Studies on the Optimization of Neuropeptides Detection in the Human Tear Film

By

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Introduction:

Dry Eye Disease (DED) stems from a disruption of the homeostasis of the tear film (TF), a thin layer of fluid covering the ocular surface. The TF consists of numerous constituents that include proteins, lipids, mucins, water, electrolytes, immunoglobulins, vitamins, cytokines, and neuropeptides. An imbalance in any of these constituents could result in an unstable tear film, contributing to the pathophysiology of DED. Among these factors, neuropeptides, small proteinaceous substances produced and released by neurons through regulated secretory routes, may have a role in the pathophysiology of DED. To understand the impact of the disease on the concentrations of calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) in the tear film, it is important to first understand the sample collection and quantification methods. The purpose of this thesis was to optimize a method to quantify the concentration of four neuropeptides using enzyme linked immunosorbent assay (ELISA). a common laboratory technique used to quantify the concentration of neuropeptides in the tear film. ELISAs have been used to determine the quantity of different components in blood and other bodily fluids in the human tear film.

The aims of each chapter were as follows:

Chapter 3: To determine the variability of two tear collection methods, basal tear collection and flush tear collection, for quantifying SP, CGRP, VIP, and NPY, and to quantify the day-to-day variability of these neuropeptides.

Chapter 4: To assess the validity of a commercially available ELISA kits for the quantification of neuropeptides.

Chapter 5: To examine the measurement variability of two commercially available ELISA kits for the quantification of SP.

Methods:

Chapter 3: Basal and flush tears (following instillation of 20 µL of saline on the ocular surface) of 8 healthy participants were collected from the right and left eyes respectively, using glass microcapillary tubes on two consecutive days. The concentrations of the four neuropeptides in the tears were determined using ELISA, for both collection methods, and for both days.

Chapter 4: Basal tears (5 µL) were collected from the temporal canthus of each eye of 3 healthy participants using glass microcapillary tubes. To assess the validity of the ELISA kit used in Chapter 3, two experiments were performed: a spike and recovery experiment, followed by a serial dilution response. In the spike and recovery experiment, 2 µL of tears from each participant were diluted in 108 µL of three known concentrations of SP, CGRP, and NPY (1 pg/mL, 10 pg/mL, and

100 pg/mL). The concentrations of neuropeptides were quantified using ELISA and the percent recovery was calculated. In the serial dilution response experiment, 4 µL of tears were collected from a single participant and was spiked into a known concentration of NPY (100 pg/mL). Serial dilutions (1:2, 1:4 and 1:8) were conducted and the percent recovery was calculated. Multiple troubleshooting and optimizing experiments to conducted to examine the effect of using a blocking agent, C18 pipette tip column, protease inhibitor, and the effect of freeze storage on neuropeptide quantification.

Chapter 5: SP from Phoenix Pharmaceuticals, SP from Cayman Chemicals and SP from Sigma-Aldrich were each formulated at 0.5 mg/mL. Their UV absorbance profile from 200 nm to 300 nm was obtained using SoftMax Pro 5.4.1 software on a SPECTRAmax M5e ROM v2.1.35. The two SP from Phoenix Pharmaceuticals and SP from Sigma-Aldrich were formulated at various concentrations (500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL, to 7.8 pg/mL and 3.9 pg/mL) and were quantified using two different ELISA kits (Phoenix Pharmaceuticals, Cayman Chemicals). A Bland Altman plot was used to quantify the agreement of the two SP, quantified by each of the ELISA kits.

Results:

Chapter 3: There was no significant difference in the concentrations of CGRP, SP, NPY, and VIP between the two collection methods (all $P > 0.19$). The difference in concentrations of CGRP, SP, NPY, and VIP between the two study days was also not significant ($P > 0.06$ for all tests).

Chapter 4: The percent recovery for spike and recovery experiment ranged between 63,953.15% to 13.74% for CGRP, 676.17% to 5.21% for SP and 412.42% to 7.51% for NPY. The initial concentration of NPY was 208 pg/mL and after the 1:2, 1:4, and 1:8 dilution, the observed recovery was 188.40 pg/mL for the 1:2 dilution, 153.60 pg/mL for the 1:4 dilution, and 204.28 pg/mL for the 1:8 dilution. In the troubleshooting experiments; there were minimal differences in the concentration of SP associated with the use of a blocking agent; there was a reduction in VIP when processed using C18 column pipette tips; using protease inhibitors reduced the amount of VIP recovered; the amount of VIP recovered was reduced in the presence of albumin; a higher amount of SP was recovered in freshly collected tears compared to tears which were stored frozen for four months.

Chapter 5: A similar absorbance profile was observed for SP from Phoenix Pharmaceuticals and SP from sigma Aldrich. A trend toward higher variability was observed at lower concentrations of SP. The Bland Altman plot shows a mean difference of -3.36%, and a 95% limits of agreement of

[-10.75, 4.01] for the Phoenix Pharmaceuticals kit, and a mean difference was -9.70%, and the 95% limits of agreement were [-14.61, -4.79] for the Cayman Chemicals kit.

Conclusion:

Chapter 3: It was anticipated that diluting to facilitate collection using the flush tears method would have yielded a lower concentration than the basal tears collection method. However, the ELISA kits found no significant difference between the two collection methods.

Chapter 4: High variation was observed in the recovered values of both the spike and recovery and the serial dilution response experiments. The troubleshooting experiments have provided some optimization steps to consider for tear sample collection and processing for the detection for neuropeptides.

Chapter 5: The agreements of two SP standards quantified with two different SP ELISA kits were poor. Greater variability in fluorescence and absorbance units was associated with lower concentrations of SP, highlighting the difficulty in quantifying SP near the detection limit of the kits.

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Dedication

To my family and friends

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Chapter 1 - Introduction

1.1 Structure and Function of neuropeptides

It is estimated that there are more than 100 billion neurons in the human body,[1] and with it, hundreds of different types of neurotransmitters to transmit information from one neuron to another. One important class of neurotransmitters in the human body are neuropeptides. Neuropeptides are defined as "… small proteinaceous substances produced and released by neurons to communicate with each other. They are signaling molecules in the brain which are involved in many physiological functions."[1] Until the early 19th century, neuropeptides were thought to be hormones, chemical messengers that are carried from one organ to the another through the bloodstream.[2] In the 1950s, these peptide hormones consisting of a small chain of amino acids were discovered in the gastrointestinal tract and later in the brain. After studying the biological effects of these peptide hormones, David de Wied in 1971 coined the term "neuropeptide".[3] Neuropeptides are secreted internally in the nervous system, where they can act as a neurotransmitter, or into the bloodstream as hormones.[2]

Neuropeptides are signaling molecules that are released by neurons to communicate with adjacent neurons and also with immune cells during inflammation.[4] Neuropeptides are released through calcium-dependent exocytosis but are not reabsorbed into the neuron once they are secreted. The inactivation of extracellular neuropeptides occurs by diffusion and

breakdown by extracellular proteases.[5-9] Neuropeptides are typically secreted in low concentrations compared to neurotransmitters and are slow-acting. The half-life of neuropeptides can vary depending on the region of the body. For example, the half-life of neuropeptides in plasma is between 2 to 7 minutes and approximately 10 to 60 minutes in cerebrospinal fluid.[2,4,10]

