observed. Although the UV detector is possibly the most versatile and useful detector in high performance liquid chromatography, it is not as widely used in SEC since many polymers do not absorb electromagnetic radiation in the UV range. Other detectors such as refractive index or light scattering are more commonly used, but quantitation and method validation are often challenging with these [70]. Thus the combination of SEC with UV detection is especially suited to this application and would be suitable for other polymer analysis in which a chromophore is present. Figure 8 shows a UV spectrum of povidone with a maximum absorbance about 213 nm.

The main goal during method development was to have an isocratic method that separates PVP from other compounds in the formulation or degradation products.

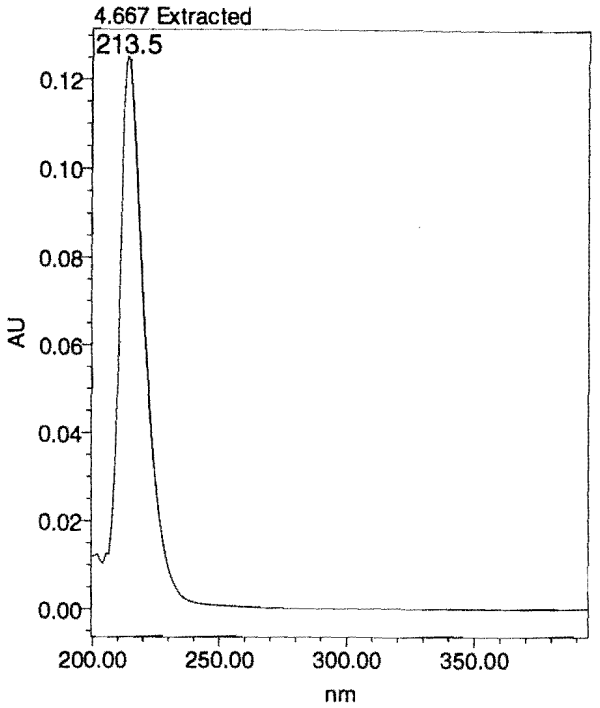
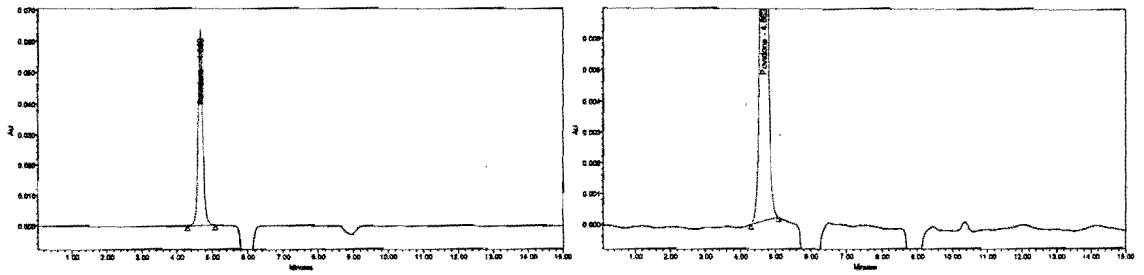


Figure 8: UV spectrum of polyvinylpyrrolidone with a UV maximum absorbance at 213 nm.

Usually in SEC method development for unknowns, the molecular weight of the analyte polymer must be independently determined to aid in column selection. In this study that was not necessary since the molecular weight range of PVP, approximately 50 kDa, was provided by the vendor. Initially, several size exclusion columns from different manufacturers were screened and TSK-gel G1000PW, 7.5 mm ID x 30 cm x 12 μm column was selected based on symmetrical peak shape of PVP. TSK-gel columns in general consist of hydrophilic polymethacrylate spherical beads with sizes ranging from 12 μm to 17 μm. These types of columns are suitable for the analysis of water soluble polymers and more specific the G1000PW is suitable for small (1000 Da and less) molecular weight polymers. As seen in Figure 9, in our method the TSK-gel G1000PW column is used in a reversed context: the analyte is of much higher molecular weight than would normally be separated by this column, eluting before the void volume disturbance, fully excluded from the stationary phase. Other compounds present, with molecular weights less than 1000, elute in the separation range of the column. While uncommon, quantifying a peak eluting before the void volume has been recently reported in a similar context for the group assay of polyvinylsulfonic acid impurities in 2-(Nomorpholino)-ethanesulfonic acid [71]. Figure 9 clearly shows a very symmetrical peak for PVP, demonstrating a satisfactory tailing factor for system performance and the validation data presented in the next section demonstrate adequate precision. The symmetrical peak of polyvinylpyrrolidone is expected based on the exclusion of the molecule from the porous space of packing material and its fast elution from the column.



(a) (b)

Figure 9: Chromatogram of polyvinylpyrrolidone (a): Full scale chromatogram (b): Expanded chromatogram

SEC separation is based on molecular size of the analyte relative to the pore size of the packing material. Mobile phase selection is important to avoid enthalpic interactions between the analyte and the packing material. There are a variety of solvents compatible with TSK-GEL columns, so the selection process depends on the chemical structure and ionic nature of the analyte. In this study since a UV detector was used the ideal mobile phase should have a low UV absorbance as well. Methanol has a low wavelength UV absorbance cutoff of about 205 nm and low background absorbance combined with good solubilizing properties for polyvinylpyrrolidone made methanol the organic solvent of choice for this method. For simplicity, premixed aqueous buffer mixture of 0.1 M sodium acetate with methanol (80:20% v:v) was selected as the mobile phase [72]. This generates a mobile phase pH of approximately 10, which assists in ensuring rapid and efficient transport of PVP through the column by ensuring that the electron pair on nitrogen in PVP does not protonate while the hydroxyl groups on the surface of the TSK-GEL column are slightly deprotonated, generating additional repulsion between the stationary phase and the analyte.

Temperature adjustment can reduce the analysis time and improve chromatographic performance. More specific as the temperature increases, the viscosity of the mobile phase decreases and the diffusivity of the analyte increases. Fast size exclusion chromatography has been discussed in the literature and temperature is one of the primary parameters adjusted to achieve faster analysis times [73-74]. Optimum chromatographic performance was obtained in this method with column temperature at 50°C.

3.3.2 Experimental Work

Figure 10 shows the analysis of PVP in a formulation (top), standard (middle) and formulation without PVP (bottom). PVP does not appear in the placebo which is simply a formulation prepared without the analyte, demonstrating that compounds other compounds that commonly appear in ophthalmic formulations will not interfere with the method. This chromatogram clearly demonstrates the reverse SEC analysis: determining a larger molecular weight compound using a column designed for small molecular weight analytes. PVP elutes first, followed by the void volume disturbance, followed by other components. In each case, chromatographic performance, was evaluated by tailing factor, peak shape, peak width, and found to be adequate.

Figure 11a shows chromatograms of several PVP polymer formulations in combination with a vinyl pyrollidone monomer. In these chromatograms, the PVP polymer is seen eluting before the void volume of the column (totally excluded from the stationary phase pores) and the monomer eluting within the analytical range of the column. Further, all of the polymer formulations elute at the same retention time in the void volume, providing the desired total analysis from a single chromatographic peak, regardless of variations in the polymer formulation. Essentially all material with a molecular weight greater than 1000 Da is included in the main peak. In Figure 11b, a chromatogram of the PVP used in this study for ophthalmic formulations in combination with its monomer is shown, demonstrating the outstanding selectivity of this system.

The specificity of the method was tested and no interference was observed for the polyvinylpyrrolidone peak from ophthalmic solution without polyvinylpyrrolidone and

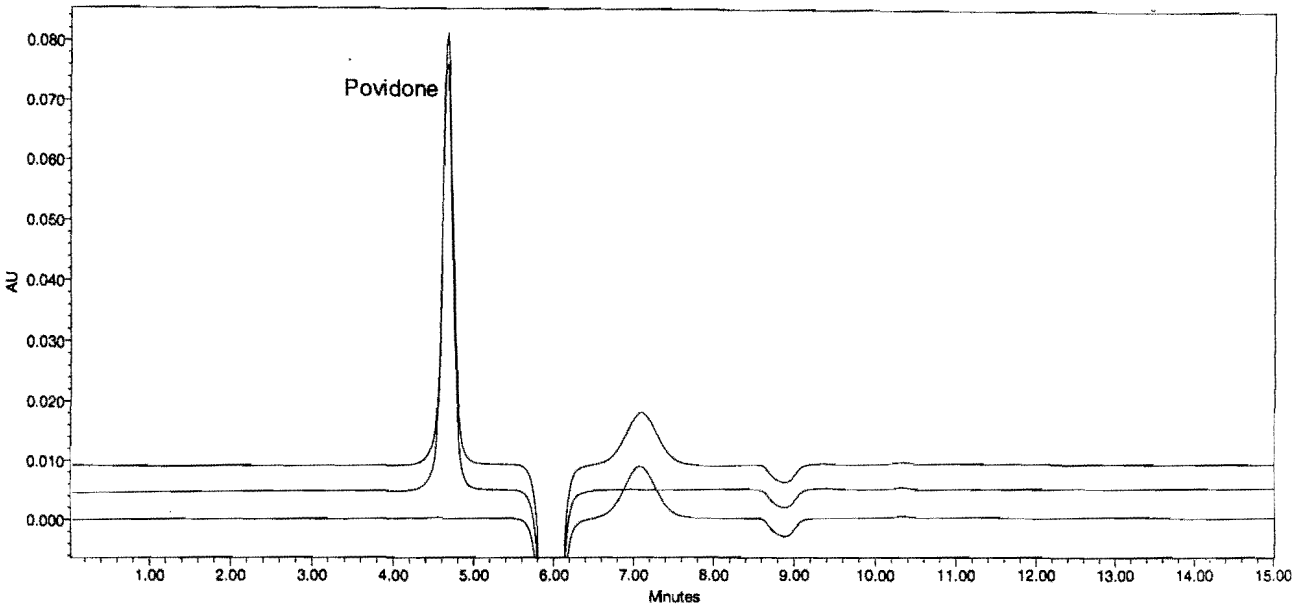
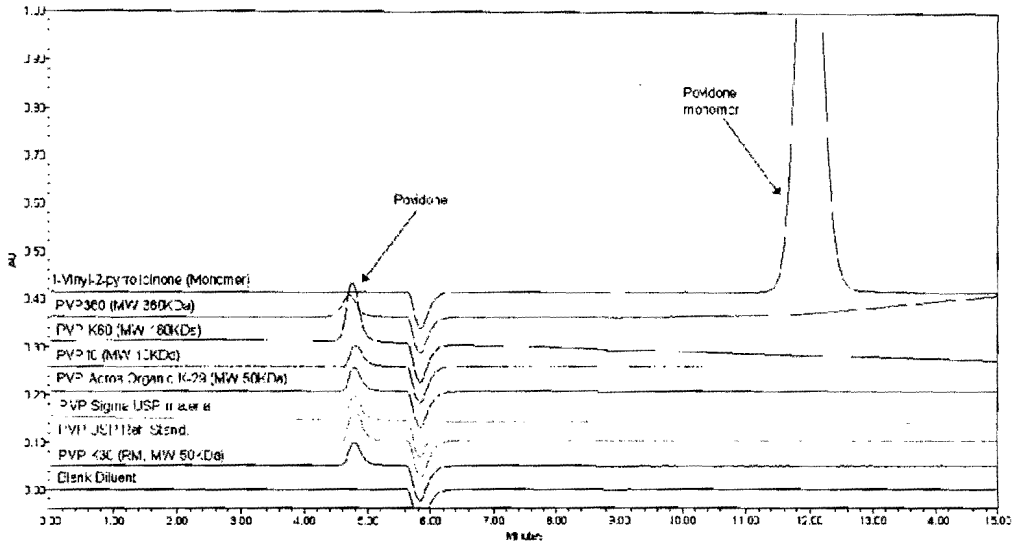
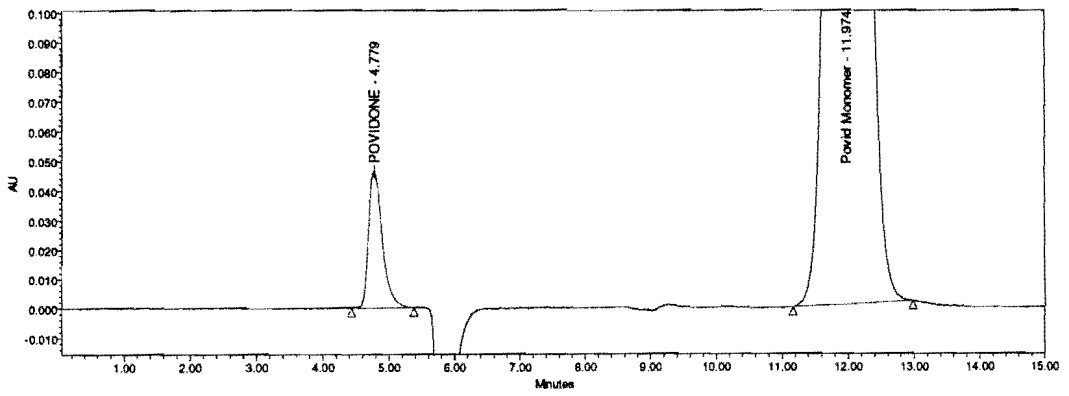


Figure 10: Overlay chromatogram of ophthalmic formulation (top), standard of polyvinylpyrrolidone (middle), and formulation without polyvinylpyrrolidone (bottom)



(a)



(b)

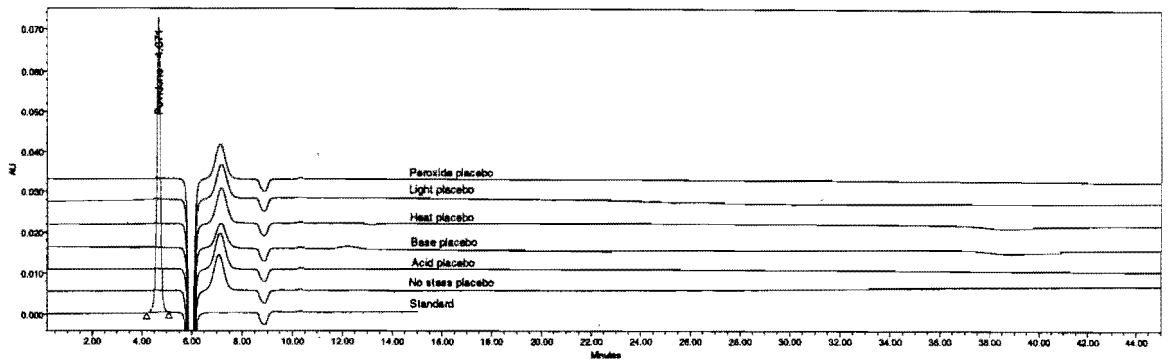
Figure 11: Overlay chromatogram of different molecular weight polyvinylpyrrolidone polymers (a) chromatogram of polyvinylpyrrolidone and its monomer

forced degradation samples. To ensure no extraneous peak co-eluted with the peak of interest, a UV diode array detector was used to double check the full UV spectrum for all peaks. Figure 12 shows overlay chromatograms of the sample without polyvinylpyrrolidone (a) sample (b) and standard (c) at different stress conditions.

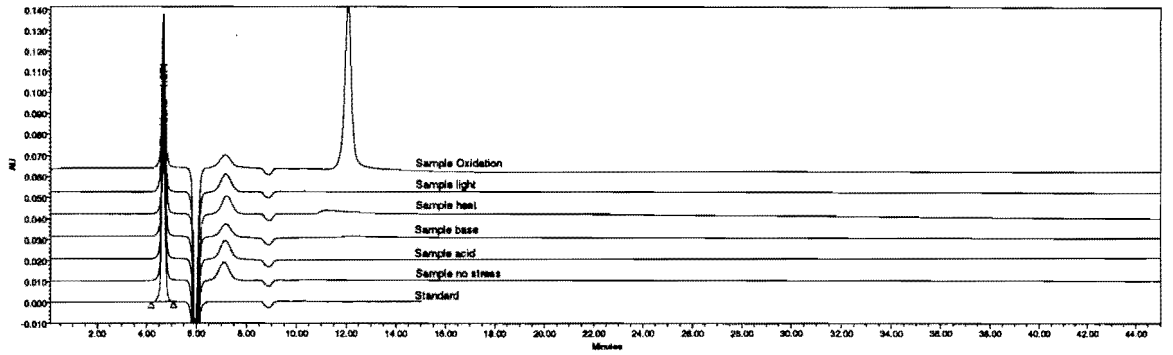
Linearity of the method was established by injecting five standard concentrations of polyvinylpyrrolidone and preparing a calibration curve by plotting PVP response versus concentration. The solutions covered a concentration range of 0.05 – 0.15%. The linearity curve for polyvinylpyrrolidone is presented in Figure 13. The solutions covered a range of 50%-150 % of theoretical concentration 0.1 mg/mL. The method was linear in this range with R^2 values of 0.9999. Concentration of linearity solutions, responses and linearity parameters such as slope, y-intercept, and coefficient of determination are listed in Table 5. No carryover was observed into blank injections immediately after the highest level linearity standard, ensuring independence of the samples.

System precision was established by six replicate measurements of a 100% theoretical standard solution of povidone. The %RSD for povidone was found to be 0.1%. Table 6a shows the results, mean and standard deviation.

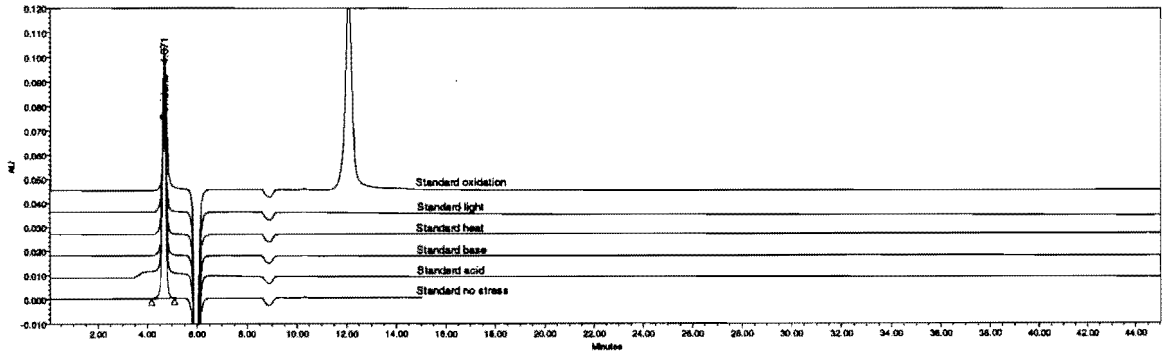
The repeatability of the method was evaluated by six identical sample preparations of a homogeneous batch and the results were found to be within the specifications. The percent relative standard deviation of the six preparations for polyvinylpyrrolidone was found to be 0.3%. In order to further validate the results, the experiment was conducted again on a different day using a different instrument, column and analyst. In addition the work was repeated at a different work site, using different instruments and different columns.