1.1.1 Mechanism of Neuropeptide production

Neuropeptides are synthesized in the soma of neurons in both the central and peripheral nervous system. Neuropeptides are typically 3 – 36 amino acids in size and generally have higher molecular weights than other neurotransmitters.[1,4,5] They are derived from proteolytic cleavage of larger precursors, which are encoded by over 70 different genes.[6,11] The precursor mRNAs are translated by the polyribosomes on the endoplasmic reticulum,[6] and the resulting protein then undergoes further processing through the Golgi complex. These precursors are then packed into large dense core vesicles (Fig 1.1) where the proteases in the vesicles cleave the precursors into the final form of the neuropeptide.[1,6,12] There are three different proteolytic processing steps that occur in the vesicle to form a mature neuropeptide. In the first step, an endopeptidase cleaves the precursor into two separate propeptides. In the second step, carboxypeptidase

cleaves residues from the c terminus of a newly formed peptide. The final process involves converting the carboxy group at the c terminus to an amide group.[6,13]

Once synthesized, these neuropeptides remain stored in large dense-core vesicles in the nerve axon and are released either into the bloodstream or nervous system.[2,12] Once released into the bloodstream these neuropeptides function by binding to G-protein-coupled receptors at cells within their target tissues.[2,1,3]

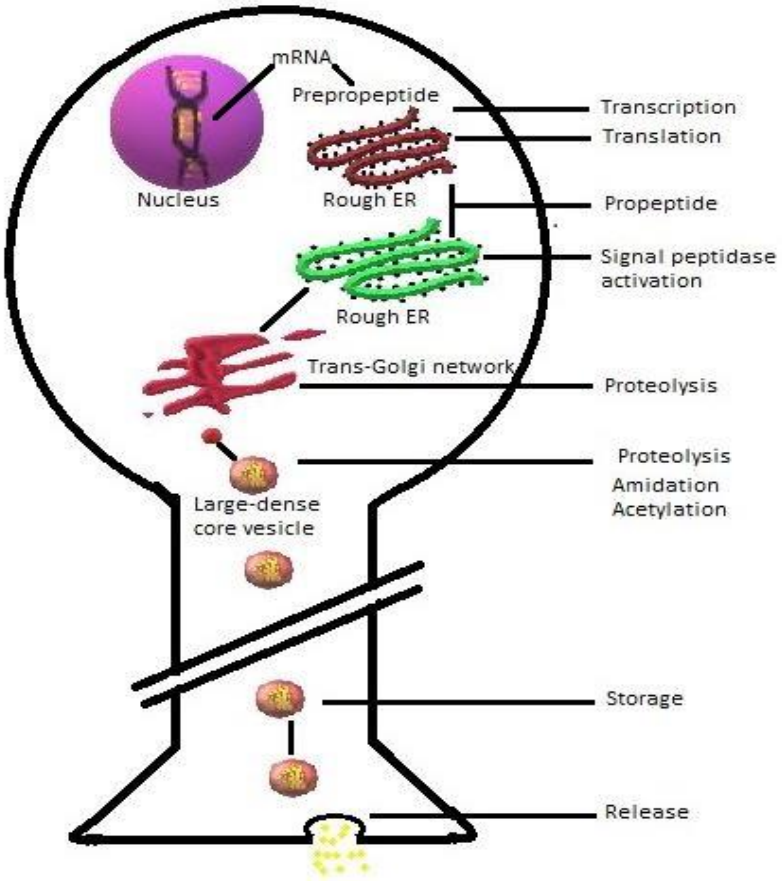

Figure 2-1. Synthesis of neuropeptides in the cell soma of neurons from the central or peripheral nervous system

1.1.2 Types of neuropeptides and their function

As previously stated, there are more than 70 genes that encode the neuropeptide precursors that collectively yield over 100 different types of neuropeptides.[1,14] These neuropeptides are categorized based on gene families (e.g., opioid, vasopressin/oxytocin, Calcitonin, etc.).[1,2,15,16] Each gene family contains neuropeptides with similar structural and functional characteristics.

With respect to the ocular surface, the neuropeptides that have attracted most attention in the literature are Calcitonin Gene-Related Peptide (CGRP), Substance P (SP), Neuropeptide Y (NPY), and Vasoactive Intestinal Peptide (VIP).[4,17-22]

1.1.2.1 Calcitonin Gene-Related Peptide (CGRP)

Calcitonin gene-related peptide is a neuropeptide that consists of 37 amino acids. It is obtained by alternative mRNA splicing of CAL1 gene on the 11th chromosome.[15] CGRP belongs to the regulatory peptide or calcitonin gene family, which also includes adrenomedullin and amylin, that work together with CGRP as vasodilators. This neuropeptide functions by binding to two G-protein coupled receptors, CGRP1 and CGRP2, which are extensively distributed in both the central and peripheral nervous systems.[15] The primary function of this neuropeptide is vasodilation.[23] For example, the relaxation of the smooth muscles in coronary arteries[4] is in part due to the activation of the CGRP1 receptor and through the modulation of potassium channels.[4,23] CGRP also plays a role in tissue injury by increasing blood flow at the site of inflammation.[24]

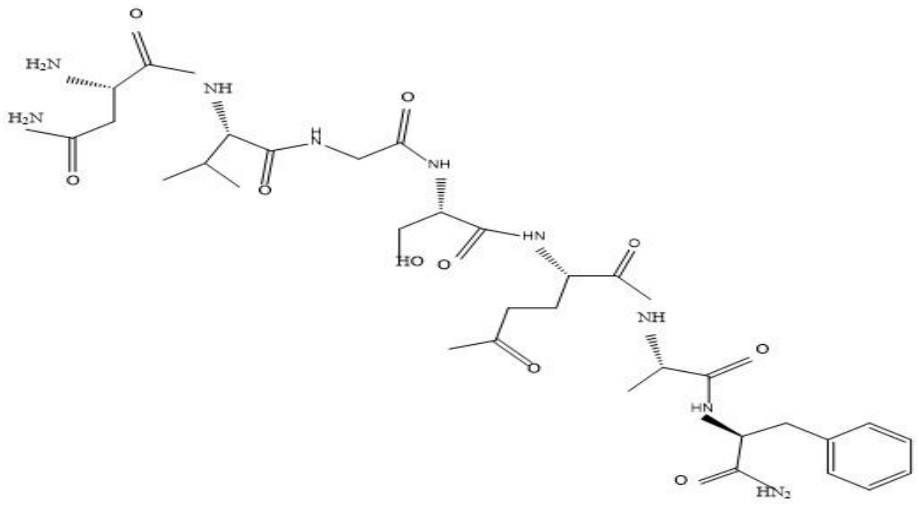

Figure 1-2. Chemical structure of Calcitonin Gene Related Peptide

1.1.2.2 Substance P (SP)

Substance P is a neuropeptide consisting of a chain of 11 amino acids. It belongs to the tachykinin family, which is a group of abundantly distributed peptides in the CNS active at low concentrations and plays a role in gastrointestinal tract motility and secretions.[16] SP functions by binding to the G-coupled protein receptor NK1, which ultimately regulate processes such as cell proliferation, neuroimmune cross-talk, and chemotaxis.[16] SP is abundantly distributed in the limbic system located in the temporal lobe.[16] This neuropeptide can act as a neurotransmitter or a neuromodulator, where it controls the physiological levels of neurotransmitters. [16]

Emotional or physical stressors can modulate the tissue concentration of SP or immunoreactivity of SP in the amygdala (regulating fear and anxiety).[16] It also plays a role in transmitting pain stimuli to the CNS. Additionally, in tissue injuries, SP can stimulate cell growth for wound healing.[25,26] In the gastrointestinal tract, SP plays a role in regulating smooth muscle cell activity and secretion of ions and fluids from gastrointestinal epithelial cells. [27,28]