(a)



(b)



(c)

Figure 12: Overlay stress studies chromatograms for (a) ophthalmic solution without polyvinylpyrrolidone (b) ophthalmic solution (C) polyvinylpyrrolidone standard

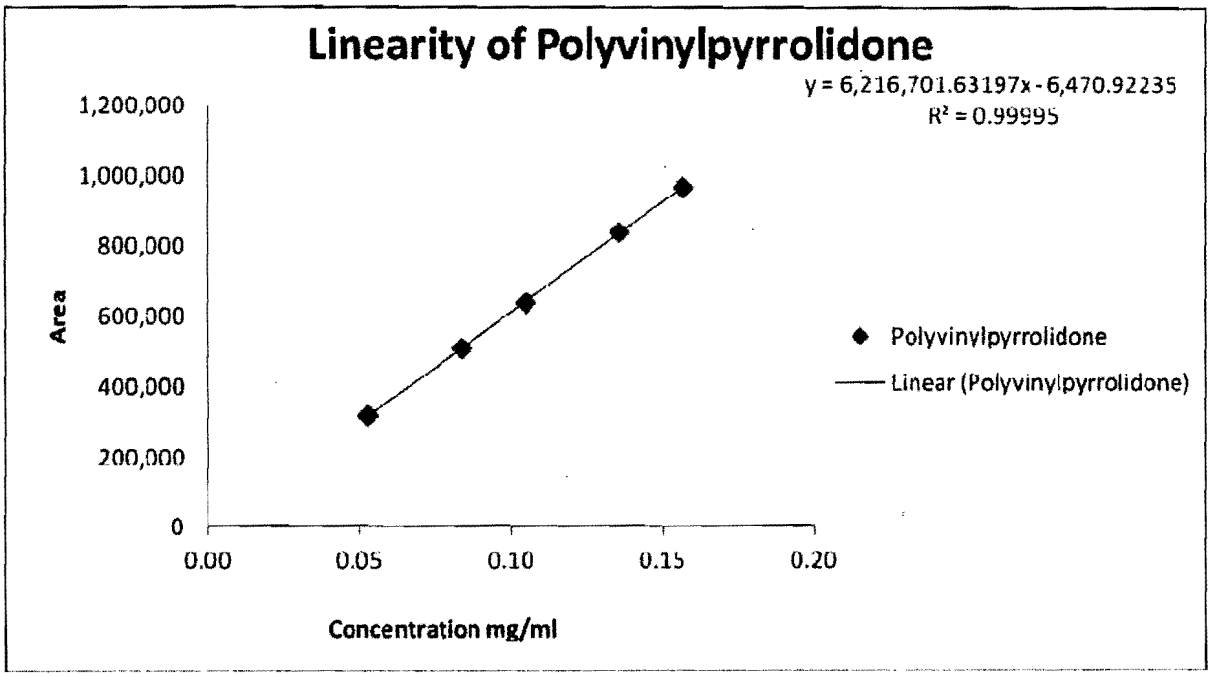


Figure 13: Linearity graph of Polyvinylpyrrolidone (PVP). PVP found to has a linear response over a range of 50%-150% of theoretical concentration 0.1 mg/mL

Target Concentration Level	Concentration (mg/mL)	Response (Peak Area)
50%	**0.05225**	**320089.74**
80%	**0.083600**	**512627.13**
100%	**0.104500**	**640589.55**
130%	**0.135850**	**838004.55**
150%	**0.156760**	**969587.72**
Slope	**62616701.63**	
Y Intercept (%)	**-6470.92**	
Correlation	**0.99995**	

Table 5: Linearity parameters of polyvinylpyrrolidone (PVP). PVP found to has a linear response over a range of 50%-150% of theoretical concentration 0.1 mg/mL

Replicate #	Peak Response (*Area*)
1	601997.31
2	601504.28
3	601485.97
4	600671.02
5	601882.20
6	601477.17
Mean	**601502.99**
% RSD	**0.1**

(a)

Replicate	% Label Claim of Povidone (%LC)			
	Intermediate Precision		Reproducibility	
	Analyst 1/Lab 1	Analyst 2/Lab 1	Lab 1	Lab 2
1	102.0	102.0	102.0	102.4
2	102.3	101.9	102.3	102.9
3	102.4	102.1	102.4	102.9
4	102.8	103.3	102.8	103.1
5	101.9	101.4	101.9	102.7
6	102.6	102.7	102.6	102.8
Mean	**102.3**	**102.2**	**102.3**	**102.8**
%RSD	**0.3**	**0.7**	**0.3**	**0.2**
Mean Agreement	**0.1**		**0.5**	

(b)

Table 6: Experimental results for (a) system precision and (b) intermediate method precision and reproducibility results

The experimental mean agreement for povidone between the two days and sites was found to be 0.1 and 0.5 respectively and is within the acceptance criteria. Results are displayed in Table 6b.

The accuracy of the method was established by assaying three different concentration levels 70%, 100%, and 130% of the theoretical concentration.

Six preparations of ophthalmic formulation without polyvinylpyrrolidone, at each level, were spiked with standard of polyvinylpyrrolidone and were injected into the HPLC system. Results are reported in Table 7a with polyvinylpyrrolidone mean recovery values varied from 100.0 to 100.8% of the prepared standard concentration. No bias was observed for polyvinylpyrrolidone, since the results for the mean recovery from all three accuracy levels were not significantly lower or higher (±1.5%) than the theoretical value. The range in which the method is shown to be linear and accurate for povidone is between 70-130% of theoretical concentration.

The method was unaffected by small, deliberate variations in chromatographic parameters and mobile phase preparation. The parameters tested were detector wavelength (+/- 4 nm), mobile phase flow rate (+/- 0.1 mL/min) and temperature (+/- 5°C). Retention time and the peak shape were not affected by these parameters. The variation in results that was observed between the normal method conditions and the changed parameters were from 0.1 to 1.6% for povidone. Table 7b shows the results that were obtained by the robustness studies of polyvinylpyrrolidone.

Different lots of polyvinylpyrrolidone raw materials were used to evaluate the impact on the assay values using polyvinylpyrrolidone other than the one used in the formulation batch. In addition, the USP reference standard of polyvinylpyrrolidone was

Prep #	Povidone		
	Mean Recovery Value %LC		
	70% Level	100% Level	130% Level
1	100.5	101.0	100.6
2	98.9	100.7	100.1
3	99.9	100.8	101.0
4	100.2	101.1	99.5
5	99.1	100.3	100.9
6	101.0	101.1	100.9
Mean	**100.0**	**100.8**	**100.5**
%RSD	**0.81**	**0.3**	**0.6**

(a)

Parameters under study	% LC of povidone	Percent difference
Control	102.8	N/A
Flow 0.9ml/min	103.6	-0.8
Flow 1.1ml/min	103.8	-1.0
Column temperature 45° C	102.7	0.1
Column temperature 55° C	103.1	-0.3
Mobile Phase - 10%	103.5	-0.7
Mobile Phase + 10%	104.4	-1.6
Wavelength at 216nm	102.9	-0.7
Wavelength at 224nm	103.6	-0.9

(b)

Table 7: Experimental results for (a) Accuracy results (Ophthalmic solution without polyvinylpyrrolidone spiked with standard solution of polyvinylpyrolidone at three levels, 70, 100, 130% of theoretical) and (b) robustness studies of polyvinylpyrolidone

evaluated to quantitate polyvinylpyrrolidone in ophthalmic solutions. One sample preparation was run with five different raw material polyvinylpyrrolidone standards and one USP reference standard as per method. Table 8a summarizes the results of this test. An attempt to explain the variability on the results the water content of each standard of polyvinylpyrrolidone was determined by Karl Fischer as per USP 30 <921> method I. It should be noted that standard 6 is the USP standard and water content was not performed for this standard because it was dried prior to its use. Table 8b summarizes the water content results of polyvinylpyrrolidone. The water content results explain the variability on the assay sample results that were observed when different standards were used. If results corrected for water content then they are a lot closer to each other and the differences between them are within experimental error of the analytical method. Table 8c shows the results taking into account the water content.

The stability of the standard and sample solutions for povidone was also evaluated. No significant change in PVP response was observed over a period of 192 hrs. Solutions stored at refrigerator and ambient temperature. Samples tested at the same time and results between the two conditions were comparable. Table 9 shows the solution stability of both standard and sample at ambient temperature.

	Standard 1 Povidone	Standard 2 Povidone	Standard 3 Povidone	Standard 4 Povidone	Standard 5 Povidone	Standard 6 Povidone
	% Label Claim					
Assay	102.7	106.8	106.4	105.1	107.0	101.3
Absolute difference from Standard 1	N/A	4.1	3.7	2.4	4.3	1.4

(a)

	Standard 1 Povidone	Standard 2 Povidone	Standard 3 Povidone	Standard 4 Povidone	Standard 5 Povidone	Standard 6 Povidone
	% water content					
Water content	3.84	6.42	6.81	4.93	7.11	N/A*
Absolute difference from Standard 1	N/A	2.58	2.97	1.09	3.27	N/A
* Water content of USP standard not determined since it is dried prior to use						

(b)

	Standard 1 Povidone	Standard 2 Povidone	Standard 3 Povidone	Standard 4 Povidone	Standard 5 Povidone	Standard 6 Povidone
	% Label Claim					
Assay	98.7	99.9	99.2	99.9	99.4	101.3
Absolute difference from Standard 1	N/A	1.2	0.5	1.2	0.7	2.6

(c)

Table 8: Evaluation of polyvinylpyrrolidone assay using different raw material (a) assay results of ophthalmic formulation using different raw materials of polyvinylpyrrolidone as a standard, (b) water content of polyvinylpyrrolidone standards, (c) assay results of ophthalmic formulation taking into account the water content of polyvinylpyrrolidone

Time Point	Standard	Sample
	% Label Claim of polyvinylpyrrolidone (%LC)	
Initial	99.5	102.0
24 Hours	99.9	102.4
4 Days	100.8	102.8
8 days	101.4	104.3

Table 9 Solution stability studies of polyvinylpyrrolidone at ambient temperature

3.4 Conclusion

A new method for the determination of total polyvinylpyrrolidone in ophthalmic solutions was developed using SEC-UV with quantitation of the analyte peak eluting before the void volume disturbance. This unique combination separated polyvinylpyrrolidone from other formulation components and allowed a simple isocratic method. Validation for this method was performed according to ICH guidelines and met all acceptance criteria. The method is precise (+/- 0.1%), accurate (+/- 1%), and linear at concentration ranges of 0.07 mg/mL to 0.13 mg/mL, typical of prepared ophthalmic solution samples for polyvinylpyrrolidone. Several unusual chromatographic situations were used together successfully in this work: SEC with UV detection and quantitation of a chromatographic peak eluting before the void volume. This method provides a model for the analysis of a polymeric component in the presence of monomeric components in a number of different types of formulations.

Chapter 4: Demulcents (PEG400 and Glycerin)

4.1 Introduction

An array of over the counter (OTC) ophthalmic solutions is available for the self treatment of minor ophthalmic disorders. Common treatments for minor eye episodes include the use of OTC ophthalmic lubricants, including artificial tears products which contain polyethylene glycol 400, and glycerin. The use of lubricants offers many advantages. From a chemical composition perspective, they are used to control the viscosity of the solutions. They also offer a medicinal benefit: to relieve pain in inflamed or irritated mucous membranes.

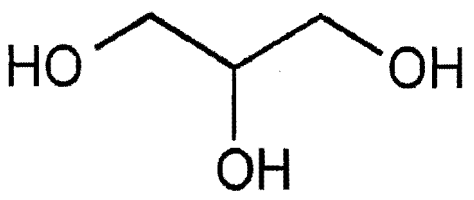
Glycerin was synthesized by the Swedish scientist K. W. Scheele in 1779, as a result of heating a mixture of olive oil with lead monoxide. He published this method in 1783 after noticing other metals and glycerides produced the same chemical reaction which yields glycerin and soap. However, it was not until 1811 when the French investigator M. E. Chevreul discovered the immeasurable properties of glycerin. He named the compound glycerin after the Greek word glykys, meaning sweet. Twelve years later he patented a new way to produce fatty acids from fats when treated with alkali in which glycerin was released. The first empirical formula of glycerin ($C_3H_8O_3$) was announced in 1835 by French investigator Pelouze. About fifty years later, in 1883, Berthelot and Lucea established the accepted formula of glycerin $C_3H_5(OH)_3$ [75-76].

The unique chemical and physical properties of glycerin including that it is not toxic to the digestive system and not a skin irritant, make glycerin applicable in a variety

of applications within the chemical, pharmaceutical, consumer, and food industries [77]. In ophthalmic solutions, glycerin is generally present at approximately 0.2% concentration in an aqueous matrix containing other formulation components and active therapeutic ingredients. Glycerin is also used as a lubricant to relieve symptoms of dry eyes such as burning, and itching, which are caused by exposure to wind, sun, heating, air conditioning, and extended computer use.

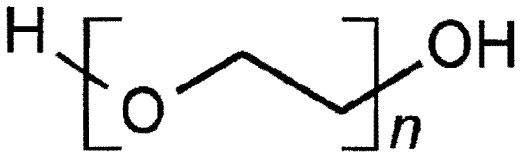
Chemically, glycerin is a very stable alcohol. It has three hydroxyl groups which can react with other chemical groups, thereby permitting the synthesis of many different derivatives with specific applications [78]. Physically, glycerin is a clear and viscous liquid miscible with water and alcohol. At low temperature glycerin does not crystallize which makes it a favorable candidate for cooling systems such as antifreeze. However, the most important property of glycerin is its ability to absorb and hold moisture from the air. This unique property, in conjunction with a glycerin-water solution makes glycerin a suitable agent for plasticizers which give products the desired shelf life, flexibility and softness. Glycerin is found in nature in the form of triglycerides and occurs naturally in beers, wines, breads and other products of grains and sugars.

As was mentioned above, glycerin has three hydroxyl groups. Two of these groups are primary and the other secondary [79]. Figure 14a shows the structure of glycerin. The two primary hydroxyl groups are more reactive than the secondary but in some reactions the secondary hydroxyl group can react before all the primary groups are exhausted. Industrially, glycerin is produced during saponification from fats and oils after



Structure name: propane-1,2,3-triol
Molecular formula: $C_3H_8O_3$
Formula weight: 92.09

(a)



Structure name: Polyethylene Glycol
Molecular formula: $C_nH_{4n+2}O_{n+1}$, n=8.2 to 9.1
Formula weight: 380-420

(b)

Figure 14: Structures of a) Glycerin and b) PEG400

Hydrolysis. It is recovered in a crude state and purified by ion exchange or distillation [75].

Glycerin is analyzed with different techniques that are labor intensive and in many instances not stability indicating. A more specific assay of glycerin in the United States Pharmacopeia is accomplished by periodate oxidation titration. Glycerin is oxidized by potassium periodate to form formic acid, then the solution is titrated with standard potassium arsenite and the liberated iodine is indicated by the starch-iodine reaction [80].

Glycerin and its derivatives have been chromatographically analyzed by a variety of techniques including thin layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE), ion chromatography and HPLC utilizing different detectors, ultra violet (UV), charged aerosol detector (CAD) [81-86]. A large number of chromatographic methods for the determination of glycerin have been also reported in biodiesel research literature [87-102]. Biodiesel is a renewable fuel from natural oils like soybean oil, rapeseed oil or animal fats and produced by transesterification reaction of these oils/fats with alcohol to yield smaller molecules (FAMES). Their properties are close to diesel fuel. However, during this process glycerin also is generated as by-product of the reaction and needs to be removed because it can be harmful to the engine. There is a maximum amount of free and total glycerin that permitted in the fuel and is limited to 0.02% and 0.24% respectively (0.25% for European) [103]. Figure 15 shows such a reaction.

Glycerin is a metabolic precursor for the synthesis of triglycerides and membrane phospholipids. The serum content of glycerin is correlated as a marker of cardiovascular

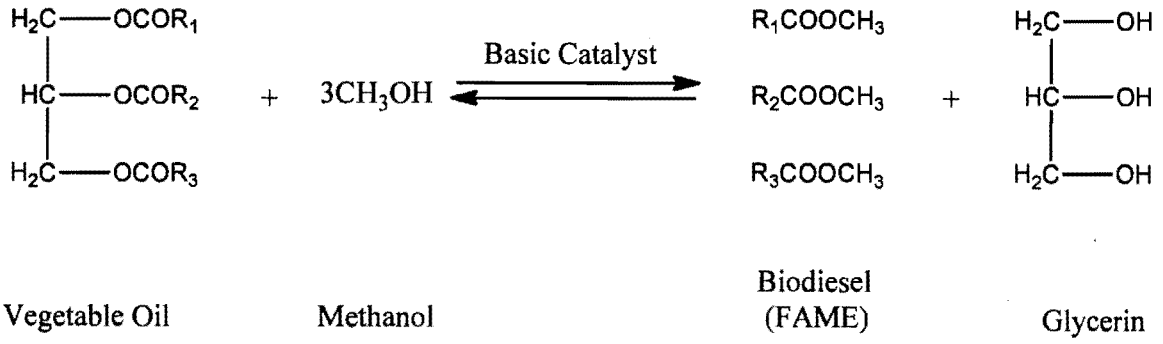


Figure 15: Transesterification reaction of triglycerides to fatty acid methyl esters [92]

disease [104] and the plasma, tissue content of glycerin is considered and index of cardiac triglyceride metabolism [105]. A significant number of publications exist in this area for the analysis of polyhydroxyl compounds using thin layer chromatography, [106-107] enzyme linked assays, [108-109] pulsed amperometric detection, [110] mass spectra analysis [111-112] and UV detection [113-118].

Polyethylene glycol is a polyether compound with many applications to consumer and pharmaceutical products. Manufactured by the polymerization of ethylene oxide with either water, mono ethylene glycol or diethylene glycol under alkaline catalysis. Ethylene glycol and ethylene glycol oligomers are preferred over water because the products of such reactions are polymers with smaller range of molecular weight (low polydispersity) [119-120].

After the desired molecular weight was reached, the reaction is terminated by adding an acid to neutralize the catalyst. The molar mass distribution of polyethylene glycol defines the different grades assigned for this chemical substance. The various grades of polyethylene glycols are not uniform compounds, but rather mixtures of similar polymer members. For that reason, controlling the polymerization is a very important step in the production of polyethylene glycol. Molar mass distribution is assigned by gel permeation chromatography (GPC) in which analysis is based on the different migration rates of the polymer mixture in microporous gel. In general, each polyethylene glycol follows by a number which is the average molecular weight of the polymer.

Polyethylene glycol with the general formula $H(OCH_2CH_3)_nOH$ can be in both liquid or solid form based on the size of the molecular weight [121]. Structurally the shorter chains, (with a degree of polymerization less than 10) form a zigzag structure,

while the longer chains are in the crystalline state, form a twisting / spiral like structure where the oxygen atoms form ether bridges at regular intervals. Figure 14b shows the structure of PEG400.

Chemically the hydroxyl end groups of polyethylene glycol have a significant role in the physical and chemical properties of these molecules and for that reason molecules of molecular weights 200-35000, are referenced as polyethylene glycols and not polyethylene oxides.