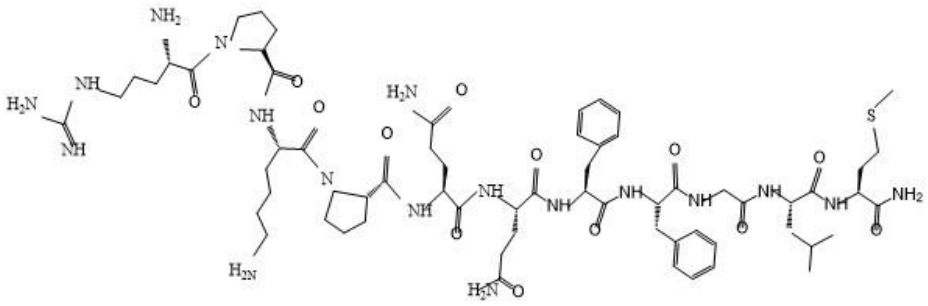

Figure 1-3. Chemical structure of Substance P

1.1.2.3 Neuropeptide Y (NPY)

Neuropeptide Y is a 36 amino acid peptide that has similarities with peptide YY and pancreatic polypeptide.[4] This neuropeptide was discovered initially in the porcine brain in 1982.[29] It is one of the most widely distributed peptides in the nervous system and plays a role in cell neurogenesis and neuroendocrine release of hormones from the hypothalamus. It is secreted with other neurotransmitters like GABA and glutamate. NPY exerts its function mainly through

G-protein coupled receptors Y1 and Y2, which are involved in the contraction of smooth muscles, and energy homeostasis.[29,30]

NPY increases food appetite by decreasing the latency between meals, increasing motivation to eat, and by delaying satiety through increasing meal size. Additionally, it facilitates storage of energy as fat.[31-33] It acts as a vasoconstrictor, it plays a role in secretion of growth hormone and insulin release, and potentially has a role in promoting obesity and diabetes.[29,32,34]

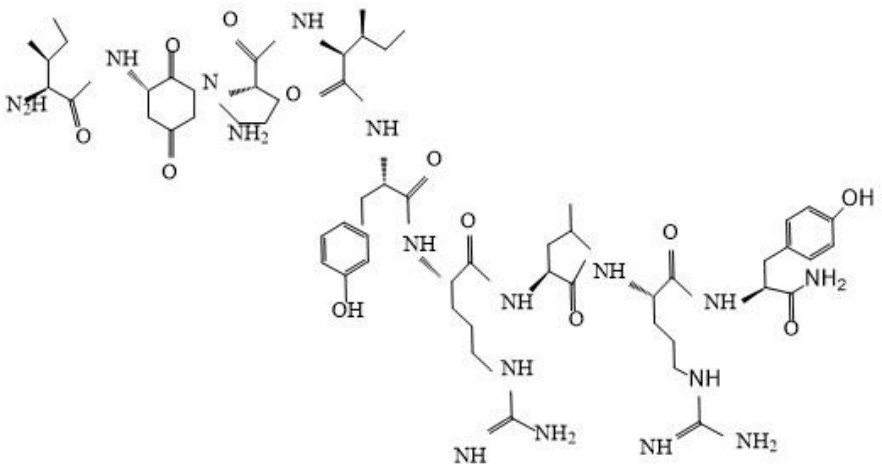

Figure 1-4. Chemical structure of Neuropeptide Y

1.1.2.4 Vasoactive Intestinal Peptide (VIP)

Vasoactive Intestinal Peptide is derived from prepro-VIP, a 170 amino acid chain precursor. Splicing of this precursor results in the active form of VIP. This neuropeptide shares structural

and functional similarities to gastrointestinal hormones.[4] VIP functions by activating two G-protein receptors VPAC1 and VPAC2 and facilitates smooth muscle dilation and constriction. It is abundant in the nervous system and in both the large and small intestines. Its function in the intestines is to relax smooth muscles and increase the secretion of water and electrolytes.[25] It also regulates the circadian rhythm and has an anti-inflammatory effect by inhibiting the production of inflammatory cytokines and chemokines from macrophages, microglial cells, and dendritic cells.[34-39]

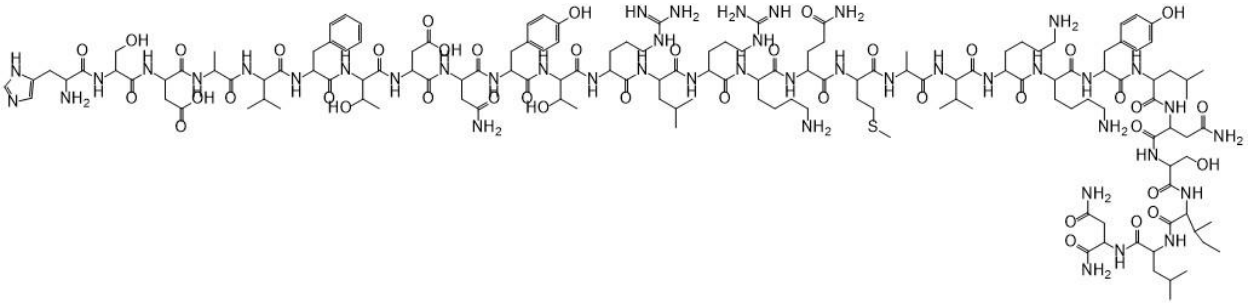

Figure 1-5. Chemical structure of Vasoactive Intestinal Peptide

1.1.3 Ocular surface neuropeptides and their functions

Neuropeptides have a role in maintaining the immune homeostasis of the ocular surface by modulating both the innate and adaptive immune response on the ocular surface.[4,40] The polymodal nociceptor nerve terminals release neuropeptides into the corneal and conjunctival epithelium.[41] Hegarty et al. showed that 3-31% of corneal nerves express SP and 15-41% of corneal nerves express CGRP.[42] Sensory nerve fibers releasing SP and CGRP are widely spread throughout the corneal stroma and epithelium.[4] In contrast, NPY nerve fibers originate from sympathetic nerves and VIP fibers originate from parasympathetic nerves. NPY and VIP nerve fibers are limited to the anterior stroma and the basal epithelial cells.[4] The distribution of SP and CGRP nerve fibers are equally abundant in the stroma and the distribution of SP is fewer than CGRP in the epithelium.[4] A study by Muller et al showed that 60% of the polymodal nociceptor sensory nerve endings release CGRP, 20% release SP, and the remaining 20% release NPY and VIP.[43] As there is not enough evidence on the parasympathetic innervation to the human cornea,[44] the release of VIP from parasympathetic nerve fibers is relatively unknown.