Physically these molecules are clear, viscous liquids or white solids which dissolve in a variety of solvents including water to form transparent solutions. They are soluble in aromatic hydrocarbons and slightly soluble in aliphatic hydrocarbons. Polyethylene glycols are very stable molecules which do not hydrolyze or otherwise deteriorate upon storage and do not support mold growth. They are compounds of low toxicity and for that reason are widely applicable in many different industries [122]. In ophthalmic solutions, PEG400 is used as a moisturizer, and is generally present at approximately 1% concentration in an aqueous matrix containing other excipients and active pharmaceutical components. PEG400 is used to adjust the viscosity of the solution and to enhance the solubility of other poorly soluble components in the formulation. The formulation viscosity is a critical parameter in ophthalmic solutions because it enables the formulation to remain in the eye longer and gives more time for the drug to exert its therapeutic activity or undergo absorption.

Smaller range molecular mass polyethylene glycols are used extensively in the pharmaceutical industry and in clinical research [123]. Therefore there are many requirements for accurate quality control and product characterization methods. It should

also be noted that chromatography is the preferred method for analyzing such compounds [124-132]. Other methods such as colorimetry, [133] turbidimetry, [134] Fourier Transform IR (FTIR) [135] can provide only bulk information about polyethylene glycols and are subject to interference. Paper chromatography methods have been used for the determination of polyethylene glycols but the method is limited in sensitivity and repeatability [136]. Capillary gel electrophoresis has also been reported in the literature for the separation of the polyethylene glycols but this method, too, has its limitations to column fragility and cost [137]. Derivatization techniques are widely used for the separation of polyethylene glycols due to the popularity of the conventional UV detector. These techniques also allow the use of fluorescent detectors, minimizing interference and increase the detection limits [138-140]. UV detection without using derivatisation was also reported in the literature but the use of sodium azide in the mobile phase was needed to minimize solvent absorbances of mobile phase components. However, the baseline drift still posed a problem [141]. An evaporative light scattering detector was successfully implemented in the determination of polyethylene glycols without derivatization [142]. More expensive mass spectrometer detectors are not widely used and are not available to every laboratory have been used for the analysis of polyethylene glycols [143-145].

Based on extensive investigation of the literature no method appeared to report the simultaneous determination of PEG400 and glycerin in ophthalmic solutions. One paper was found for the determination of PEG400 in ophthalmic solution but it was using two different columns in series to separate PEG400 from other components in the formulation. It should be noted, that the method applicability in this work was limited to

a specific type of ophthalmic formulation and the length of each column used was 300 mm which made the instrument arrangements a bit more complicated. In addition the mobile phase used in this publication was methanol and the buffer system consisted of by three different components, making the pre-run system preparation time consuming [146].

In this work a new method is described for the analysis of PEG400 and glycerin in ophthalmic solutions. The analytical procedure is a simple, direct HPLC method that is based on Size Exclusion Chromatography (SEC). The sample is diluted with water and injected into a HPLC instrument equipped with TOSOH Bioscience TSKgel G1000PW, 7.5 mm ID x 30 cm, 10 μm column at 50°C. Quantitation is achieved by comparison of peak heights of PEG400 and glycerin in the sample to that of a known concentration reference standard. PEG400 and glycerin are separated from other components in the formula and detected by a Waters Refractive Index (RI) detector.

4.2 Experimental

4.2.1 Reagents and Chemicals

The raw material for PEG400 was purchased from P&G Chemical (Cincinnati, OH, USA) and the raw material for glycerin was purchased from Clariant Produkte (Gendorf, Germany). HPLC water grade was purchased by J.T. Baker (Phillipsburg, NJ, USA).

Laboratory formulations of typical opthalmic solutions were prepared in water at 0.57 mg/mL and 0.13 mg/mL concentration of PEG400 and glycerin respectively. They

were diluted by adding 5 mL of formulation to a 100 mL volumetric flask and diluting to the mark with water prior to HPLC analysis. The standard working concentrations of the standard solutions were 0.57 mg/mL and 0.13 mg/mL for PEG400 and glycerin respectively. They were prepared by weighing 130 mg of glycerin into 50 mL volumetric flask and diluted to the mark with water (glycerin stock solution). Working standard concentrations were prepared by weighing 114 mg of PEG400 into 200 mL volumetric flask and pipette 10 mL of glycerin stock mix well and diluted to the mark with water. The working standard concentrations were 0.57 mg/mL and 0.13 mg/mL for PEG400 and glycerin respectively.

4.2.2 Instrumental Conditions

An Alliance HPLC system equipped with a 2695 separation module with a differential refractive index detector model 4210 was used for all experiments. Data collection and processing was performed using an Empower chromatographic data acquisition system. (Waters Corporation, Milford, Massachusetts, USA) The chromatographic column was a TSKgel G2000PW, 7.5 mm ID x 30 cm, 12 μm column (TOSOH Bioscience, Tokyo, Japan). The flow was kept at 1.0 mL/min during the length of the run and the column temperature was 50°C. The refractive index detector settings were: sensitivity 64, filter (TC) 3, cell temperature 35°C. The injection volume was 10 μL and the mobile phase was water vacuum degassed for fifteen minutes prior to use.

4.2.3 Testing Parameters

Testing of the chromatographic method was performed in accordance with International Conference on Harmonisation (ICH) guidelines and typical operating procedures for pharmaceutical analysis. The test parameters are presented in the same order as they were investigated during the method validation. Specificity was determined by exposing ophthalmic solution samples, placebos, and standards of PEG400, glycerin to stress conditions of acid, base, hydrogen peroxide, light, and heat. Subsequently, the solutions were analyzed according to the method presented in this paper to ensure no extraneous peak co eluted with the actives. A refractive index detector was used during validation to ensure that no interfering compounds co-eluted with PEG400 and glycerin. Accuracy was measured by spiking ophthalmic solutions without PEG400 and glycerin with PEG400 and glycerin at three working levels of 70%, 100%, and 130% of the standard concentration. Actual concentrations tested for PEG400 / glycerin are: 0.399 mg/mL / 0.089 mg/mL, 0.57 mg/mL / 0.13 mg/mL, 0.74 mg/mL / 0.169 mg/mL. Six preparations were performed at each level and assayed as per method conditions. The average result from each individual level was compared to its respective theoretical concentration value to check for any potential bias.

To ensure that the method was linear in the working concentration range, five concentration level solutions of PEG400 and glycerin, corresponding to 0.287-0.862 mg/mL for PEG400 and 0.064-0.192 mg/mL for glycerin (about 50-150% of the expected analyte concentration) were prepared and injected. In addition, the ability of the system to retain and carryover the analyte into subsequent injections were evaluated by injecting a

blank solution (diluent only) in duplicate immediately after the 150% linearity level. To ensure performance of the system before and during the analysis, system performance parameters, as defined in USP/NF, were established as a direct result of ruggedness and robustness experiments [147].

System precision was determined using six replicate measurements of a 100% theoretical concentration standard solution (0.57 mg/mL and 0.13 mg/mL for PEG400 and glycerin concentration respectively) containing PEG400 and glycerin, with an acceptance criterion of the RSD being less than 2.0%. Repeatability was determined by six identical sample preparations of the same lot. To determine agreement among test results obtained from multiple samplings of the same lot of samples on different days using different instruments, columns and analysts, six identical samples from the same lot were prepared and analyzed.

Robustness was determined by examining small variations in: flow rate (± 0.1 mL/min), column temperature (± 5°C). These parameters were changed one at a time. System suitability and sample runs were both conducted with unchanged method parameters and modified parameters. In addition a quantitative comparison study was performed between the raw material that was used to prepare the batch and other raw materials of PEG400 and glycerin including a USP reference standard. Three sample preparations were run and quantitated with four different standards as per the method. Further, to assess sample stability, standard and sample solutions were stored at room temperature and tested at initial, 24 hrs, 48 hrs, 72 hrs, 168 hrs and for some formulations up to 216 hrs. The solutions were tested against a freshly prepared standard at each time point.

4.3 Results and Discussion

4.3.1 Developmental Work

The physical and chemical properties of PEG400 and glycerin have been well established. The UV detector is possibly the most versatile and useful detector in high performance liquid chromatography, however it is not as widely used in SEC since many polymers do not absorb electromagnetic radiation in the UV range [35]. Utilization of UV detection is possible if derivatization is being used. However, it would be difficult to derivitize both analytes at the same time and analyze them simultaneously, not to mention the added complexity of the sample preparation. For these reasons and the fact that both compounds do not absorb electromagnetic radiation in the UV range, differential refractive index detection was selected for this work. Refractive Index is a universal detector and is widely used in size exclusion chromatography (SEC) for compounds without significant chromophore [70]. Detection is achieved by monitoring changes in the refractive index of the mobile phase in the presence of PEG400 and glycerin molecules. Thus the combination of SEC with refractive index detection is especially suited to this application.

The main goal during method development was to have an isocratic method that separates PEG400 and glycerin from other compounds in the formulation or degradation products. In this study that was not necessary since the molecular weight range of PEG400 and glycerin were well defined, approximately 400 g/mol (range 380-420 g/mol) for PEG400 and 92.09 g/mol for glycerin and that information was furnished by the

vendor. Initially, several size exclusion columns from different manufacturers were screened and TSK-gel G2000PW, 7.5 mm ID x 30 cm x 12 μm column was selected based on symmetrical peak shape of PEG400 and glycerin. TSK-gel columns in general consist of hydrophilic polymethacrylate spherical beads with sizes ranging from 12 μm to 17 μm. These types of columns are suitable for the analysis of water soluble polymers and more specific the G2000PW is suitable for small (2000 Da and less) molecular weight polymers. As seen in Figure 16, in our method the TSK-gel G2000PW column is used in the expected context with smaller molecular weight analytes (glycerin) eluted later than analytes with higher molecular weight (PEG400). Other compounds present in the formulation, with molecular weights less than 2000, elute in the separation range of the column and if they are are smaller than glycerin will be eluted substantially later. If they are larger than PEG400 will be eluted earlier than the PEG400. In many of the sample chromatograms that were obtained, an additional peak was observed prior to PEG400 peak which we believe to be hydroxypropyl methylcellulose (HPMC): another polymer (higher in molecular weight than PEG400) used in ophthalmic solutions. Figure 16 shows a chromatogram of this run with all three peaks (HPMC, PEG400, glycerin) separated.

In general, polymers outside the upper limit of the exclusion volume range of the column will elute before the void volume. While uncommon, quantifying a peak eluting before the void volume has been recently reported in a similar context for the group assay of polyvinylsulfonic acid impurities in 2-(N-morpholino)-ethanesulfonic acid and polyvinylpyrrolidone [35, 71].

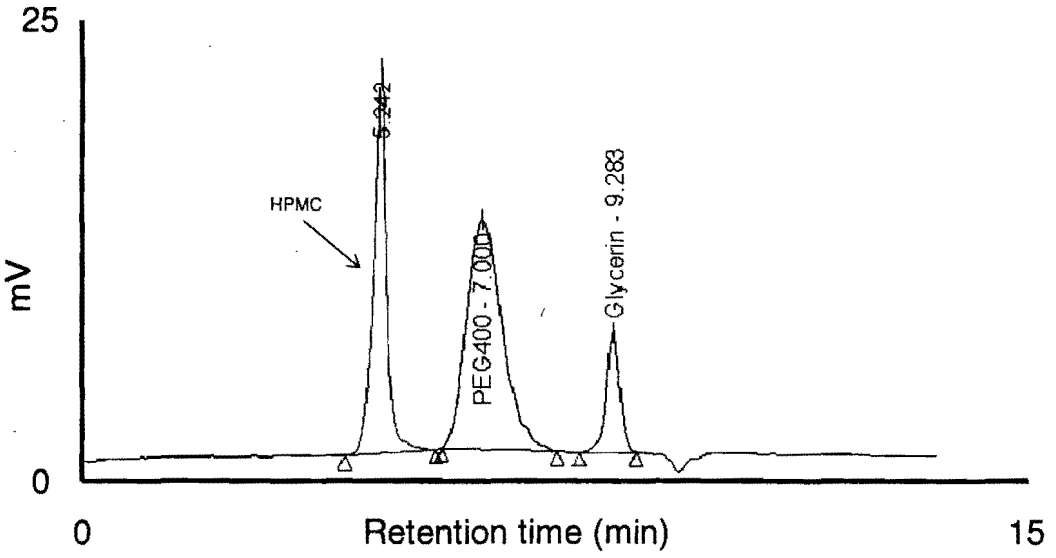
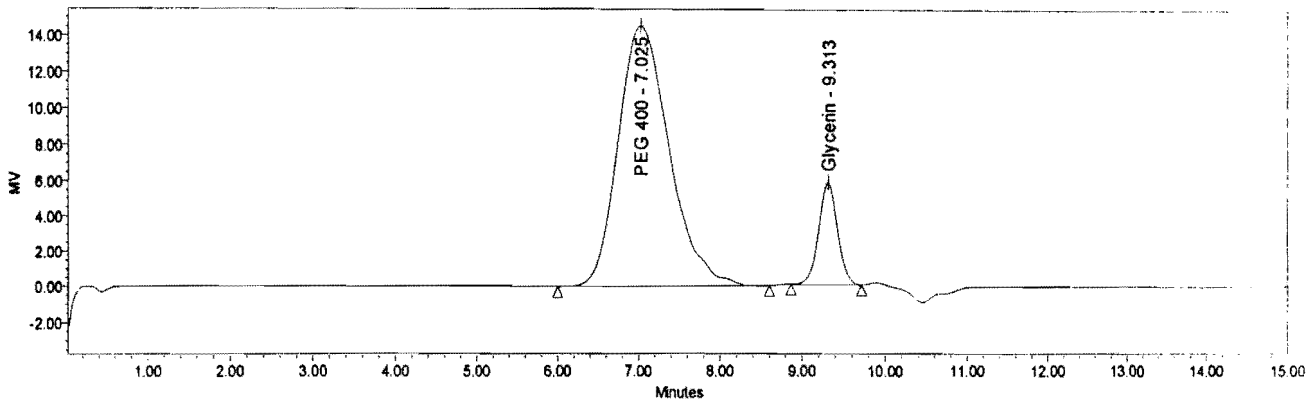


Figure 16: Sample chromatogram with hydroxypropyl methylcellulose (HPMC), PEG400 and glycerin

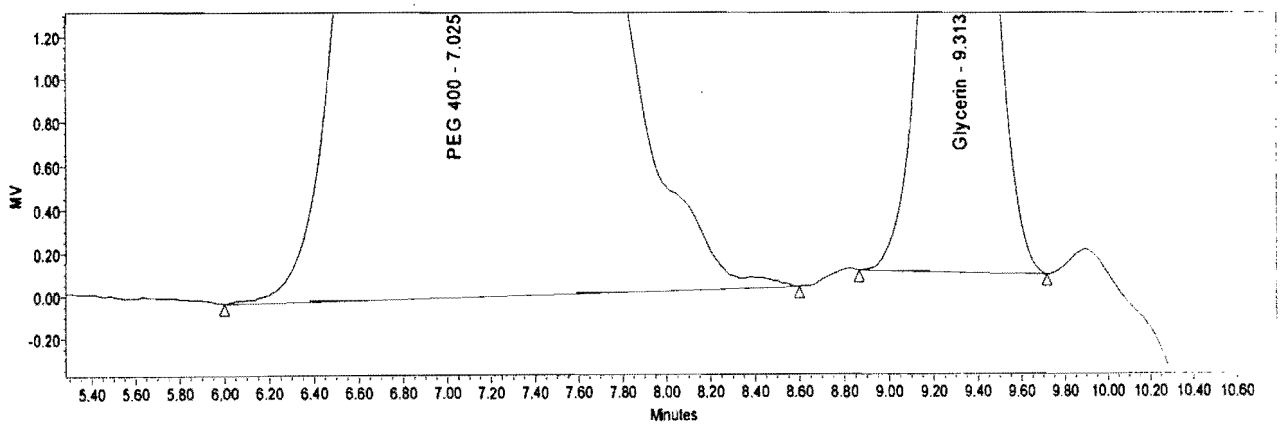
Figure 17 clearly shows a very symmetrical peak for PEG400 and glycerin, demonstrating a satisfactory tailing factor for system performance. The validation data presented below demonstrate adequate precision.

In an ideal SEC method separation is based on molecular size of the analyte relative to the pore size of the packing material without any interactions between the analyte and the packing material. However, a small number of weakly charged groups at the surface of the packing material can cause changes in elution order or peak distortion. Mobile phase selection is important to avoid enthalpic interactions between the analyte and the packing material. There are a variety of solvents compatible with TSK-GEL columns, so the selection process depends on the chemical structure, ionic nature and solubility of the analytes [72]. In this study, water was used as the ideal mobile phase because both PEG400 and glycerin are soluble in water. A good peak shape was observed, with the expected elution order, which is a clear indication that there are no interactions between the packing material and the analytes. To avoid microbial growth in the column which can cause erroneous peaks and peak distortion in future analyses the column rinsed with 0.5% sodium azide prior to storage [124].

Temperature adjustment can reduce the analysis time and improve chromatographic performance. More specifically, as the temperature increases, the viscosity of the mobile phase decreases and the diffusivity of the analyte increases. Fast size exclusion chromatography has been discussed in the literature and temperature is one of the primary parameters adjusted to achieve faster analysis times [73-74]. Optimum chromatographic performance was obtained in this method with column temperature at 50°C.