Recent studies show that both SP and CGRP promote corneal wound healing.[4,19] SP restores corneal sensitivity and promotes wound healing in diabetes.[4,34] VIP is involved in controlling the secretion of electrolytes from the lacrimal gland, conjunctiva, and the secretion

of mucin from conjunctival goblet cells.[45] The precise role of NPY is still ill-defined, but may play a role in cell proliferation and neurogenesis.[4]

1.1.4 Mechanism of neuropeptide production during inflammation

Several studies have shown that neuropeptides are involved in the processing of noxious stimuli.[1,2,4,34,46,47] When nociceptors from nerve endings are stimulated, a signal is transmitted through the spinal cord and to the brain through neural pathways. The terminus of sensory nerves releases CGRP and SP and activates the innate immune system (e.g., mast cells, basophil, neutrophil, macrophages) and adaptive immune cells (e.g., T lymphocytes, B lymphocytes).[4] The immediate response is vasodilation and increased vascular permeability.[48] When the inflammation terminates the neutrophils undergo programmed cell death and are engulfed by macrophages and the anti-inflammatory process starts.[49]

On the ocular surface the cornea and conjunctiva contain sensory nerves that are divided into mechanoreceptors (low threshold), mechano-nociceptors (high threshold), polymodal nociceptors, and cold receptors.[50] About 70% of the nerves are polymodal nociceptors that are stimulated by mechanical force, chemical irritants, inflammatory cells, and from plasma leakage from the blood vessels.[50,51] Approximately 20% of sensory nerves contain mechanoreceptors and mechano-nociceptors that activates on high mechanical force that can damage the cornea, and

the remainder 10 – 15% have cold receptors. The polymodal nociceptor nerve terminals releases neuropeptides into the corneal and conjunctival epithelium.[50,52]

These polymodal nociceptors conduct impulses in an antidromic direction in which the impulses are conducted from the axon terminal to the cell soma resulting in synthesis and release of neuropeptides from nerve endings.[52] This antidromic stimulation occurs due to tissue injury or inflammation that releases inflammatory mediators. These inflammatory mediators change the electrical activity of the nerve resulting in release of neuropeptides.[50] These neuropeptides facilitate vasodilation, vascular permeability, cytokine release, and release of innate and adaptive immune cells.[50,53,54] The excited nerve endings stimulate the non-injured branches of the axon to release neuropeptides. [55]

1.2 Methods for quantifying neuropeptides in the tear film

In the reported literature, several methods have been described detailing the collection and quantification of neuropeptides from the tear film.[17,18,20,21,56] The collection of basal tears[57,58] could be facilitated from the temporal canthus using glass capillary tubes.[20,21] Other methods include placing a micro-sponge at the inferior conjunctival fornix to soak up tears[19] or the use of Schirmer strips or filter paper strips that draw the tears from the inferior conjunctival fornix.[20]

Collecting basal tears from patients with dry eye disease (DED) is difficult as there are minimal amounts of tears on the ocular surface. This difficulty is compounded by the rapid evaporative loss of tears.[58] To overcome this difficulty, Bjerrum and Prause introduced the "eye-flush" tear collection method, where a fixed volume of sterile saline is instilled onto the ocular surface and the effluent is collected using a microcapillary tube.[59] This tear collection method has been used to reduce tear collection time and collect a significantly higher volume of sample. While adding saline would dilute the tear film constituents, Markoulli et al. showed that relative protein concentrations remain the same between basal and flush tears.[56]

The most common method for quantifying neuropeptides in the tear film is through an enzyme-linked immunosorbent (ELISA) assay.[17-22,56,60] An ELISA assay is a plate-based technique where an antibody is coated to the bottom of the plate. A sample is added, and the analyte being quantified binds with the antibody attached to the plate. Next, a second antibody carrying an absorbent dye or fluorescent molecule is added and binds to the analyte. An enzyme conjugate is then added to each well to amplify the signal. Following this, a substrate solution is added to react with the enzyme conjugate and the fluorescence or absorbance is measured.

Other methods for detecting and quantifying neuropeptides include mass spectrometry (MS)[18] and high performance liquid chromatography (HPLC).[17] Mass spectrometry is an analytical technique that determines the mass to charge ratio of fragmented, ionized analytes in a sample.

These ions are separated based on their unique mass to charge ratio and are presented as a mass spectrum. An advantage of this technique is that it is highly sensitive and only requires a small amount of sample volume.[61] For quantifying neuropeptides, MS identifies neuropeptides based on reference values in MS fragmentation databases.[62] HPLC uses a high pressure pump to drive the sample through a column that separates the components to identify, quantify, or purify the individual components. A chromatogram is generated to identify the eluted peptide from the column.[63]

1.3 Neuropeptides in Contact Lens (CL) wear

There are over 140 million contact lens (CL) wearers worldwide, of which up to 51% discontinue wear due to discomfort.[64] The reason behind the discomfort is multifactorial, one of which may be due to inflammation.[51] The mechanical interaction of the lens on the ocular surface could stimulate nociceptors in the cornea and lids. The bacterial adherence, presence of denatured proteins, and use of lens care solutions may induce corneal or conjunctival toxicity, or papillary conjunctivitis.[65,66] These conditions can lead to the release of inflammatory mediators.[51]

Neuropeptides are important neurotransmitters on the ocular surface and may have a role in CL-related discomfort.[21] Substance P may induce neurogenic inflammation in the cornea or conjunctiva when exposed to allergens, irritants, pathogens, or upon injury.[22] A recent study

by Golebiowski et al. showed that there was no significant difference in concentration of neuropeptides between contact lens wearers and non-lens wearers, however this may be due to the small sample size of the study.[21] Additional studies on the role of neuropeptides in CL discomfort could potentially be valuable to elucidating the mechanisms behind discomfort and CL discontinuation.

1.4 Neuropeptides in Dry Eye Disease (DED)

The Tear Film and Ocular Surface Society Dry Eye Workshop II (TFOS DEWS II) definition of dry eye disease (DED) is "… a multifactorial disease of the ocular surface which is characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles."[67] There are more than 344 million people worldwide who suffer from DED.[68,69] Studies have shown that there are alterations in nerve morphology in patients with DED and a decrease in the production of neurotransmitters.[60,70,71] In aqueous deficient dry eye, as seen in Sjögren's syndrome, there is a decrease in tear production due to inflammation of the lacrimal gland.[60] Decreased tear production stimulates the ocular surface nerve endings and gives rise to symptoms of dryness and burning. This results in the release of neuropeptides and which leads to the activation of immune cells and inflammation.[4,60] There are

very few studies examining the relationship between DED and neuropeptides.[4] A study by Lambiase et al. showed a significant decrease in the concentrations of CGRP and VIP in patients with dry eye when compared with healthy individuals, but no significant decrease in concentrations of SP and NPY in patients with dry eye when compared with healthy individuals. The reduction may be attributed to impaired lacrimal function.[60] There is a need to better understand the mechanism and role of neuropeptides in DED.

Table 3-1. Summarsing the mean results, tear collection method, analytical method and type of participants recruited in the literature.

Author	Participants	Tear collection method	Analytical method	Neuropeptide	Mean results
Fujishima et al.[22]	a) Allergic conjunctivitis	Micropipette	ELISA	SP	**SP** - 107.7 pg/mL
	b) Atopic dermatitis	Micropipette	ELISA	SP	**SP** - 65.0 pg/mL
	c) Healthy normals	Micropipette	ELISA	SP	**SP** - 70.9 pg/mL
Varnell et al.[18]	a) Healthy normals	Micropipette	ELISA	SP	**SP** - 125 pg/mL

Yamada et al[17]	a) Healthy normals	Micropipette	ELISA	SP	**SP** - 306.0 pg/mL
Sacchetti et al.[19]	a) Allergic conjunctivitis	Dry sharp tip microsponge	ELISA	SP, CGRP, NPY, VIP	**SP** - 2.1 ng/mL **CGRP** - 5.3 ng/mL **NPY** - 3.1 ng/mL **VIP** - 4.1 ng/mL
Sacchetti et al.[19]	b) Healthy normals	Dry sharp tip microsponge	ELISA	SP, CGRP, NPY, VIP	**SP** - 3.4 ng/mL **CGRP** - 6.7 ng/mL **NPY** - 3.5 ng/mL **VIP** - 5.0 ng/mL
Lambiase et al[60]	a) Sjögren syndrome DED	Dry sharp tip microsponge	ELISA	CGRP, NPY	**CGRP** - 6.0 ng/mL **NPY** - 1.5 ng/mL
Lambiase et al[60]	b)Non-Sjögren syndrome DED	Dry sharp tip microsponge	ELISA	CGRP, NPY	**CGRP** -3.0 ng/mL **NPY** - 4.6 ng/mL
	c) Ocular cicatricial pemphigoid	Dry sharp tip microsponge	ELISA		**CGRP** - 2.3 ng/mL **NPY** - 1.5 ng/mL

	d) Healthy normals	Dry sharp tip microsponge	ELISA	SP, CGRP, NPY, VIP	**CGRP** - 6.0 ng/mL **NPY** - 4.3 ng/mL **SP** - 2.3 ng/mL **VIP** - 4.5 ng/mL
	e) Dry eye disease	Dry sharp tip microsponge	ELISA	SP, CGRP, NPY, VIP	**CGRP** - 3.6 ng/mL **NPY** - 3.1 ng/mL **SP** - 2.6 ng/mL **VIP** - 2.1 ng/mL
Golebiowski et al.[21]	a) CL wear	Glass capillary tube	ELISA	CGRP, SP	**CGRP** - 14.8 ng/mL **SP** - 4.74 ng/mL
	b) Non-CL wear	Glass capillary tube	ELISA	CGRP, SP	**CGRP** - 24.71 ng/mL **SP** - 4.16 ng/mL
Markoulli et al[20]	a) Healthy normals	Flush-tear using 20 µL and 60 µL saline Schirmer's strips	ELISA	SP	F-20 - **SP-**13.1 ng/mL F-60 - **SP**-9.1 ng/mL SS - **SP** 14.9 ng/mL

Chapter 2 – Thesis Rationale

The role of neuropeptides and their role in the inflammatory process has been studied extensively.[2] However, the focus on the role of neuropeptides in ocular surface disease related to both dry eye and contact lens wear has only come to light recently.[4,17-22,60] Most of the studies are focused specifically on either SP or CGRP, but few studies that discuss the function or the concentration of all neuropeptides in the tear film.[19,60]

The general role of neuropeptides in the human body is in maintaining metabolism, food intake and inflammation.[25,27,34,46] There is a need to understand the role of these neuropeptides on the ocular surface, how they function, and their relationship to inflammatory ocular surface diseases. Studies have shown that the concentrations of SP and CGRP decrease during severe DED and increase in allergic conditions, but there is minimal information on the alteration of the level of VIP and NPY during other ocular surface diseases or inflammation.[60]

There is large variation in the concentration of neuropeptides in the tear film as reported in the literature. For example, Lambiase et al. showed that the concentrations of CGRP in the tear film of healthy humans was between 2.5 ng/mL and 10.5 ng/mL,[60] whereas a study by Golebiowski et al. reported the concentrations of CGRP to be between 1.98 ng/mL and 75.03 ng/mL.[21] While the tear collection methods were different for both studies, the concentrations

were both determined using commercially-available ELISA kits. It was not known whether this variability was due to natural physiological variability, the tear collection method, or the technique used to quantify the concentration of the neuropeptides. In order to determine the source of this variability, there is a need to accurately and precisely measure the concentration of neuropeptides in the tear film. This shifts the focus to examining the techniques for neuropeptide quantification. Moreover, there has been no studies evaluating the validity of the ELISA kits for the measurement of neuropeptides. This thesis focused on the quantification and validation of neuropeptide ELISA kits. In order to build on the current work in the literature, this thesis focused on four neuropeptides that were commonly studied in the tear film CGRP, SP, NPY, and VIP.[4,17-22,60]

Chapter 3 – Method optimization to measure the level of four neuropeptides in the human tear film

3.1 Introduction

There are several methods described for tear collection and for measuring the concentration of neuropeptides in the tear film.[17,19-22,56,60] The amount of SP and CGRP have been previously studied,[17,18,20,21] but the concentration of NPY and VIP in the tear film is still relatively unknown. A study by Sacchetti et al. showed an increase in concentration of all four neuropeptides (SP, GCRP, VIP, NPY) in subjects with allergic conjunctivitis compared to healthy individuals.[19] A study by Lambiase et al. showed that the concentration of these neuropeptides decreased in DED.[60]

There is large variation in the concentration of neuropeptides reported in the literature[21,60], and it remains unclear if these differences are true patient differences, or whether they may be due to differences in the tear collection technique, or the ELISA kit used for quantification.[21,60] To highlight this, Markoulli et al. showed no significant difference in concentration of tear film SP when the tears were collected with either a 20 µL or a 60 µL saline flush, where the 3-fold dilution of the tear film was expected to significantly dilute the concentration of SP.[20]

The purpose of this study was to replicate the work of Markoulli et al and determine the variability between basal and flush tears for not only SP, but also for CGRP, VIP, and NPY. Additionally, the study aimed to quantify the day-to-day variability of the four neuropeptides.

3.2 Methods

This was a prospective cross-sectional study that enrolled a total of 10 healthy asymptomatic, non-CL wearers between 18 and 40 years of age. This study received ethics approval by the Office of Research Ethics at the University of Waterloo prior to participant enrollment.

All participants completed the Ocular Surface Disease Index (OSDI) questionnaire,[72] and those with an OSDI score of greater than 12 were excluded. For basal tear collection, participants were asked to blink three times and 5 µL of tears were collected from the temporal canthus of the right eye using a 10 µL glass capillary tube (Wiertrol II, Thermo Fisher Scientific, Ottawa, ON, CA). For flush tear collection, 20 µL of sterile saline were instilled into the left eye and 5 µL of tears were collected from the temporal canthus using a 10 µL glass capillary tube. The collected tears were immediately transferred to a microtube (Thermo Fisher Scientific, Ottawa, ON, CA) and stored at -80°C. The participants were asked to return the following day and tears were collected at the same time of the day using the same technique.

Figure 3-1. Tear collection from the temporal canthus of the left eye using glass capillary tube

Figure 3-2. Tears transferred to microtube from glass capillary tube

3.2.1 Neuropeptides Quantification

The concentrations of each neuropeptide were determined using ELISA kits specific for each neuropeptide (CGRP, SP, NPY, VIP) (Phoenix Pharmaceuticals, Burlingame, CA, USA). Standards were prepared in accordance with the manufacturer instructions (Fig 3-3).[73] The plates were pre-coated with the capture antibody. 50 µL of prepared tear samples were added in duplicates at a 1:50 dilution. Then, 25 µL of the antibody-enzyme conjugate was added to each well and the plate was then incubated for 24 hours at 4°C. Excess reagent was removed by washing the plate three times with buffer and the substrate solution was added. After 20 minutes of incubation, the reaction was stopped and the fluorescence of each well was read at 325 nm for excitation and 420 nm for emission using SoftMax Pro 5.4.1 on SPECTRAmax M5e ROM v2.1.35 (Molecular Devices, San Jose, CA 95134, USA).