(a)



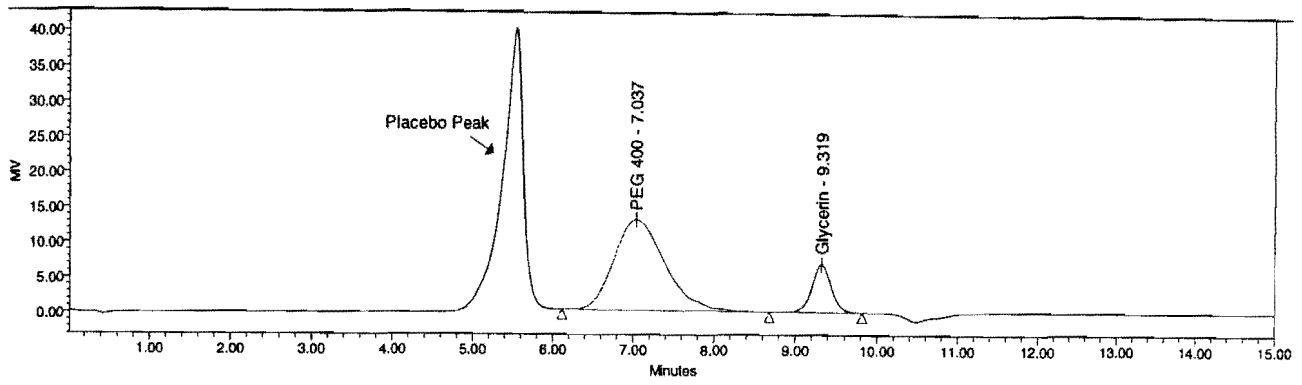
(b)

Figure 17: Chromatogram of PEG400 and glycerin standard (a): Full scale chromatogram (b): Expanded chromatogram

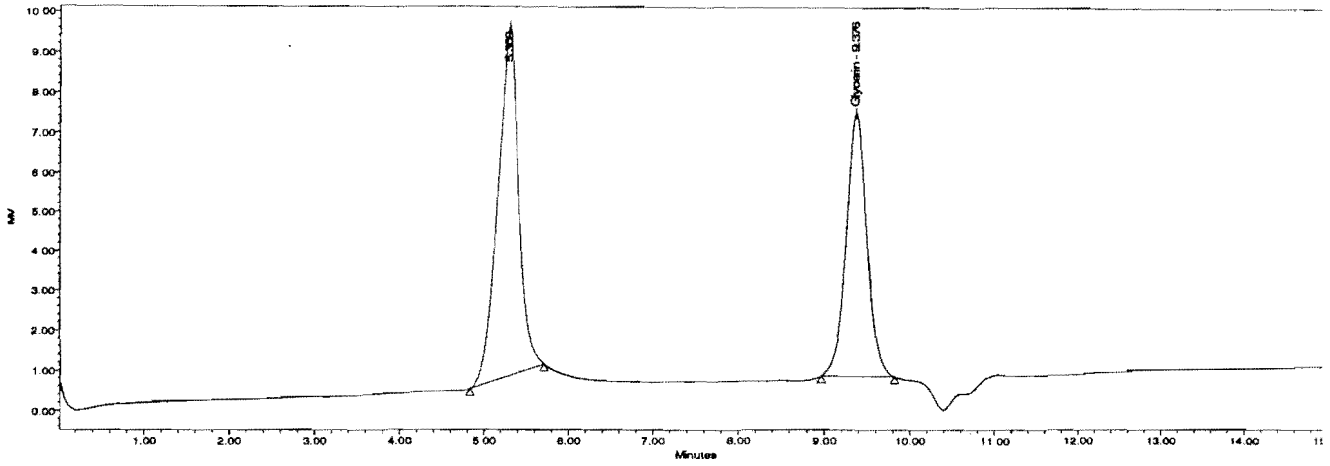
4.3.2 Experimental Work

Figure 18 shows the analysis of PEG400 and glycerin in several different formulations that contain both PEG400 and glycerin (top), only glycerin (middle) and only PEG400 (bottom). PEG400 and glycerin do not appear in the placebo chromatograms because it is simply a formulation prepared without the analytes used to demonstrate that other compounds that commonly appear in ophthalmic formulations will not interfere with the method. Figure 19 shows overlay standard chromatograms with ophthalmic solutions that doesn't contain PEG400 and glycerin, glycerin (top) and PEG400 (bottom). These chromatograms clearly demonstrate that SEC can be used for the separation of PEG400 and glycerin from other formulation components. Chromatographic performance of the system was evaluated for each run by measuring the precision of six injections of PEG400 / glycerin working standard, tailing factor, resolution between analytes or any excipient that elutes close to method analytes.

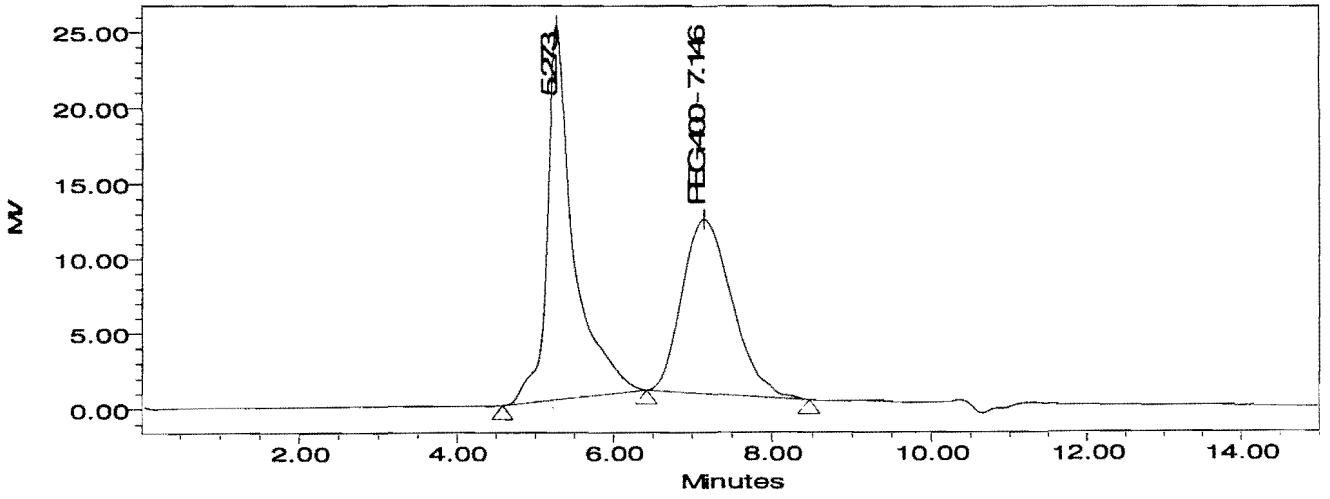
The specificity of the method was tested by stressing standards of PEG400 / glycerin and ophthalmic solutions with and without PEG400 / glycerin. No interference was observed for the PEG400 and glycerin peaks from the forced degradation samples and standards. During these studies equivalent amounts as per method of ophthalmic solution samples, ophthalmic solution without PEG400, glycerin, and PEG400, glycerin standard were stressed for the following conditions: (1) heat at 75°C for 1 hr; (2) 5 mL of 1 N hydrochloric acid at 75°C for 1 hr; (3) 5 mL of 1 N sodium hydroxide at 75°C for 1 hr; (4) 5 mL of 30% hydrogen peroxide at 75°C for 1 hr; and (5) light exposure for 24 hrs as per ICH option 1.



(a)

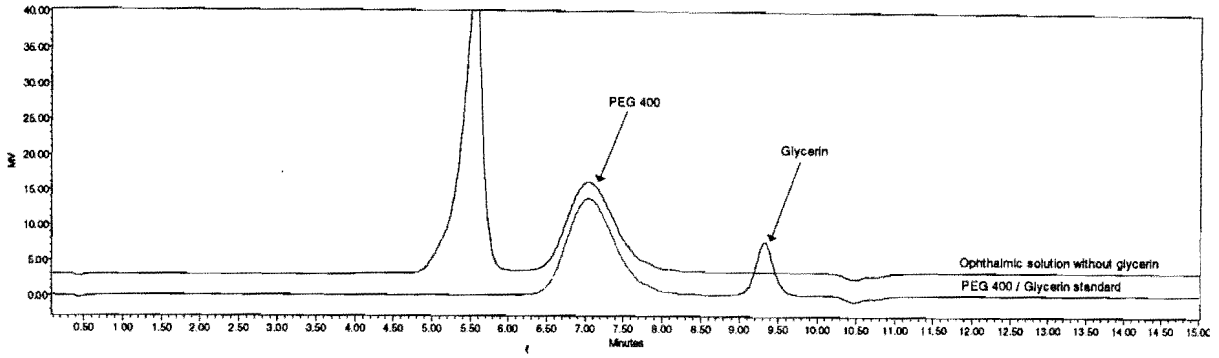


(b)

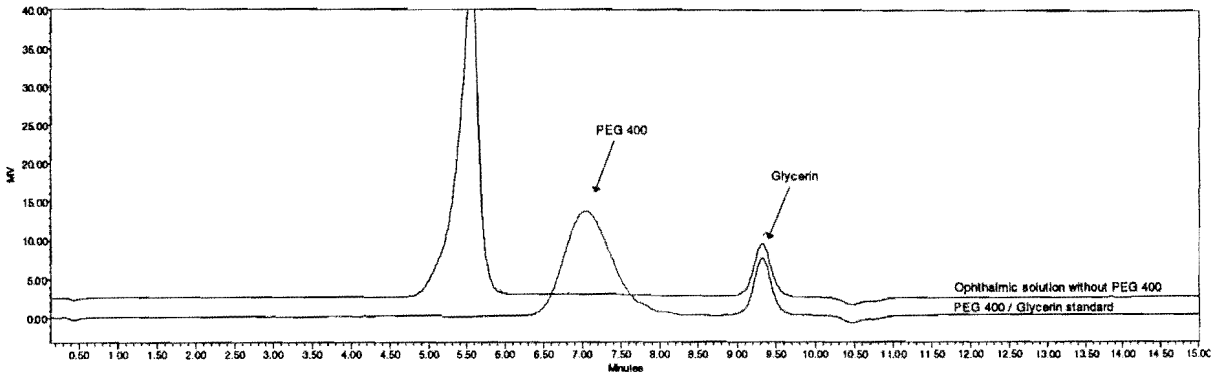


(c)

Figure 18: Different sample chromatograms (a) contain both PEG400 and Glycerin, (b) only glycerin, (C) only PEG400



Glycerin

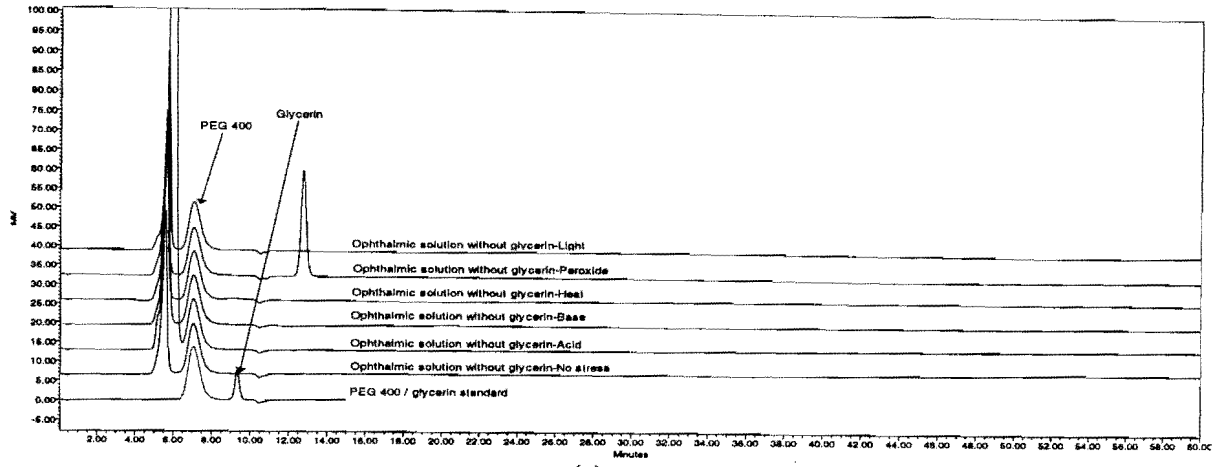


PEG400

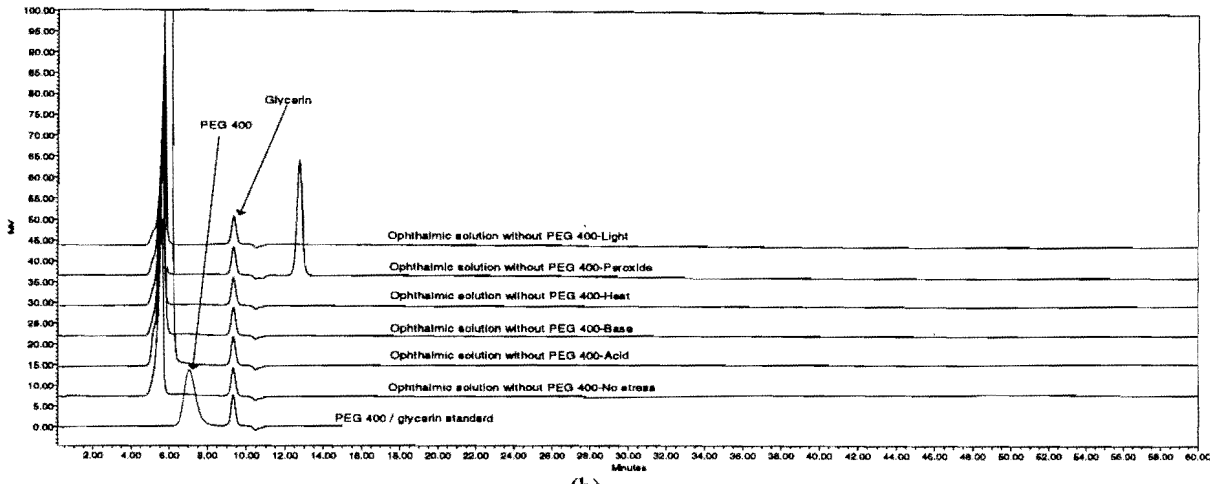
Figure 19: Overlay chromatograms of working standard with placebo: glycerin (top), PEG400 (bottom)

Samples during this option were exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square meter. Subsequently the solutions were cooled to room temperature, neutralized (if needed) and analyzed according to the test procedure. To ensure that the method is stability indicating and no extraneous peaks co-eluted with the peaks of interest, the stress samples of ophthalmic solutions and standards were run for 60 minutes. No interference was observed during stressing of these samples. A peak that was observed at an approximate retention time of 12 minutes during stressing (with hydrogen peroxide in all of the samples) is attributed to peroxide solution and is not related to degradation of PEG400 and glycerin. Figure 20 shows overlay chromatograms of ophthalmic solutions without glycerin (a) without PEG400 (b) and complete ophthalmic solution sample (c) at different stress conditions.

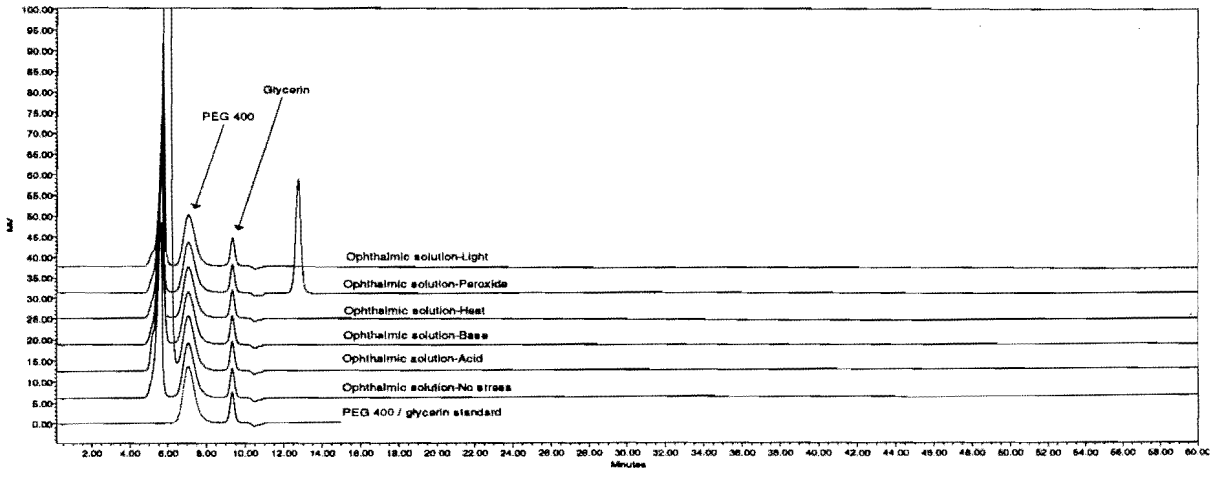
Linearity of the method was established by injecting five standard concentrations of PEG400, glycerin and preparing a calibration curve by plotting PEG400, glycerin response versus concentration. The solutions covered a concentration range of 0.287 – 0.8622 mg/mL for PEG400 and 0.0642 – 0.1925 mg/mL for glycerin. The linearity curve for PEG400 and glycerin are presented in Figures 21 and 22. The solutions covered a range of 50%-150 % of theoretical concentration 0.128 mg/mL and 0.5748 mg/mL of glycerin and PEG400 respectively. The method was linear in this range with R^2 values of 0.9999. Concentration of linearity solutions, responses and linearity parameters such as slope, y-intercept, and coefficient of determination are listed on Tables 10 and 11. No carryover was observed into blank injections immediately after the highest level linearity standard, ensuring independence of the samples.



(a)



(b)



(c)

Figure 20: Overlay chromatograms of ophthalmic solutions without glycerin (a) without PEG400 (b) and complete ophthalmic solution sample (c) at different stress conditions

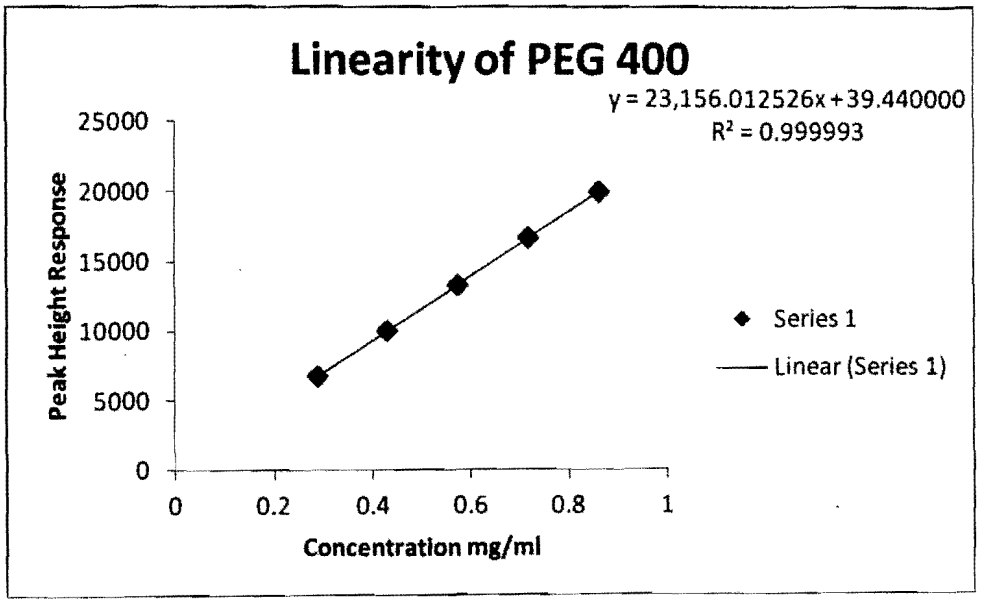


Figure 21: Linearity graph of PEG400

Target Concentration Level	Concentration (mg/mL)	Response (Peak Height)
50%	**0.2874**	**6707.58**
80%	**0.4311**	**10017.79**
100%	**0.5748**	**13326.56**
130%	**0.7185**	**16683.16**
150%	**0.8622**	**20012.49**
Slope	**23156.01**	
Y Intercept (%)	**39.44**	
Correlation	**0.999993**	

Table 10: PEG400 linearity parameters

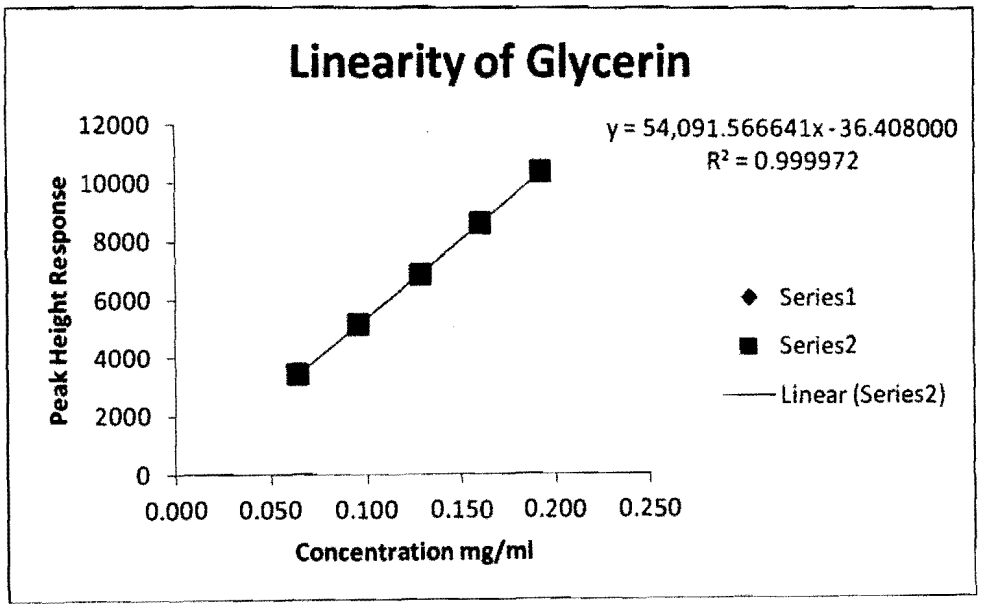


Figure 22: Linearity graph of glycerin

Target Concentration Level	Concentration (mg/mL)	Response (Peak Height)
50%	**0.064150**	**3442.01**
80%	**0.096225**	**5173.94**
100%	**0.128300**	**6878.86**
130%	**0.160375**	**8637.95**
150%	**0.192450**	**10384.94**
Slope	**54091.567**	
Y Intercept (%)	**-36.408**	
Correlation	**0.999972**	

Table 11: Glycerin linearity parameters

System precision was established by six replicate measurements of a 100% theoretical standard solution of PEG400 and glycerin. The %RSD for PEG400 and glycerin was found to be 0.1% and 0.4% respectively. Table 12a shows the system precision results, mean and standard deviation.