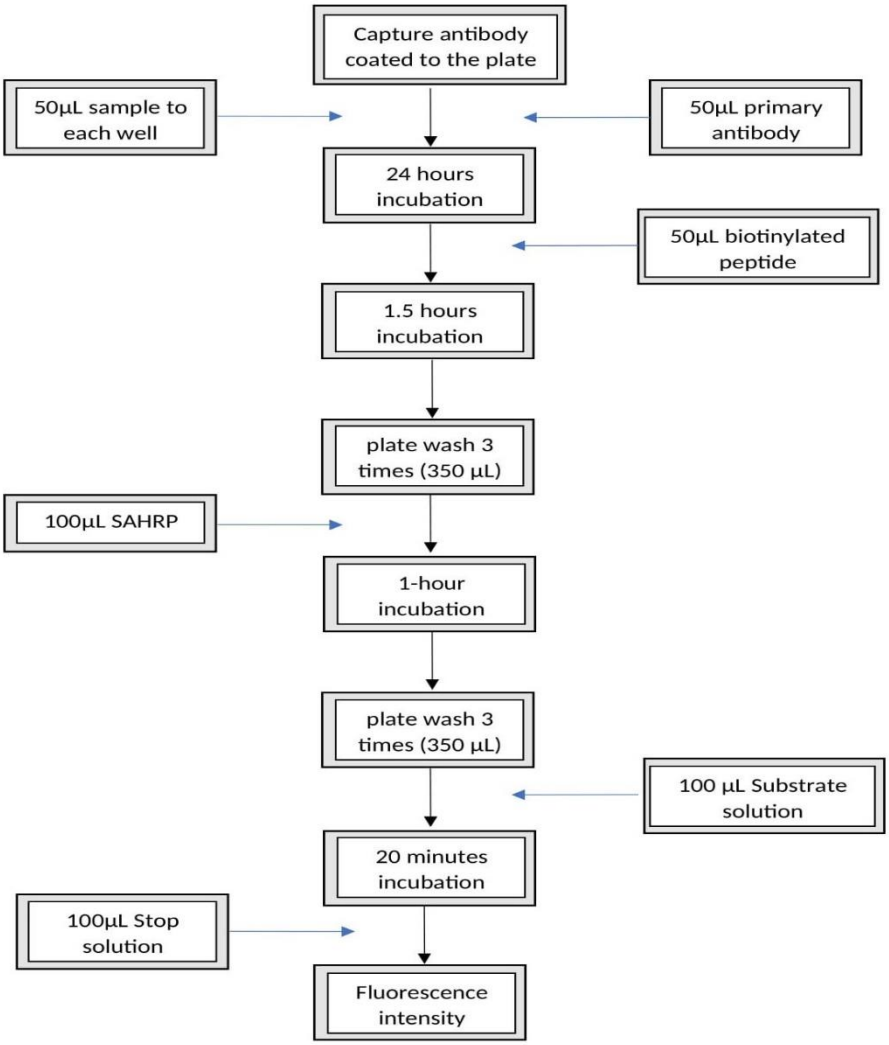

Figure 3-3. Neuropeptide ELISA Procedure (Image obtained from Neuropeptide ELISA kit protocol from Phoenix Pharmaceuticals)

3.2.2 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 8.3.0. (San Diego, CA, USA). Shapiro-Wilk normality test was used to test data the distribution of the data for normality. Unpaired t-test was used to test the difference in the concentration of each of the four neuropeptides between basal and flush tears, and between the concentrations on day 1 and day 2. A P-value of < 0.05 was considered statistically significant.

3.3 Results

A total of 8 participants (2 males and 6 females) completed the study, with the mean age of 28 ± 5 years. Two participants were excluded from the study due to OSDI scores of 18.75 and 14.58. The average OSDI score was 4.92 ± 2.32.

There was no significant difference in the concentrations of CGRP, SP, NPY, and VIP collected with either basal or flush tears (all $P > 0.19$) and no significant difference in the concentration of each of the neuropeptides between day 1 and day 2 (all $P > 0.06$).

3.3.1 Calcitonin Gene Related Peptide (CGRP)

The concentration of CGRP from basal tears was 2.88 ± 1.18 ng/mL on day 1 and 3.32 ± 1.18 ng/mL on day 2. The difference in concentration was not statistically significant ($P = 0.47$) (Fig 3-

4). The amount of CGRP in flush tears was 3.21 ± 1.39 ng/mL on day 1 and 2.28 ± 1.37 ng/mL on day 2. The difference was not statistically significant (P = 0.19). There was no significant difference in CGRP concentrations between basal and flush tears for both day 1 (P = 0.61) and day 2 (P = 0.12). Fig 3-5 shows individual participant data for basal and flush tears between day 1 and day 2.

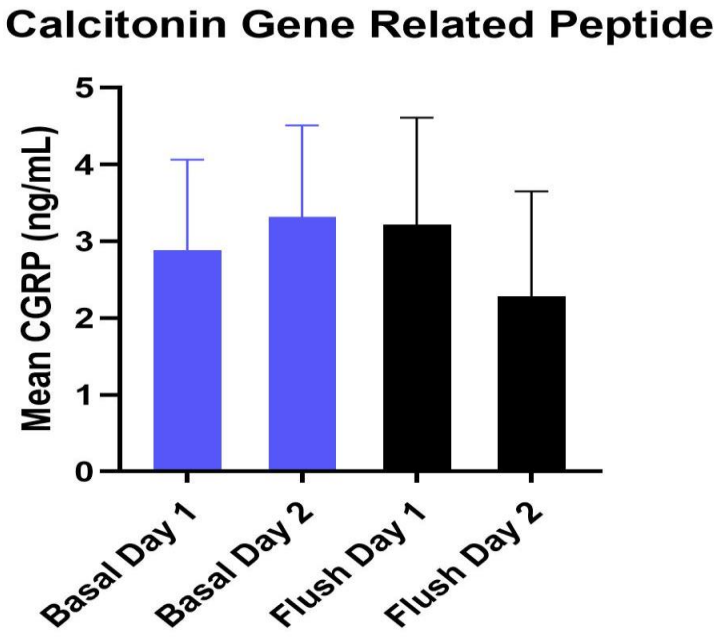

Figure 3-4. Mean CGRP levels in basal and flush tears on day 1 and 2

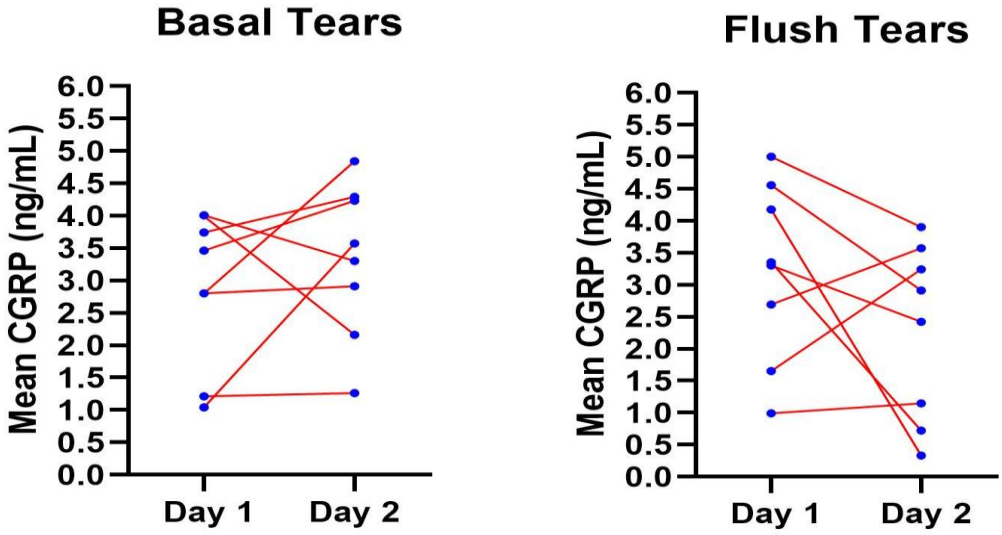

Figure 3-5. Change in CGRP concentration for each participant on day 1 and day 2 for basal/flush tear collection