Repeatability was evaluated by six identical sample preparations of a homogeneous batch and the results were found to be within the specifications. The percent relative standard deviation of the six preparations for PEG400 and glycerin was found to be 0.5% and 0.2% respectively. In order to further validate the results, the experiment was conducted again on a different day using a different instrument, column and analyst. In addition the work was repeated at a different work site, using different instruments and different columns. The experimental mean agreement for PEG400 between the two days and sites was found to be 1.6% and 0.4% respectively. This is within the acceptance criteria. Results are displayed in Table 12b. The experimental mean agreement for glycerin between the two days and sites was found to be 1.2% and 0.2% respectively; also within the acceptance criteria. Results are displayed in Table 12c.

The accuracy of the method was established by assaying three different concentration levels 70%, 100%, and 130% of the theoretical concentration. Six preparations of ophthalmic solutions without PEG400 and glycerin, were spiked at each level with standard of PEG400 and glycerin and were injected into the HPLC system. Results are reported in Table 13a with PEG400 mean recovery values varying from 99.7% to 100.0 % of the prepared standard concentration. No bias was observed for PEG400, because the results for the mean recovery

Replicate #	Peak Response *(PEG400)*	Peak Response *(Glycerin)*
	Peak Height	
1	14567.82	5840.56
2	14520.11	5872.62
3	14510.00	5869.59
4	14528.96	5892.72
5	14540.22	5910.14
6	14517.93	5890.99
Mean	**14530.84**	**5879.44**
% RSD	**0.1**	**0.4**

(a)

Replicate	% Label Claim of PEG400 (%LC)			
	Intermediate Precision		Reproducibility	
	Analyst 1/Lab 1	Analyst 2/Lab 1	Lab 1	Lab 2
1	100.9	100.2	100.9	101.0
2	101.7	99.8	101.7	101.7
3	102.0	100.6	102.0	101.5
4	102.6	99.9	102.6	101.4
5	101.5	99.9	101.5	101.5
6	101.4	100.1	101.4	101.0
Mean	**101.7**	**100.1**	**101.7**	**101.3**
%RSD	**0.5**	**0.3**	**0.5**	**0.3**
Mean Agreement	**1.6%**		**0.4%**	

(b)

Replicate	% Label Claim of Glycerin (%LC)			
	Intermediate Precision		Reproducibility	
	Analyst 1/Lab 1	Analyst 2/Lab 1	Lab 1	Lab 2
1	99.2	101.0	99.2	99.2
2	99.9	100.3	99.9	99.7
3	99.3	101.7	99.3	100.0
4	99.7	101.0	99.7	99.5
5	99.6	100.7	99.6	99.3
6	99.4	99.6	99.4	98.6
Mean	**99.5**	**100.7**	**99.5**	**99.4**
%RSD	**0.2**	**0.7**	**0.2**	**0.5**
Mean Agreement	**1.2%**		**0.2%**	

(c)

Table 12: Experimental results (a) system precision results for PEG400 and glycerin, (b) Intermediate method precision and reproducibility results for PEG400, (c) Intermediate method precision and reproducibility results for glycerin

Prep #	PEG400		
	Mean Recovery Value %LC		
	70% Level	100% Level	130% Level
1	99.9	99.7	99.8
2	99.6	100.0	99.7
3	100.0	100.1	100.4
4	100.0	99.9	98.7
5	99.0	100.3	100.0
6	99.6	100.0	100.0
Mean	**99.7**	**100.0**	**99.8**
%RSD	0.4	0.2	0.6

(a)

Prep #	Glycerin		
	Mean Recovery Value %LC		
	70% Level	100% Level	130% Level
1	99.6	99.2	99.6
2	100.2	99.9	99.7
3	100.3	100.0	100.0
4	99.5	100.2	99.7
5	100.0	100.0	100.1
6	99.0	99.9	100.3
Mean	**99.8**	**99.9**	**99.9**
%RSD	0.5	0.4	0.3

(b)

Table 13: Experimental accuracy results of (a) ophthalmic solution without PEG400 spiked with PEG400 standard at three levels, 70, 100, 130% of theoretical (b) ophthalmic solution without glycerin spiked with glycerin standard at three levels, 70, 100, 130% of theoretical.

from all three accuracy levels were not significantly lower or higher (±1.5%) than the theoretical value. Results are reported in Table 13b with glycerin mean recovery values varied from 99.8% to 99.9% of the prepared standard concentration. No bias was observed for glycerin, because the results for the mean recovery from all three accuracy levels were not significantly lower or higher (±1.5%) than the theoretical value. The range in which the method is shown to be linear and accurate for PEG400 and glycerin is between 70-130% of theoretical concentration.

The method was unaffected by small, deliberate variations in chromatographic parameters and mobile phase preparation. The parameters tested were mobile phase flow rate (+/- 0.1 mL/min) and temperature (+/- 4°C). The peak shape of PEG400 and glycerin were not affected by these parameters but in general higher variability results were observed for the PEG400 robustness study. The variation in results that was observed between the normal method conditions and the changed parameters were from 0.2 to 2.3% for PEG400 and 0.1 to 0.3% for glycerin. Table 14a and 14b show the results that were obtained by the robustness studies of PEG400 and glycerin respectively.

Two different lots of glycerin raw materials were used to evaluate the impact to the assay values using glycerin other than the one used in the formulation batch. In addition, USP reference glycerin standard and Sigma Aldrich glycerin were evaluated to quantitate glycerin in ophthalmic solutions. Three sample preparations were run with four different glycerin standards as per method. Table 15a summarizes the results of this study. In attempt to explain the variability on the results the water content of each standard of glycerin was investigated by assaying water content via Karl Fischer as per United States

Parameters under study	% LC of PEG400	Percent difference
Control	98.8	N/A
Flow 0.9ml/min	101.1	2.3
Flow 1.1ml/min	99.1	0.3
Control	101.5	N/A
Column temperature 46° C	101.3	0.2
Column temperature 54° C	101.2	0.3

(a)

Parameters under study	% LC of glycerin	Percent difference
Control	97.7	N/A
Flow 0.9ml/min	97.4	0.3
Flow 1.1ml/min	97.4	0.3
Control	98.5	N/A
Column temperature 46° C	98.8	0.3
Column temperature 54° C	98.4	0.1

(b)

Table 14: Results of robustness studies for (a) PEG400 and (b) glycerin

	Standard 1 Raw Material	Standard 2 Raw Material	Standard 3 Sigma Aldrich	Standard 4 USP
	% Label Claim of glycerin			
Assay prep-1	101.6	97.0	97.2	97.6
Assay prep-2	101.8	97.5	97.2	97.5
Assay prep-3	101,5	96.8	96.4	96.9
Mean	**101.7**	**97.1**	**96.9**	**97.4**
Absolute difference from Standard 1	N/A	**4.6**	**4.8**	**4.3**

(a)

	Standard 1 Raw Material	Standard 2 Raw Material	Standard 3 Sigma Aldrich	Standard 4 USP
	% water content			
Water content	4.31	0.06	0.13	1.03
Absolute difference from Standard 1	N/A	-4.25	-4.18	-3.28

(b)

	Standard 1 Raw Material	Standard 2 Raw Material	Standard 3 Sigma Aldrich	Standard 4 USP
	% Label Claim of glycerin			
Assay prep-1	101.6	101.3	101.4	100.9
Assay prep-2	101.8	101.8	101.4	100.8
Assay prep-3	101,5	101.1	100.6	100.2
Mean	**101.7**	**101.4**	**101.2**	**100.6**
Absolute difference from Standard 1	N/A	**0.3**	**0.5**	**1.1**

(c)

Table 15: Experimental results of (a) assay ophthalmic formulation using different sources of glycerin as a standard (b) Water content of glycerin standards (c) Assay of ophthalmic formulations corrected for water content

Pharmacopeia 30 <921> method I. Table 15b summarizes the water content results of glycerin. The water results explain the variability that was observed for the assay values when different standards were used. Correcting the assay values for water content brings the assay results within agreement to each other. Table 15c has the glycerin results corrected for water content. Standard glycerin will be evaluated for water content prior of using it for the analysis of ophthalmic solutions.

Two different lots of PEG400 raw materials were used to evaluate the impact on the assay values of using different materials of PEG400. In addition, USP reference PEG400 standard was evaluated by quantitate PEG400 in ophthalmic solutions. Three sample preparations were run with three different PEG400 standards as per method. Table 16a summarizes the results of this study. Even though the results were within experimental error from each other, additional verifications were performed. For example, water content of each standard of PEG400 was determined by Karl Fischer as per USP 30 <921> method I, to asure that there is no bias to assay results. Table 16b summarizes the water content results of PEG400. A small amount of water was found by Karl Fischer titration but assay results should not change much if they corrected. Water determination for PEG400 standards prior to sample analysis was not necessary. Correction of assay values for water content did not change the results drastically and results are within the method variability and agreement to each other. Table 16c has the PEG400 results corrected for water content.

The stability of the standard and sample solutions for PEG400 and glycerin was also evaluated. No significant change in PEG400 and glycerin response was observed

	Standard 1 Raw Material	Standard 2 Raw Material	Standard 3 USP
	% Label Claim of PEG 400		
Assay prep-1	100.3	99.2	100.3
Assay prep-2	99.9	98.7	100.7
Assay prep-3	99.6	99.4	100.9
Mean	**99.9**	**99.1**	**100.7**
Absolute difference from Standard 1	N/A	**0.8**	**0.8**

(a)

	Standard 1 Raw Material	Standard 2 Raw Material	Standard 3 USP
	% water content		
Water content	0.82	0.09	0.44
Absolute difference from Standard 1	N/A	-0.73	-0.38

(b)

	Standard 1 Raw Material	Standard 2 Raw Material	Standard 3 USP
	% Label Claim of glycerin		
Assay prep-1	100.3	99.9	100.7
Assay prep-2	99.9	99.4	101.1
Assay prep-3	99.6	100.1	101.3
Mean	**99.9**	**99.8**	**101.0**
Absolute difference from Standard 1	N/A	**0.1**	**1.1**

(c)

Table 16: Experimental results of (a) assay ophthalmic formulation using different sources of PEG400 as a standard (b) Water content of PEG400 standards (c) Assay of ophthalmic formulations corrected for water content

over a period of 192 hours. Solutions were stored at refrigerator and ambient temperature. Samples tested at the same time against freshly prepared standards of PEG400 and glycerin and results between the two conditions were comparable. Reference tables 17 and 18 for the solution stability of both standard and sample respectively at ambient temperature.

4.4 Conclusion

A new method for the simultaneous determination of total PEG400 and glycerin in ophthalmic solutions was developed using SEC with refractive index detector. This fast, simple isocratic method separates PEG400 and glycerin from other excipients in the formulation. Validation for this method was performed according to ICH guidelines and met all acceptance criteria. The method is precise (+/- 0.5%), accurate (+/- 1%), and linear at concentration ranges of 0.064 mg/mL to 0.192 mg/mL for glycerin and 0.287 mg/ml to 0.862 mg/mL for PEG400, typical of prepared ophthalmic solution samples for glycerin and PEG400. This method provides a model for the simultaneous analysis of polymeric components such as PEG400 in the presence of other components such as glycerin in a number of different types of formulations.

Time Point	PEG400		Glycerin	
	Response (%LC)	% Difference from Initial	Response (%LC)	% Difference from Initial
Initial	99.7	N/A	99.9	N/A
24 Hours	100.2	-0.5	100.1	-0.2
48 Hours	100.5	-0.8	100.2	-0.3
72 Hours	100.7	-1.0	99.9	0.0
168 Days	101.1	-1.4	100.3	-0.4

(a)

Time Point	PEG400		Glycerin	
	Response (%LC)	% Difference from Initial	Response (%LC)	% Difference from Initial
Initial	99.7	N/A	99.9	N/A
24 Hours	100.4	-0.7	101.1	-1.2
48 Hours	100.8	-1.1	99.9	0.0
72 Hours	100.4	-0.7	100.6	-0.7
168 Days	101.4	-1.7	101.0	-1.1

(b)

Table 17: Standard solution stability studies at (a) ambient temperature (b) refrigerated

	PEG400		Glycerin	
Time Point	**Response (%LC)**	**% Difference from Initial**	**Response (%LC)**	**% Difference from Initial**
Initial	101.8	N/A	104.0	N/A
24 Hours	102.7	0.9	104.7	0.7
48 Hours	102.4	0.6	104.6	0.6
72 Hours	102.3	0.5	104.2	0.2
196 Days	102.3	0.5	104.5	0.5

(a)

	PEG400		Glycerin	
Time Point	**Response (%LC)**	**% Difference from Initial**	**Response (%LC)**	**% Difference from Initial**
Initial	101.8	N/A	104.0	N/A
24 Hours	103.0	1.2	104.6	0.6
48 Hours	102.6	0.8	104.6	0.6
72 Hours	102.6	0.8	104.2	0.2
8 Days	102.2	0.4	104.5	0.5

(b)

Table 18: Sample solution stability studies at (a) ambient temperature (b) refrigerated

Chapter 5: Ophthalmic Active Components

5.1 Introduction

In pharmaceutical work active components are defined by the International Committee on Harmonization (ICH Q7A) as "any substance or mixture of substances intended to be used in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient in the drug product". Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease or to affect the structure and function of the body. Any ophthalmic solution is composed of two aspects. The first is the active component which is the central ingredient or the therapeutic agent that is meant to produce the desired effect in the body. The second is the rest of the components in the formulation that are used to deliver the active components or play a role in solution stability but that do not have a direct therapeutic purpose in the ophthalmic solution.

There is a variety of over the counter ophthalmic solutions that can be applied or instilled in the eye for the treatment of symptoms such as itching, irritation, redness, dryness, tearing, conjunctival edema, burning and discharge. There are different active components for the symptoms above and in some instances they might be multiple actives for the treatment of several symptoms at once.

General categories of active components in ophthalmic solutions are vasoconstrictor, hypertonicity agent, emollient, astringent, and buffering agent [148]. A vasoconstrictor is a topically pharmacologic agent that when applied to the mucous

membranes of the eye causes constriction of conjunctival blood vessels. There are five active components in this category that can be found in ophthalmic solutions: oxymetazoline hydrochloride, naphazoline hydrochloride, tetrahydrozoline hydrochloride, ephedrine hydrochloride, and phenylephrine hydrochloride. Hypertonicity agents exert an osmotic pressure greater than that present in body tissues and fluids, so that water is drawn out from the body tissues and fluids across semipermeable membranes. Applied topically in the eye, they create an osmotic pressure which draws water out of the cornea. Sodium chloride at levels of 2% to 5% is one of the most common hypertonic agents. Emollients are usually fat or oil, which applied locally to eyelids protect and soften tissues to prevent drying and cracking. Examples of the most used emollients in ophthalmic formulations are lanolin, mineral oil, petrolatum, and paraffin. An astringent is a therapeutic local agent which precipitates protein and helps to clear mucous from the outer surface of the eye. Zinc sulfate is an example of an astringent in ophthalmic solutions and can be found at 0.25% concentration. A buffering agent stabilizes pH when acid or base is introduced to the eye in the form of body fluids and tears.

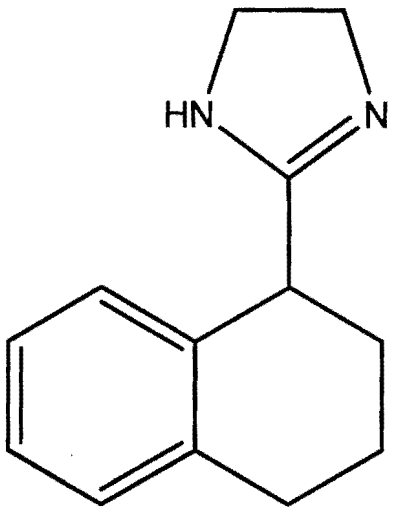
Analysis of the active component, along with possible degradants and impurities is critical to ophthalmic solutions due to the efficacy of the active component and due to regulatory requirements. Recently not only the purity profile but the impurity profile has become essential for the safety of the ophthalmic formulations because small amounts of unwanted impurities can influence the safety and efficacy. Different pharmacopoeias such as United States Pharmacopoeia, and British Pharmacopoeia, which regulate ophthalmic solutions, are incorporating limits for acceptable levels of known impurities

of active components.

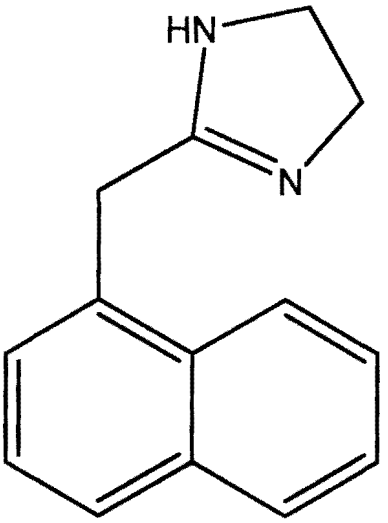
In this chapter, oxymetazoline hydrochloride was chosen to be studied extensively and additional active components which have similar structures (tetrahydrozoline hydrochloride and naphazoline hydrochloride) were also evaluated to establish common degradation pathways through stress degradation studies.

One challenge in the analysis of ophthalmic solutions is the measurement of oxymetazoline hydrochloride (OXY) and its known degradation product (N-(2-aminoethyl)-2-[4-(1,1dimethylethyl)-3-hydroxy-2,6-dimethylphenyl]-acetamide) (OXY-DEG). Although a number of HPLC methods have been developed to determine OXY in a variety of products, [149-153] none of these methods also determined OXY-DEG, which appears at much lower concentration. In this work, a straightforward, isocratic reversed-phase HPLC-UV method was sufficient to quantify both OXY and OXY-DEG in a single analysis, although their concentrations are orders of magnitude different. Forced degradation studies performed during method validation revealed that OXY-DEG is likely a base hydrolysis product of OXY. In addition, other ophthalmic active components with the same imidazoline structure were stressed and tested to verify degradation pathway similar to the unknown of OXY. Figure 23 shows the structures of tetrahydrozoline and naphazoline that were used during stress studies to confirm the degradation pathway.

A simple rapid isocratic reversed-phase high performance liquid chromatography (HPLC) method was developed for the determination of oxymetazoline hydrochloride (OXY) and its known degradation product (N-(2-aminoethyl)-2-[4-(1,1-dimethylethyl)-3-hydroxy-2,6-dimethylphenyl]-acetamide) in ophthalmic solutions.



(a)



(b)

Tetrahydrozoline
Structural name:
2-Tetralin-1-yl-4,5-dihydro-1H-imidazole
Molecular formulation: $C_{13}H_{16}N_2$
Formula weight: 200.28

Naphazoline
Structural name: 2-(naphthalen-1-ylmethyl)-4,5-dihydro-1*H*-imidazole
Molecular formulation: $C_{14}H_{14}N_2$
Formula weight: 210.274

Figure 23: Imidazoline structures (a) Tetrahydrozoline (b) Naphazoline

Oxymetazoline hydrochloride (OXY) is a long acting vaso-constrictor that acts directly to reduce the swelling of the nasal membranes, relieving decongestion in the subject. In the eye, imidazole decongestants such as OXY are used to constrict conjunctival blood vessels. More specific ophthalmic solutions that contain OXY have been shown to improve the relief of redness in the eye due to minor eye irritations [147]. OXY is an imidazole derivative with a general formula: $[C_{16}H_{24}N_2O]\cdot HCl$, and chemical name3-[(4,5-dihydro-1*H*-imidazol-2-yl)methyl]-6-(1,1-dimethylethyl)-2,4-dimethylphenol hydrochloride. The chemical structure of oxymetazoline hydrochloride is presented in Figure 24.

OXY is an ingredient in a variety of over-the-counter products such as nasal sprays and ophthalmic solutions. It was developed at E. Merck Darmstadt, Germany from xylometazoline in 1961 [154]. OXY has sympathomimetic properties meaning it constricts the blood vessels of the nose and sinuses through the activation of alpha adrenergic receptors. However, the treatment should not be continued for more than seventy two hours since excessive or improper use of topical decongestants can cause rhinitis medicamentosa which is increased nasal congestion [155]. Recently, it has been reported that OXY inhibits and resolves inflammatory reactions in human neutrophils [156]; a type of white blood cell filled with neutrally-staining granules, tiny sacs of enzymes that help the cell to kill and digest microorganisms.

Ophthalmic solutions and nasal sprays generally contain OXY at levels of 0.025% to 0.05% respectively [147, 157]. Many high performance liquid chromatography analytical methods have been developed to determine OXY in a variety of products [149-153, 158-159].

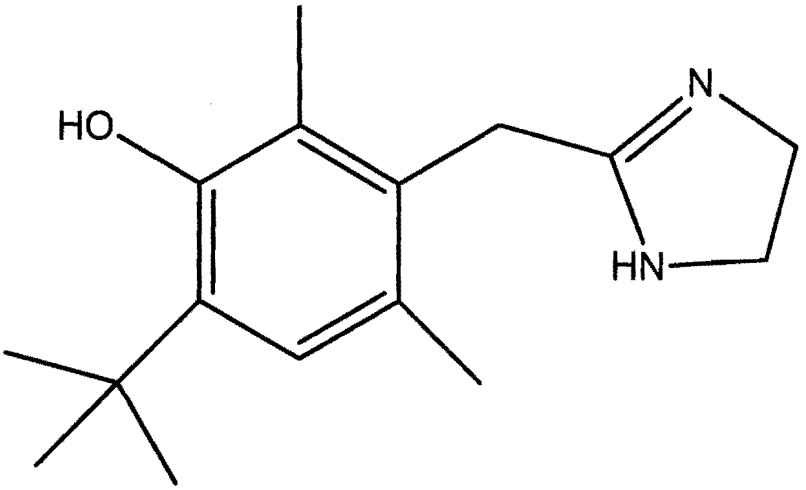


Figure 24: Oxymetazoline structure

However, none of these methods also determined the known degradation product of Oxymetazoline: N - (2 - aminoethyl) - 2 - [4 - (1,1 – dimethylethyl) - 3 – hydroxyl - 2,6 - dimethylphenyl] - acetamide (OXY-DEG).

The European Pharmacopeia (EP) lists OXY-DEG as a known impurity of oxymetazoline hydrochloride with chemical name N-(2-aminoethyl)-2-[4-(1,1-dimethylethyl)-3-hydroxy-2,6-dimethylphenyl]-acetamide [160]. The chemical structure of the impurity is shown in Figure 25. However, during the stress studies of the method validation, it was determined that this compound is a hydrolysis product of oxymetazoline hydrochloride which is consistent with information published in literature [161].

The method of determination of the OXY-DEG as described in EP is different than the one in this publication. More specific the differences are in the column dimensions, stationary phase, column temperature, flow, wavelength, and mobile phase. The USP method is only suitable for the assay determination of OXY and doesn't determine the OXY-DEG. In addition, in that USP assay method the chromatographic parameters are different than the current method. The EP assay method the determination of OXY was done with titration and not with an HPLC [160, 162].

In this work we describe new HPLC methods for the analysis of the oxymetazoline hydrochloride and its degradation product in ophthalmic solutions. The new methods are stability indicating methods and were successfully validated based on the International Conference on Harmonization guidelines [163].

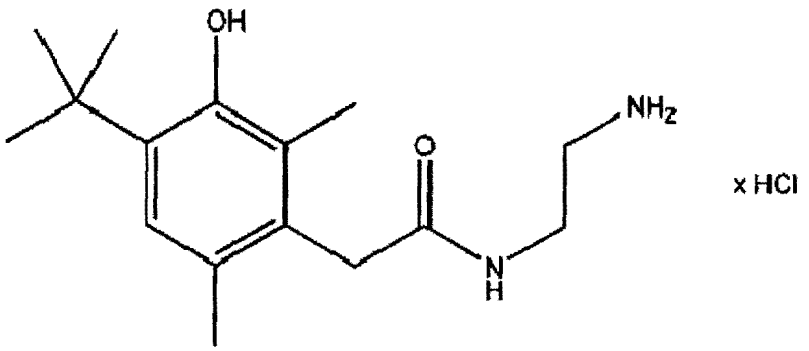


Figure 25: Structure of oxymetazoline degradation product (N - (2 - aminoethyl) - 2 - [4 - (1,1 – dimethylethyl) - 3 – hydroxyl - 2,6 - dimethylphenyl] – acetamide)

5.2 Experimental

5.2.1 Reagents and Chemicals

Standards of oxymetazoline HCl and its degradation product were obtained from LGC (LGC GmbH, Im Biotechnologiepark, TGZ II, D 14943 Luckenwalde, Germany). Samples and placebo were provided by the Johnson and Johnson product development group (Morris Plains, NJ, USA). ACS reagent grade sodium acetate trihydrate and reagent grade glacial acetic acid were purchased from Sigma Aldrich (Saint Louis, Missouri, USA). HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Deionized Water was used and obtained from VWR International, LLC (West Chester, Pennsylvania, USA).

5.2.2 Equipment

An Alliance HPLC system (Waters Corporation, Milford, Massachusetts, USA) was used for method development and validation. The Alliance LC system was equipped with 2695 separation module, 2487 UV detector and 996 photodiode array detector. Data collection and processing was done using Empower (Waters Corporation, Milford, MA USA).

5.2.6.9.2 Solution Stability: Standard and sample solutions were stored at room temperature and tested at initial, 72 hrs and 240 hrs of standing. The solutions were tested against a freshly prepared standard at each time point.

5.3 Results and Discussion

The analytical method for the degradation product of oxymetazoline hydrochloride is slightly different than the assay method of OXY since the exact parameters were not suitable to evaluate both compounds simultaneously in one method. More specifically, the differences were in detector wavelength and injection volume. These are not unusual since the target concentration level and UV absorbance between the OXY and degradation product can be different. However, the determination of OXY and degradants can be done in a single injection with the use of dual wavelength or photodiode array UV detectors. Additional validation of the method was done at 100 µl injection volume. More specific the following parameters were evaluated: system precision, method precision, accuracy and linearity. The degradation method is also capable of separating xylometazoline hydrochloride from OXY and OXY-DEG. However, xylometazoline was not included into validation since it was not observed during the stressing studies at specificity. Figure 26 shows an overlay chromatogram of ophthalmic solution sample with mixture standard OXY-DEG and xylometazoline hydrochloride.

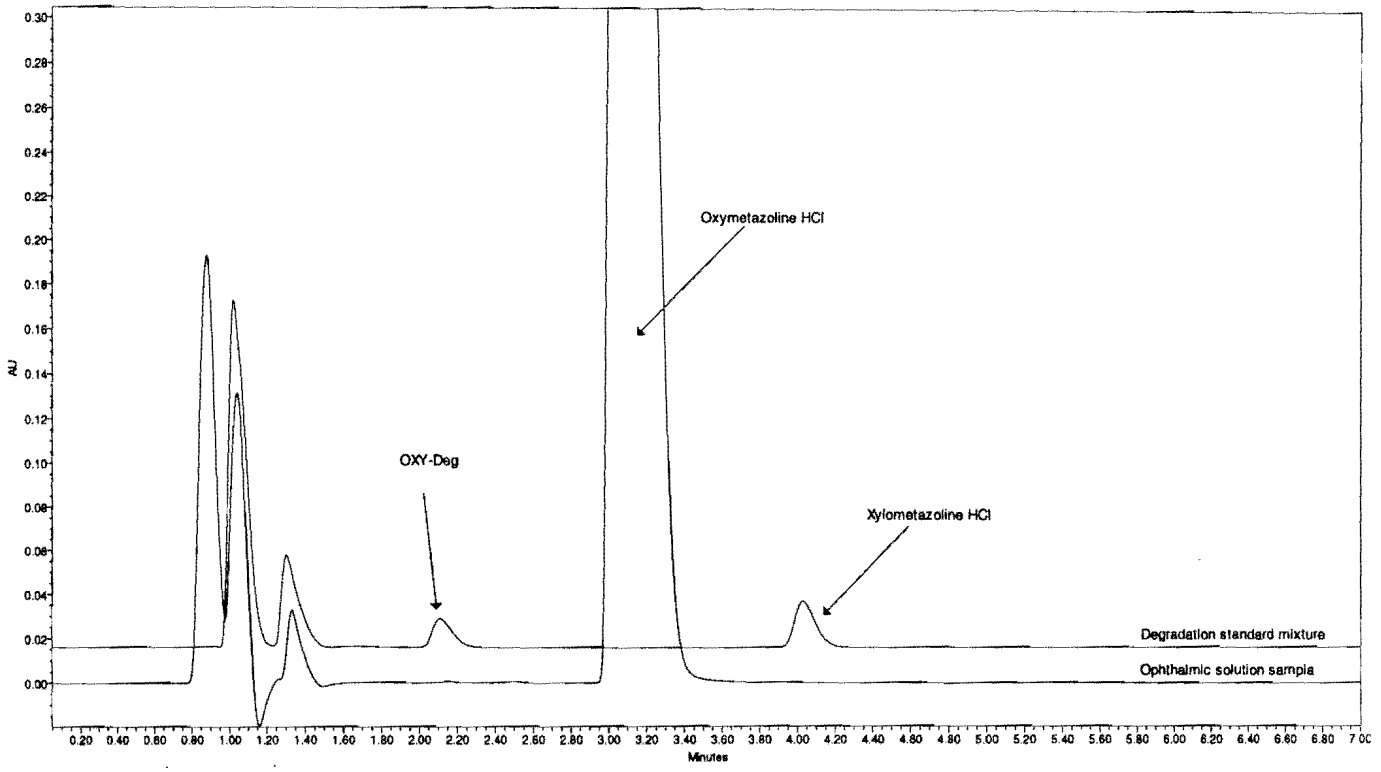


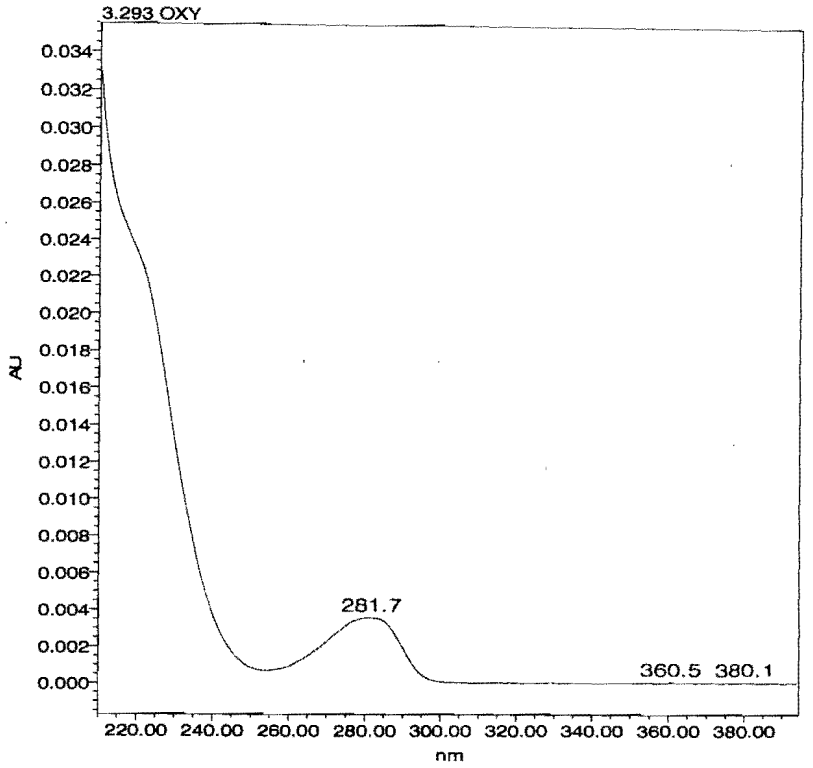
Figure 26: Overlay chromatogram of ophthalmic solution sample and degradation standard mixture

5.3.1 Developmental Work

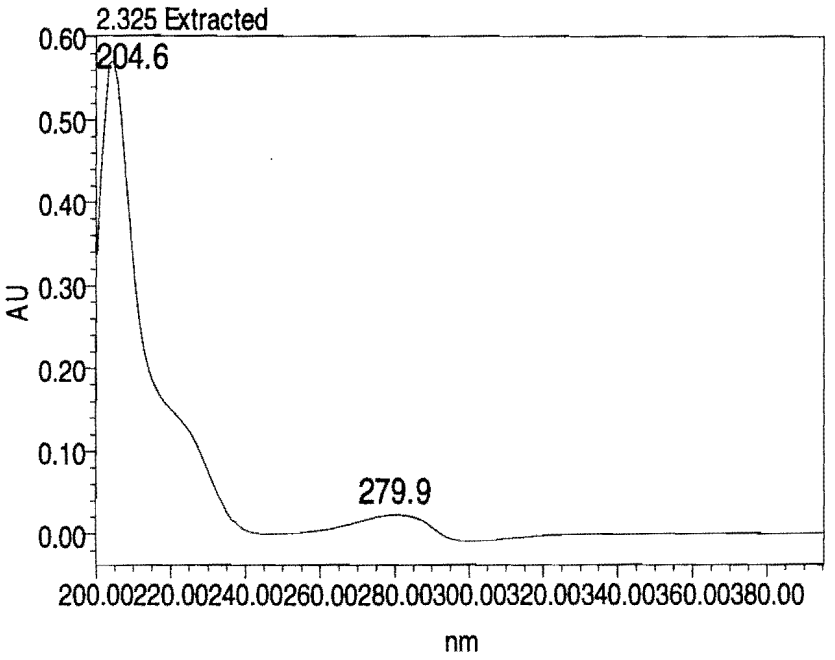
The physical and chemical properties of oxymetazoline hydrochloride are well established, since that molecule has been known since its synthesis in 1961 [149]. UV detection was selected since both oxymetazoline HCl and its degradation product have a chromophore at UV spectrum range. Figure 27 shows UV spectra of (a) oxymetazoline and (b) OXY-DEG.

The initial goal during the method development was to have one method for the determination of oxymetazoline and its degradation product. However, it was observed that oxymetazoline response was not linear at wavelength of 225 nm and injection volume of 100 μL. Optimum wavelength selection (λ=225 nm) for the degradation method was done based on the maximum absorbance of degradation product. The wavelength (λ=280 nm) of the assay method was selected based on the second maximum absorbance of the oxymetazoline. Nevertheless, the determination of OXY and its degradation product can be done in a single chromatographic run with the use of photodiode array detector.

Ideally in reversed phase HPLC method development, several columns with different stationary phases C_8, C_{18}, Cyano, Phenyl, should be tested based on separation ability in the desired pH range. However, in cases that there is familiarity with the active ingredient the use of a single column can be sufficient for method development. In this case, the cyano stationary phase (Agilent, Zorbax SB-CN, 3.5 μm, 150 mm x 4.6 mm) proved capable of adequately separating all components, so testing of other columns was not necessary.