3.3.2 Substance P (SP)

The concentration of SP in basal tears was found to be 2.61 ± 0.93 ng/mL on day 1 and 2.70 ± 1.04 ng/mL on day 2. The difference in concentration was not statistically significant (P = 0.86) (Fig 3-6). The amount of SP in flush tears was found to be 1.76 ± 0.72 ng/mL on day 1 and 2.34 ± 0.95 ng/mL on day 2. This difference was not statistically significant (P = 0.19). There was no significant difference in SP concentrations between basal and flush tears for day 1 (P = 0.06) and day 2 (P = 0.47). Fig 3-7 shows individual participant data for basal and flush tears between day 1 and day 2

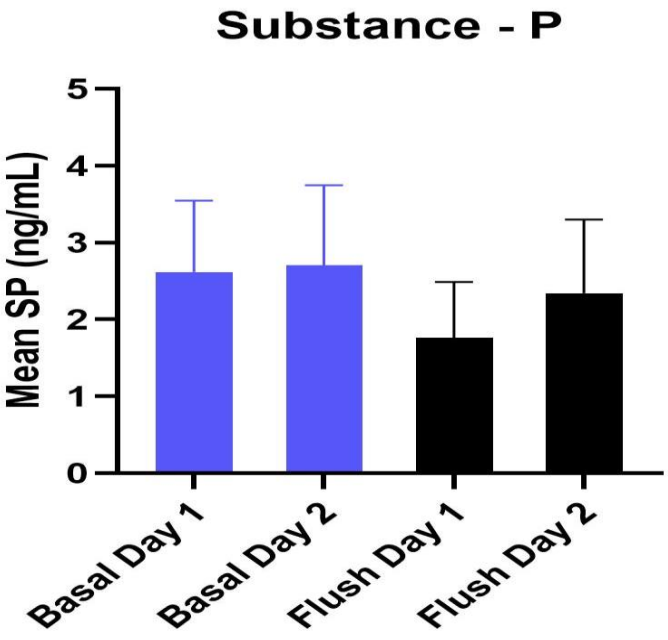

Figure 3-6. Mean SP levels in basal and flush tears between day 1 and 2

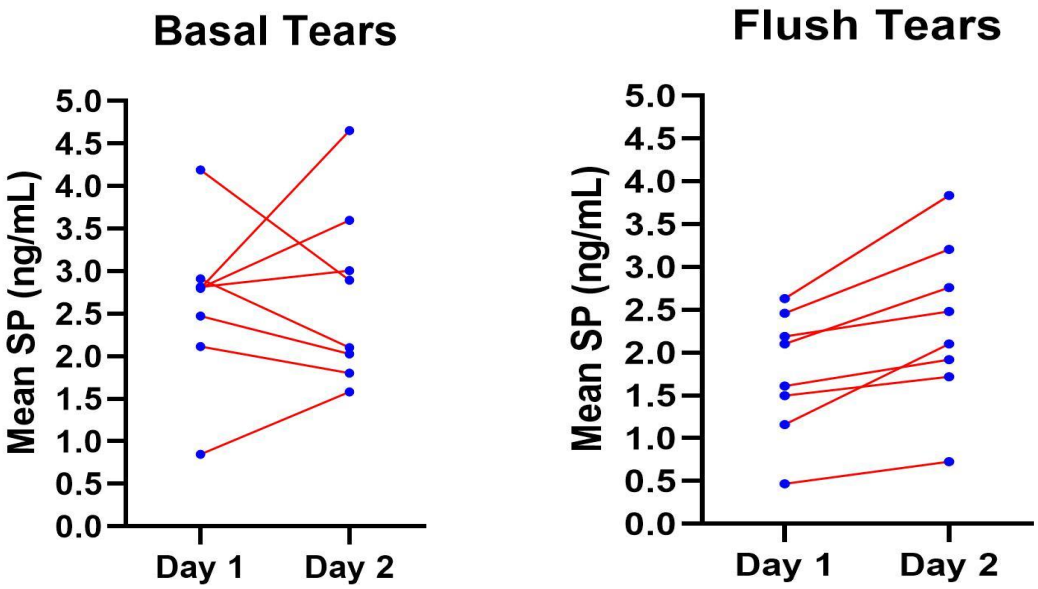

Figure 3-7. Change in SP concentration for each participant on day 1 and day 2 for basal/flush tears collection

3.3.3 Neuropeptide Y (NPY)

The concentration of NPY in basal tears was found to be 1.91 ± 0.84 ng/mL on day 1 and 2.01 ± 0.76 ng/mL on day 2. The difference in concentration was not statistically significant (P = 0.81) (Fig 3-8). The amount of NPY in flush tears was found to be 1.66 ± 0.72 ng/mL on day 1 and 1.38 ± 0.62 ng/mL on day 2. The difference was not statistically significant (P = 0.42). There was no significant difference in NPY concentration between basal and flush tears for day 1 (P = 0.53) and

day 2 (P = 0.09). Fig 3-9 represent individual participant data for basal and flush tears between day 1 and day 2.