(a)

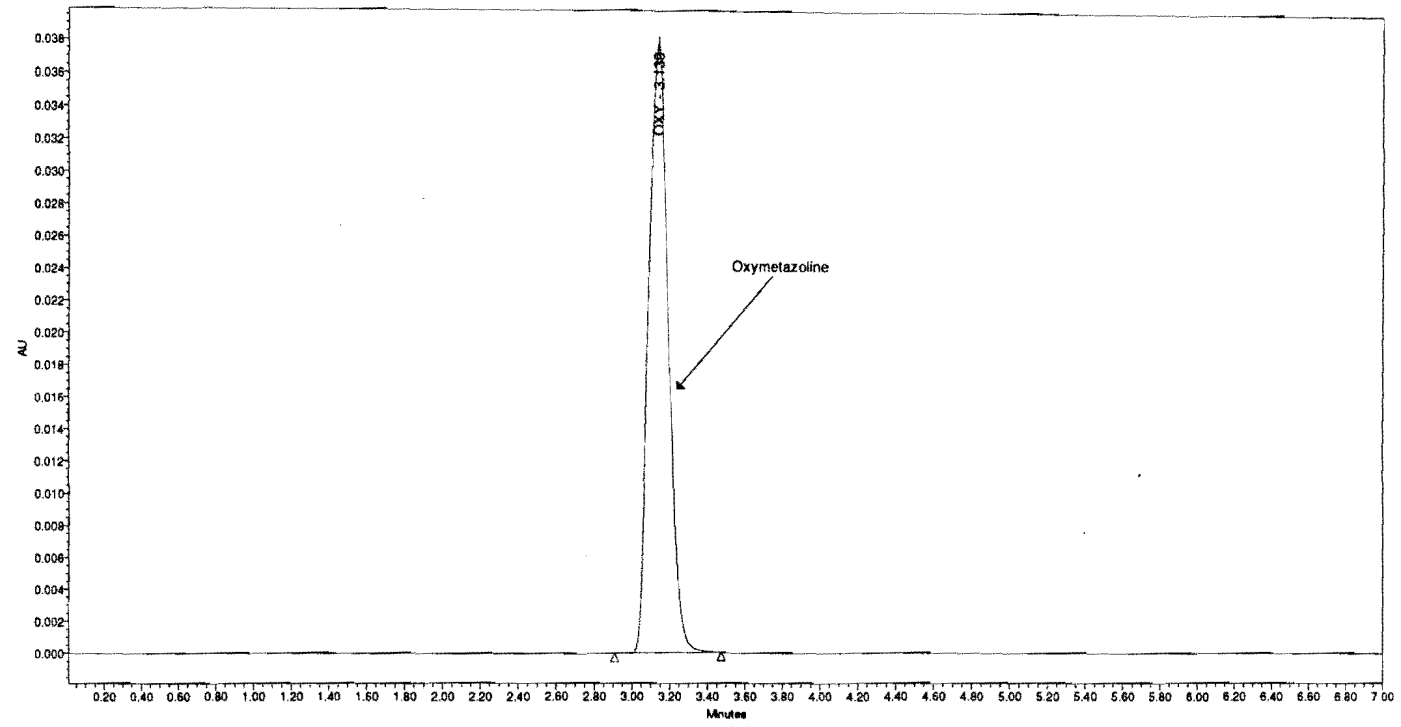


(b)

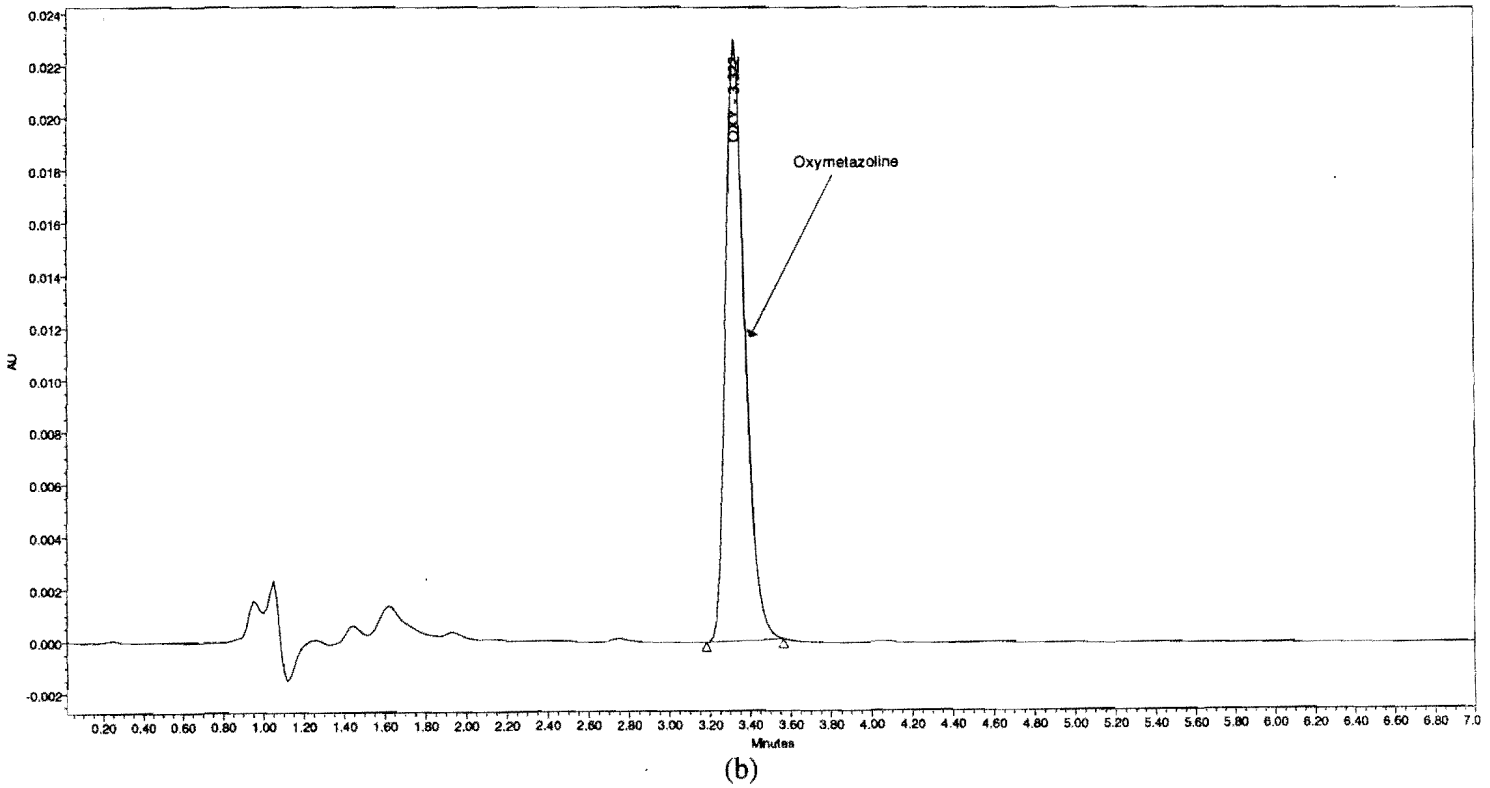
Figure 27: UV spectrum of (a): OXY (b): OXY-DEG

In general, retention of less polar compounds declines with cyano columns while polar compounds maintain retention. Many comparable studies were reported between cyano and different stationary phases. In one such study, different cyano columns were characterized in terms of selectivity. Comparison between cyano, C8, and C18 stationary phases was not practical because cyano columns required weaker mobile phases with changes in separation selectivity. Due to those differences replacement of C8, and C18 columns with an equivalent cyano column will not be possible [166].

System performance parameters were selected to provide confidence that the methods are capable of determining oxymetazoline HCl and OXY-DEG in ophthalmic solutions. Different concentration levels of oxymetazoline HCl were injected as part of the system suitability for the two methods. Typical chromatograms for oxymetazoline HCl standard solution are shown in Figure 28. The selected system performance parameters were injection precision of OXY, tailing factor and signal to noise ratio of OXY at the quantitation level. In addition, check standard conformity and bracketing standard conformity were proved during each chromatographic run. All of these tests produced results within the guidelines of good laboratory practices for system performance. The system performance at 100 μL injection volume was based on the precision of six injections of a 100% standard at concentration 0.1 mg/mL. Tailing factor and signal to noise ratio were not evaluated as part of the system suitability. The tailing factor that was observed during the validation at this injection volume was greater than the previous acceptance criteria of 0.8-2.0. This is not unusual since the amount of OXY in the column is ten times higher than the 10 μL injection. Also, during this injection adequate signal to noise ratio was observed which make this parameter unessential to



(a)



(b)

Figure 28: Chromatograms of oxymetazoline for (a): Assay method (b): Degradation method

monitor as part of the system suitability. Typical chromatogram for the oxymetazoline HCl standard is shown in Figure 29. In addition, check standard and bracketing standard conformity were proved during validation. Samples bracketed with two different standards to accommodate the simultaneous determination of OXY and unknown degradants in the sample. The standard concentrations were 0.1 mg/mL and 0.001 mg/mL for assay and degradation respectively. System suitability tests produced results within the guidelines of our standard operating procedures.

5.3.2 Experimental Work

The specificity of the methods (assay and degradation) was tested and no interference from inpurities or degradants was observed for the oxymetazoline hydrochloride and its degradation product peaks from placebo and forced degradation samples using acid, base, light, hydrogen peroxide and heating. To ensure no extraneous peak co-eluted with the OXY or its degradation product, a UV diode array detector was used to check peak purity using the full UV spectrum at several points on each peak [167]. Figure 30 shows overlay chromatograms at 225 nm of OXY standard stressed with acid, base, heat, light and peroxide showing no interference between OXY, OXY-DEG, excipients and impurities. It should be noted that during the oxidation stressing a peak that was observed around retention time of 1.1 minutes is due to the hydrogen peroxide solution and not oxymetazoline degradant.

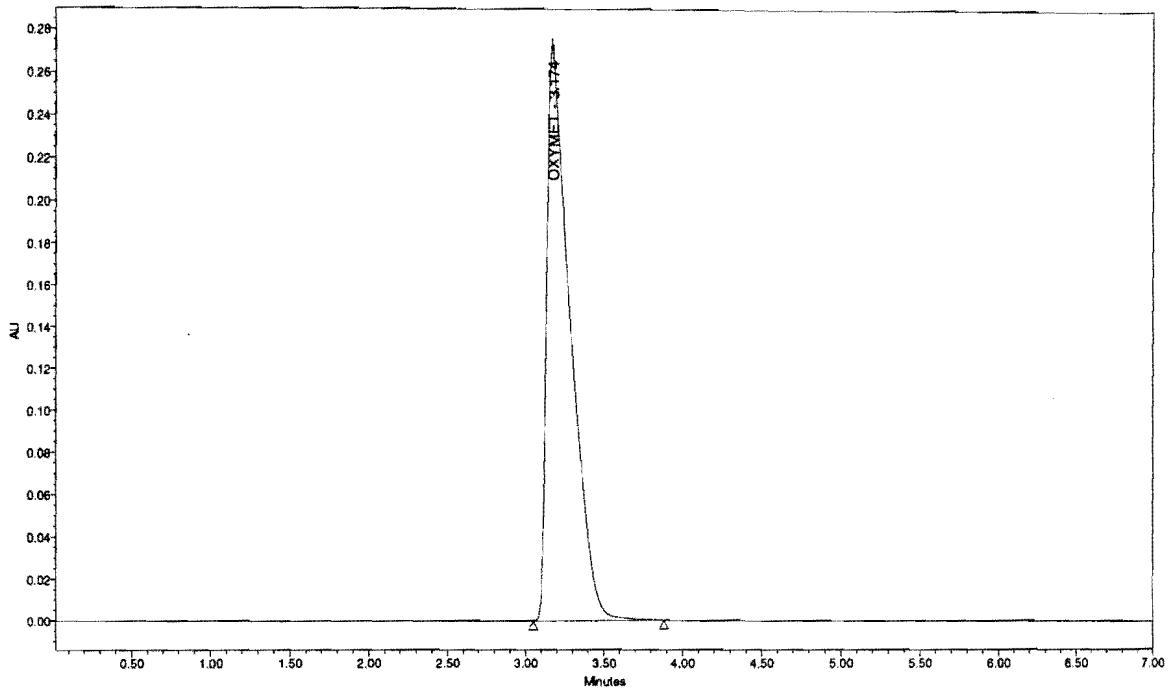


Figure 29: Typical chromatogram for oxymetazoline HCl standard of 100 µL injection volume

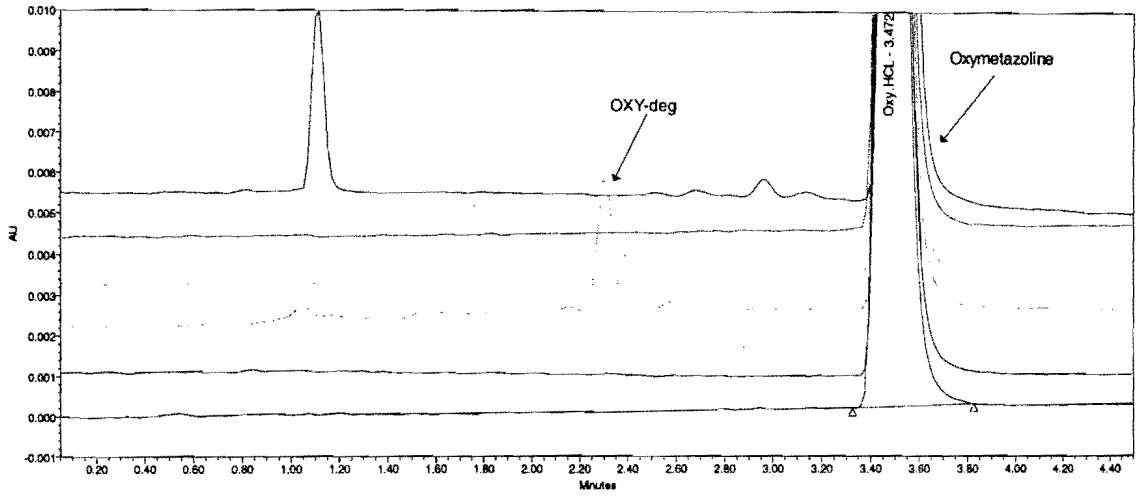


Figure 30: Overlay chromatogram of OXY standard. From the bottom: Control, Acid, Base, Heat, Light and Oxidation

OXY-DEG was observed during the stressing conditions of the oxymetazoline standard with base. The standard was subjected to 0.1 N of sodium hydroxide at 75°C for one hour. The solution was cooled to room temperature, neutralized with equivalent amount of 0.1 N hydrochloric acid and analyzed chromatographically. Hydroxide anion in the solution attack and attach to the carbon at the double bond of the imidazole ring. Furthermore the attached hydroxyl group converted into a ketone and causes the imidazole ring to open. That compound is the hydrolysis product of oxymetazoline hydrochloride. Figure 31 shows the base hydrolysis reaction of oxymetazoline.

Linearity of the method was established. Five standard concentrations of oxymetazoline hydrochloride and OXY-DEG were prepared and injected. Oxymetazoline was injected at different concentration levels for each method of assay and degradation. A linearity graph was prepared by plotting the concentration versus the response of the actives. The solutions for the oxymetazoline assay method covered a range of 50-150 % of the hypothetical formulation concentration with corresponding concentration range of 0.05 to 0.15 mg/mL. Table 19 and Figure 32 show the linearity parameters and the linearity graph for oxymetazoline assay method at wavelength 280 nm respectively.

For the degradation method the OXY solutions cover a range of 70-130% of the theoretical formulation concentration and 0.1-3.0% range for OXY-DEG relative to oxymetazoline concentration. The range of the OXY-DEG was determined based on the ICH guidelines on impurities in new drug substances and new drug products [168-169]. The corresponding concentration range is 0.0007 to 0.0013 mg/mL and 0.0001 to 0.003 mg/mL for the OXY and OXY-DEG respectively. The methods were linear in these ranges with R^2 values of 0.999 in both OXY and OXY-DEG.

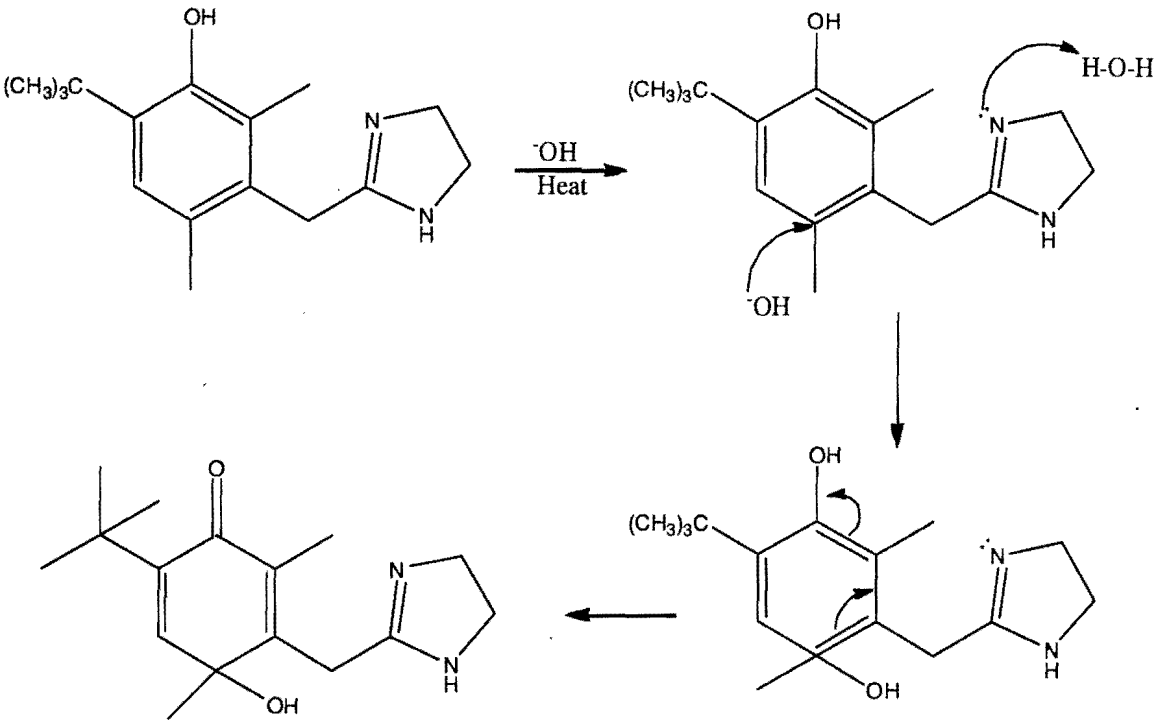


Figure 31: Formation of hydrolyzed product of oxymetazoline

Target Concentration Level	Concentration (mg/mL)	Response (Peak Area)
50%	**0.0501**	**138465.97**
80%	**0.08016**	**221667.44**
100%	**0.1002**	**276757.8**
120%	**0.12024**	**333323.64**
150%	**0.1503**	**414777.48**
Slope	**2761494.17**	
Y Intercept (%)	**296.75**	
Correlation	**0.99997**	

Table 19: Linearity parameters for Oxymetazoline assay (λ=280 nm)

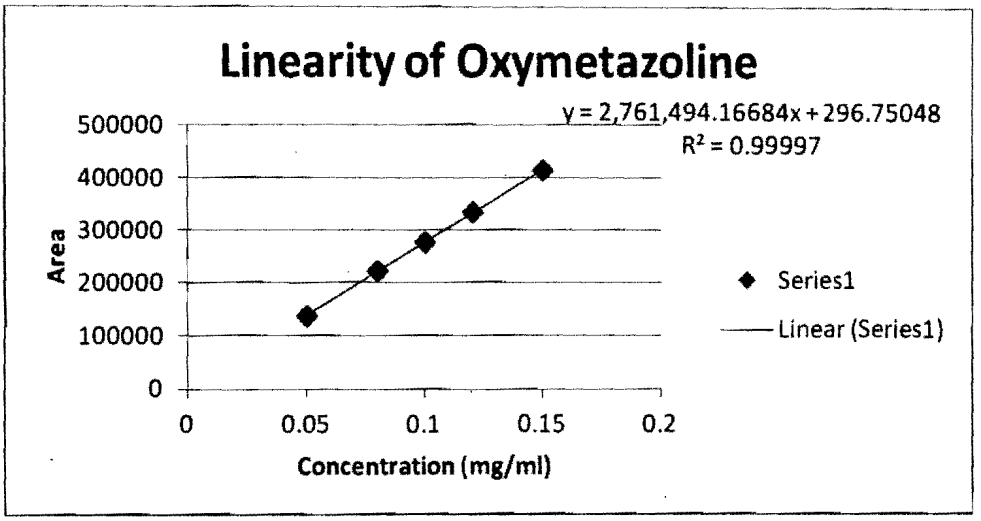


Figure 32: Linearity graph of oxymetazoline assay

Table 20 and Figure 33 show the linearity parameters and the linearity graph for oxymetazoline degradation method at wavelength 225 nm respectively. No carryover was observed into blank injections immediately after the highest level linearity standard of oxymetazoline, ensuring independence of the samples. In addition, oxymetazoline HCl was found to be linear with 100 µL injection volume at the same five concentration levels as the 10 µL injection volume. A linearity graph was prepared by plotting the concentration versus the response of oxymetazoline. Table 21 and Figure 34 show the linearity parameters and the linearity graph for oxymetazoline assay method with injection volume 100 µL and wavelength 280 nm.

This wide linearity calibration range allows the analysis of a variety of possible formulations containing oxymetazoline HCl and is not limited only to ophthalmic solutions. It should be noted that method applicability should be explored for each formulation for possible interferences from the different formulation components that were used.

System precision was established by six replicate measurements of a hypothetical formulation containing oxymetazoline HCl. The %RSD for OXY were found to be 0.8 and 0.1 for the assay and degradation methods respectively. Furthermore, to make the method a QC friendly OXY and OXY-DEG were measured in a single injection. The system precision was evaluated at 100 µl injection volume. Six replicate measurements of a 100% theoretical standard solution containing oxymetazoline hydrochloride were injected. The error contributed by the system, independent of the sample preparation, was less than the acceptance criteria of 2.0 %.

arget Concentration Level	Concentration (mg/mL)	Response (Peak Area)
70%	**0.000708**	**111788.48**
80%	**0.00081**	**127613.78**
90%	**0.000911**	**143316.57**
100%	**0.001012**	**160537.21**
130%	**0.001316**	**206790.74**
Slope	**156710120.41**	
Y Intercept (%)	**915.35**	
Correlation	**0.9997**	

Table 20: Linearity parameters for oxymetazoline degradation method (λ=225 nm)

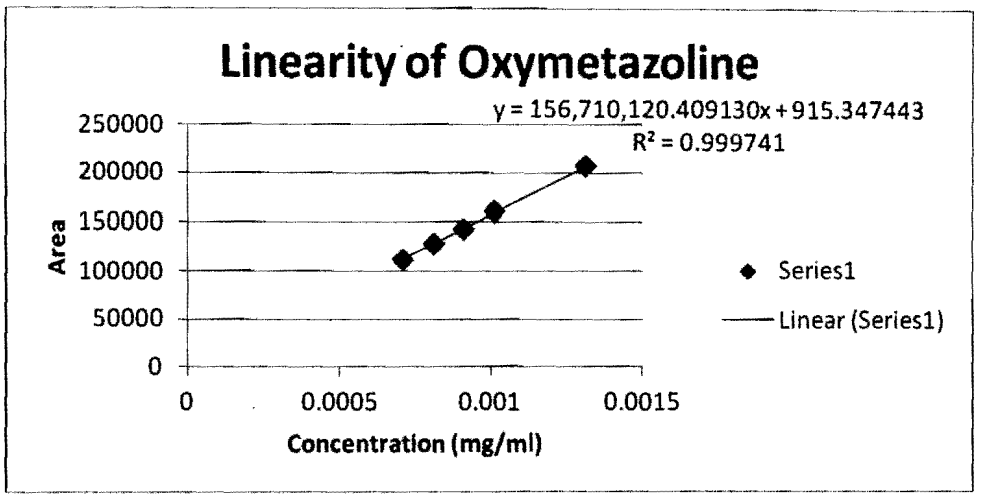


Figure 33: Linearity graph of oxymetazoline degradation method

Target Concentration Level	Concentration (mg/mL)	Response (Peak Area)
50%	**0.05127**	**1436090.49**
70%	**0.07169**	**2011388.93**
100%	**0.10242**	**2871951.50**
120%	**0.12290**	**3437903.64**
150%	**0.15362**	**4281918.60**
Slope	**27799761.12**	
Y Intercept (%)	**18880.01**	
Correlation	**0.99996**	

Table 21: Linearity parameters for oxymetazoline degradation method (λ=225 nm) with 100 µL injection volume

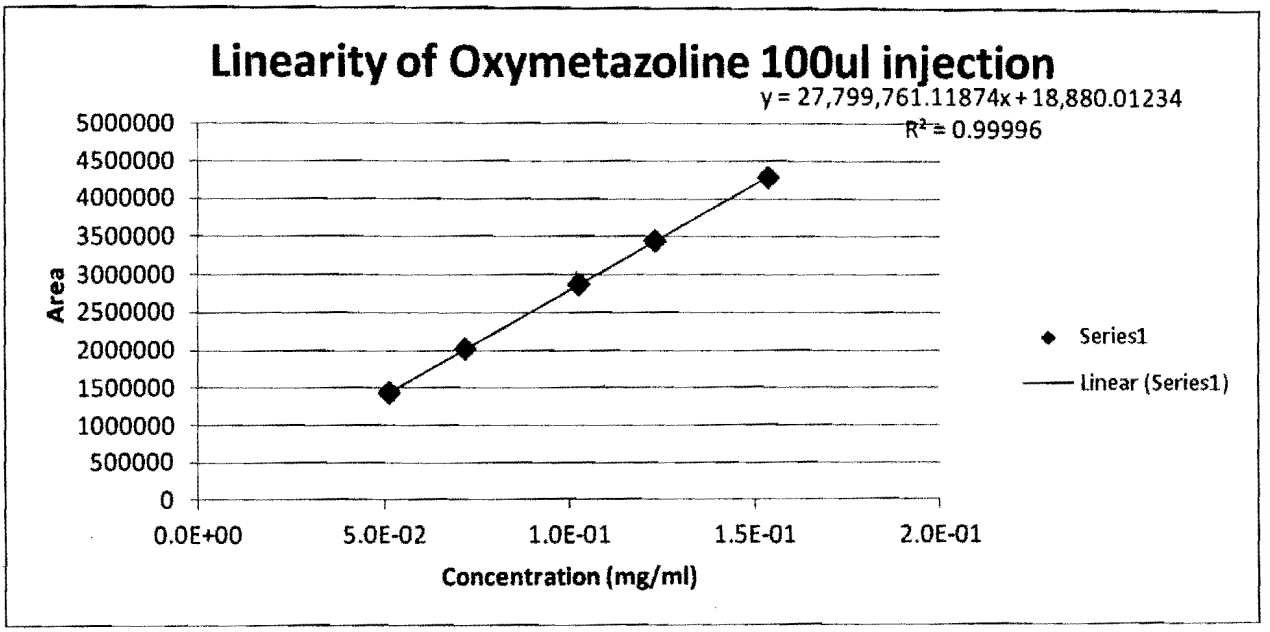


Figure 34: Linearity graph of oxymetazoline assay method at 280 nm and 100 µL injection volume

The %RSD for assay oxymetazoline was found to be 0.8 and 0.1 for 10 µL and 100 µL injection volumes respectively and the %RSD for oxymetazoline in degradation method was found to be 0.1%. In both methods (assay & degradation) including the two different injection volumes for the assay method the %RSD was well below the 2.0% acceptance criteria. Comparison tables of the system precision results between the two methods and the two injections volumes are shown on Table 22a.

The repeatability of the method was evaluated by six identical sample preparations of a homogeneous batch of an ophthalmic formulation and the results were found to be within specification. The percent relative standard deviation of the six preparations for both OXY injections of 10 µl and 100 µl were found to 0.2%. Comparison table of the results between the two injections volumes are shown on Table 22b. The repeatability of the method was also evaluated for the OXY-DEG by six identical sample preparations of a homogeneous batch of a real formulation and the results were found to be within acceptance criteria. The percent relative standard deviation of the six preparations of OXY-DEG was found to be 0.5%. In order to further validate the results, the experiment was conducted again on a different day, using a different mobile phase, instrument and column. The experimental mean agreements between the two tests were found to be 0.2 and 2.6 for OXY and OXY-DEG respectively, both of which are within the acceptance criteria. Further all individual and combined assay results for both experiments were acceptable. Detailed results of OXY and OXY-DEG are displayed in Table 22c. All the repeatability results were within acceptance criteria of 3.0%.

	Oxymetazoline Assay standard 0.10 mg/mL		Oxymetazoline Degradation standard 0.10 mg/mL
Replicate #	**Area (10uL)**	**Area (100uL)**	**Area (100µL)**
1	279044.40	2877393.58	152361.39
2	278249.36	2877490.53	152834.99
3	274391.35	2878025.81	152749.44
4	274136.23	2877960.56	152629.47
5	273974.85	2879907.73	152350.66
6	276623.72	2880944.21	152564.84
Mean	**276069.98**	**2878620.40**	**152581.80**
% RSD	**0.8**	**0.1**	**0.1**

(a)

	Oxymetazoline Theoretical Assay Value: 100% LC	
Replicate #	**%LC (10 µL)**	**%LC (100 µL)**
1	100.6	100.9
2	100.6	100.6
3	100.8	100.8
4	100.9	100.5
5	101.2	100.6
6	100.5	100.6
Mean	**100.8**	**100.7**
% RSD	**0.2**	**0.2**

(b)

Replicate	% Label Claim (%LC)			
	Oxymetazoline hydrochloride		OXY-Degradant	
	Experiment 1	**Experiment 2**	**Experiment 1**	**Experiment 2**
1	100.6	100.6	109.2	108.6
2	100.6	100.8	110.0	105.5
3	100.8	100.2	108.6	105.8
4	100.9	100.5	108.5	107.6
5	101.2	100.6	108.9	105.1
6	100.5	100.6	108.7	106.4
Mean	**100.8**	**100.6**	**109.0**	**106.4**
%RSD	**0.2**	**0.2**	**0.5**	**1.3**
Mean Agreement	**0.2%**		**2.6%**	

Table 22: Experimental results for (a) Comparison of oxymetazoline hydrochloride system precision data between 10 µL and 100 µL injection volumes (b) Comparison of oxymetazoline HCl method precision data between 10 µL and 100 µL injection volume (c) Comparison of oxymetazoline HCl and OXY-DEG for method precision by two different experiments

Accuracy was established by assaying three different concentration levels 70%, 100%, and 130% of the hypothetical formulation concentration of OXY. Six preparations of ophthalmic solutions without oxymetazoline were spiked at each level, with standard of oxymetazoline and then solutions were injected into HPLC system for the assay method. For the degradation method accuracy was established by assaying four different degradant concentration levels 0.1%, 1.0%, 1.5% and 3.0% of the hypothetical formulation concentration of oxymetazoline hydrochloride. Six preparations of ophthalmic solutions without oxymetazoline, at each level, were spiked with standard of OXY-DEG and then solutions were injected into HPLC system. Results are reported in Table 23. OXY mean recovery values varied from 100.0 to 100.4 % LC and OXY-DEG mean recovery values varied from 103.6 to 109.0. No bias was observed for the OXY since the results for the mean recovery from all three accuracy levels were not significantly lower or higher (±1.5%) than the theoretical value. The accuracy of the oxymetazoline HCl analytical assay method at 100 µl injection volume was determined at three working levels 60%, 100% and 140% of the theoretical concentration. Six preparations of OXY placebo were spiked with a standard at the three working levels of the theoretical concentration. The results for all three average values met acceptance criteria and were comparative to the results of the 10 µL injection volume. OXY mean recovery values were varied from 99.5 to 100.5 and no bias was observed from all three levels. Comparison of the results between the two injection volumes at each accuracy level are shown on Table 24.

The RRF was determined by spiking OXY-DEG linearity solutions at three levels (0.1, 1.0 and 3.0%) with OXY at the standard concentration. Injections were made in

Prep #	Oxymetazoline HCl			OXY-Degradant			
	Mean Recovery Value %LC			Mean Recovery Value %LC			
	70% Level	100% Level	130% Level	0.1% Level	1.0% Level	1.5% Level	3.0% Level
1	99.8	99.7	99.7	109.2	109.8	103.5	102.7
2	99.9	100.5	100.2	110.0	104.2	104.0	104.0
3	100.2	101.1	99.8	108.6	104.0	104.0	104.2
4	100.2	100.7	100.1	108.5	104.1	103.8	103.1
5	100.2	100.4	101.2	108.9	104.9	105.3	103.9
6	100.0	100.0	101.0	108.7	104.6	104.9	104.0
Mean	**100.0**	**100.4**	**100.3**	**109.0**	**105.3**	**104.3**	**103.6**
%RSD	**0.2**	**0.5**	**0.6**	**0.5**	**2.2**	**0.7**	**0.6**

Table 23: Accuracy results (ophthalmic solution without oxymetazoline was spiked at three different levels for assay of OXY and four levels for the OXY-DEG)

	Oxymetazoline HCl	
Replicate #	**70% Recovery (10µl)**	**60% Recovery (100µl)**
1	99.8	100.2
2	99.9	99.9
3	100.2	100.1
4	100.2	100.2
5	100.2	99.2
6	100.0	100.5
Mean	**100.0**	**100.0**
% RSD	**0.2**	**0.5**

(a)

	Oxymetazoline HCl	
Replicate #	**100% Recovery (10ul)**	**100% Recovery (100ul)**
1	99.7	99.9
2	100.5	100.2
3	101.1	99.7
4	100.7	100.1
5	100.4	100.0
6	100.0	100.3
Mean	**100.4**	**100.0**
% RSD	**0.5**	**0.2**

(b)

	Oxymetazoline HCl	
Replicate #	**130% Recovery (10 µl)**	**140% Recovery (100 µl)**
1	99.7	99.4
2	100.2	100.0
3	99.8	99.6
4	100.1	99.6
5	101.2	99.5
6	101.0	99.6
Mean	**100.3**	**99.6**
% RSD	**0.6**	**0.2**

(c)

Table 24: Comparison table of oxymetazoline HCl accuracy data between 10 µL and 100 µL injection volume: a) Level 60%, b) Level 100%, c) Level 140%

triplicate and the average response was used for the determination of the RRF. That value was corrected for the free base since the salt form of the degradant was used during the validation. The RRF value was determined to be 0.6993.

The LOD and LOQ were determined by analyzing low concentration linearity standard solutions of OXY-DEG in triplicate to confirm acceptable results. The LOD was found to be 0.0001 mg/mL for signal to noise ratio more than three and the LOQ was found to be 0.000025 mg/mL for signal to noise ratio more than twelve.

The methods were unaffected by small, deliberate variations in chromatographic parameters and mobile phase preparation. The parameters under testing were wavelength, flow, column temperature, mobile phase ratio and pH of the buffer. The robustness results have shown that the retention time and the peak shape were not affected by the parameters under testing. The variation that was observed between the normal method conditions and the changed parameters were from 0 to 1.3% for OXY assay. The stabilities of the standard and sample solutions for both OXY and OXY-DEG were evaluated. The standard of OXY is stable over a period of 168 hours and 240 hours in the assay method and shows no significant decrease in response over a period of 168 hours for OXY assay method in both sample and standard. The results are displayed in Table 25.

Solution stability for Assay method			Solution stability for degradation method		
Time Point	Oxymetazoline HCl Standard		Time Point	Oxymetazoline HCl Standard	
	Response	% Difference from Initial		Response	% Difference from Initial
Initial	100.7	-	Initial	100.0	-
24 Hours	101.3	0.6	96 Hours	98.2	1.8
72 Hours	102.2	1.5	240 Hours	99.7	0.3
168 Hours	101.4	0.7			
Time Point	Oxymetazoline HCl Sample		Time Point	OXY-Degradant	
	Response	% Difference from Initial		Response	% Difference from Initial
Initial	99.9	-	Initial	106.8	-
24 Hours	100.6	0.7	96 Hours	104.8	2.0
72 Hours	101.7	1.8	240 Hours	105.1	1.7
168 Hours	99.8	0.1			

Table 25: Solution stability results for Oxymetazoline standard for both assay and degradation methods. Solution stability of Oxymetazoline and Oxy-DEG in the sample.

5.4 Conclusion

In this work, new methods for the determination of oxymetazoline hydrochloride and its degradation product in ophthalmic solutions are described. Simple dilution followed by isocratic HPLC with UV-detection is employed, generating a very straightforward method that might be applicable in a variety of applications. The method fully separates the oxymetazoline HCl from its degradant and other excipients. Testing parameters for these methods was performed according to ICH guidelines and met all acceptance criteria. The methods are precise, accurate, and linear at concentration ranges encompassing our hypothetical formulations and the known concentrations of many products containing these substances.

Overall Conclusions

Over the counter ophthalmic solutions enable people to effectively treat symptoms of redness, dry eye, tear generation and other irritations of the eye. They are used worldwide and they are more affordable than their counterpart prescription ophthalmic solutions. As formulations get more complex with multiple components, targeting multiple symptoms, there is an increasing need for new analytical methods that are low cost, simple to operate, use straightforward instrumentation, while maintaining similar requirements to techniques employing more sophisticated instrumentation.

The importance of preservatives in over the counter ophthalmic solutions is well known given the environment in which these solutions are kept. Often, users they stored the multidose bottles in medicine cabinets, purses, and pockets, exposed to extreme conditions of heat and humidity provide a good environment for microbial growth. Benzalkonium chloride, one of the most commonly preservative systems in ophthalmic solutions was studied in this work. Separation of the benzalkonium chloride homologues from other formulation components was done with a simple, fast isocratic reversed phase chromatography method using UV detection. The separation of the homologues is dependent on mobile phase ratio, pH and buffer strength. The method was applicable to several different over the counter formulations that were use benzalkonium chloride as a preservative system.

Lubricants are important substances to ophthalmic formulations because they are used to control solution viscosity and they are offer medicinal benefits by relieving pain in inflamed or irritated mucous membranes. Due to their importance in ophthalmic

solutions several lubricants (polyvinylpyrrolidone, PEG400 and glycerin) were studied in this work.

The polyvinylpyrrolidone peak was eluted and separated before the void volume disturbance from other formulation components. Several unusual chromatographic situations were used together successfully in this work: Size exclusion chromatography with UV detection and quantitation of a chromatographic peak eluting before the void volume. This method provides a model for the analysis of a polymeric component in the presence of monomeric components in a number of different types of formulations.

A simple size exclusion chromatography method using refractive index detection was presented. PEG400 and glycerin were separated simultaneously from other formulation components. In addition, the method appears to be capable of separating another demulcent, hydroxylpropyl methylcellulose (HPMC) from formulation components. The method provides a model for the simultaneous analysis of polymeric components in the presence of other formulation components in a number of different types of formulations.

There are different active components in ophthalmic solutions for the treatment of variety of symptoms. A major category of active components in ophthalmic solutions is vasoconstrictors and they are used for the treatment of redness in the eye. A well known vasoconstrictor that used in the ophthalmic solutions is oxymetazoline. A new simple, fast, isocratic HPLC with UV-detection method for the determination of oxymetazoline hydrochloride and its degradation product in ophthalmic solutions was described. The method fully separates the oxymetazoline HCl from its degradant and other ophthalmic components and it might be applicable in a variety of other formulations. Stress studies of

other vasoconstrictors (naphazoline, tetrahydrozoline) with similar chemical structure indicate that they exhibit the same degradation pathway.

In this work, several straight forward techniques for the analysis of components in ophthalmic solutions, each of which represents an improvement over previous literature techniques, are demonstrated.

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