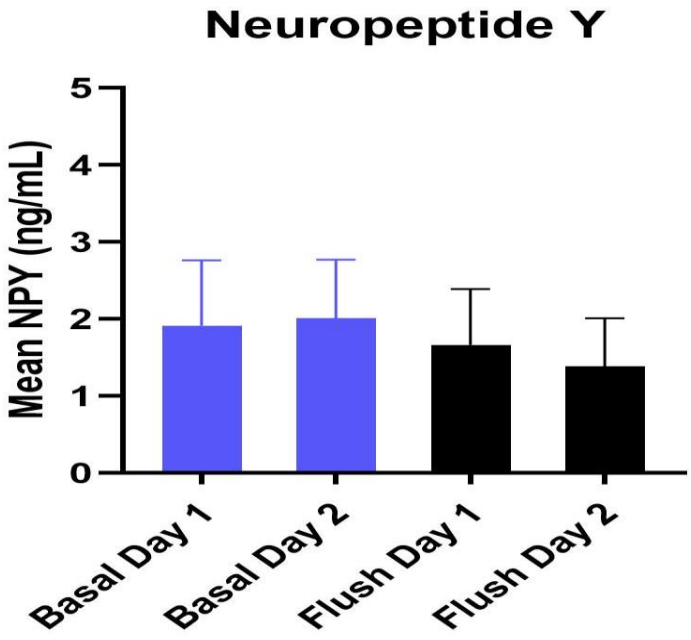

Figure 3-8. Mean NPY levels in basal and flush tears between day 1 and 2

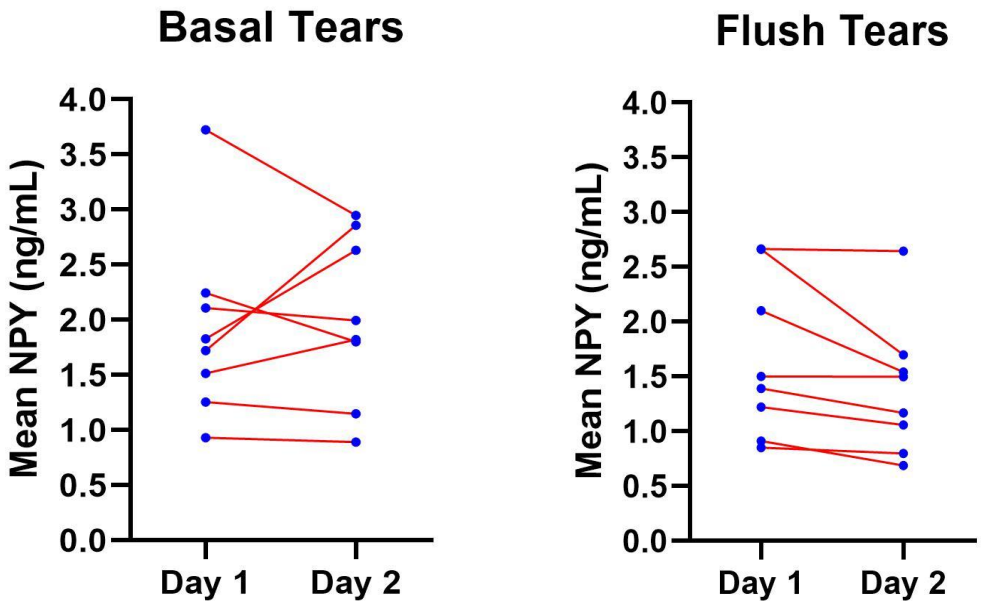

Figure 3-9. Change in VIP concentration for each participant on day 1 and day 2 for basal/flush tears collection

3.3.4 Vasoactive intestinal peptide (VIP)

The concentration of VIP in basal tears was found to be 3.05 ± 0.85 ng/mL on day 1 and 3.03 ± 0.80 ng/mL on day 2. The difference in concentration was not statistically significant (P = 0.97) (Fig 3-10). The amount of VIP in flush tears was found to be 2.77 ± 1.53 ng/mL on day 1 and 2.60 ± 1.04 ng/mL on day 2. This difference was not statistically significant (P = 0.82). There was also no significant difference in VIP concentrations between basal and flush tears for day 1 (P = 0.71)

and day 2 (P = 0.44). Fig 3-11 shows the individual participant data for basal and flush tears between day 1 and day 2.

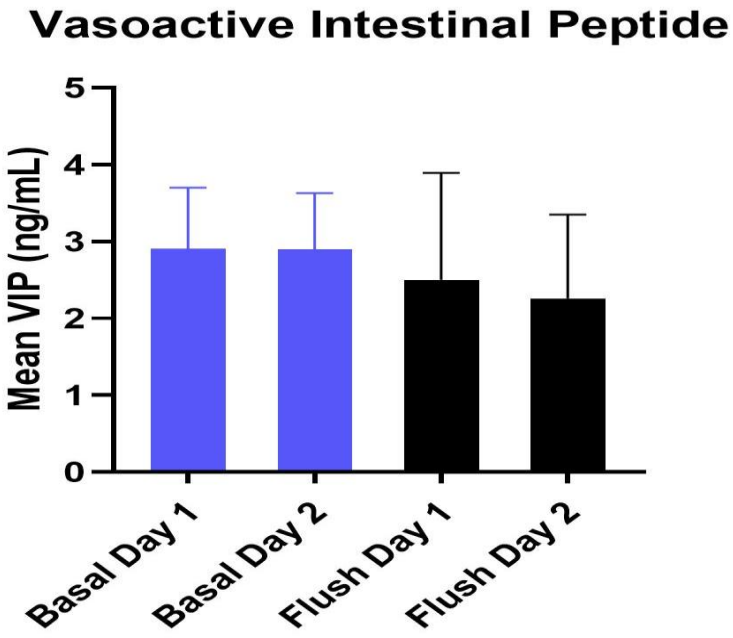

Figure 3-10. Mean VIP levels in basal and flush tears between day 1 and 2

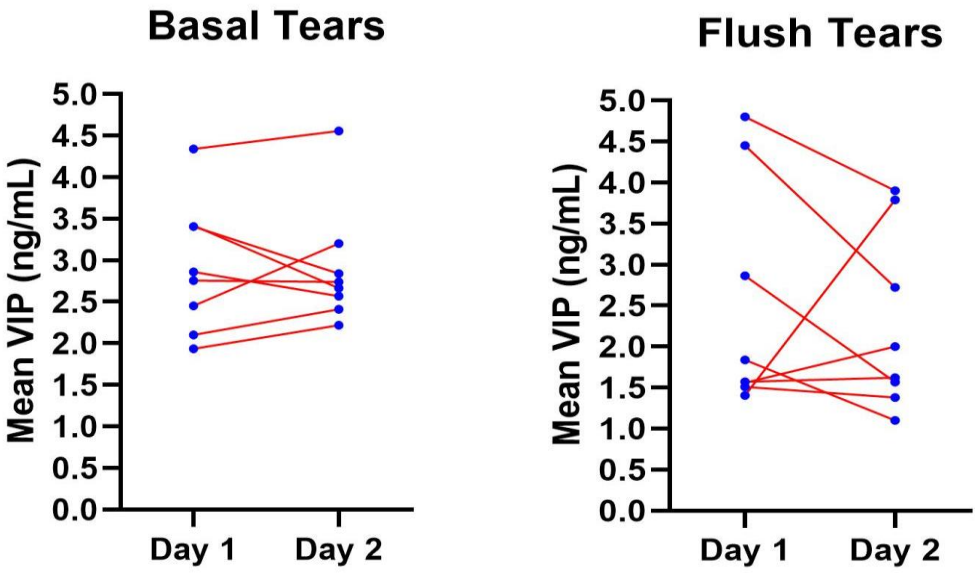

Figure 3-11. Change in VIP concentration for each participant on day 1 and day 2 for basal/flush tears collection

3.4 Discussion and conclusion

This study found no significant difference in the concentrations of CGRP, SP, NPY and VIP using two different tear collection methods, and no significant difference in the concentrations of neuropeptides between two consecutive days.

This study reflects the findings in Markoulli et al, where they also observed no significant difference in the concentrations of SP between basal tear and flush tear collection, using both 20 µL and 60 µL of saline.[20] Similarly, in this study, no significant difference was observed between basal and flush tears (20 µL) for all four neuropeptides. Using flush tears for collection was previously shown to significantly dilute protein concentrations within collected tears, however the relative protein concentrations were comparable to that of basal tears.[56] Given that no reductions were observed in neuropeptide concentration for Markoulli et al and in this study,[20] a possible explanation is that the ELISA assays may not be optimal for tear film samples. Another factor to consider is that a sample size of only 8 participants in this study may have been too low to detect a difference. To rule out the former, further work to elicit the nature and quality of the ELISA kits was required.

Chapter 4– Method Optimization

4.1 Introduction

The ELISA is a commonly used laboratory technique to determine the quantity of different components in blood and other bodily fluids. Recent studies have used this technique for the quantification of neuropeptides in the tear film.[17-22] To determine if these ELISA kits are internally valid in the quantification of neuropeptides in bodily fluids, there are two types of tests that could be used. In one of the tests, a known amount of analyte is added (spiked) to the sample that is being tested. Then, an ELISA is conducted to quantify the spiked sample (recovery) to determine how close the observed value is from the expected. The second test to determine the internal validity is through conducting a serial dilution of a known analyte. In this method, a sample of known concentration is serially diluted, and the response (which should reflect the dilution factor), is measured. The observed and expected values from these tests should be within 80-120% of each other.[74] The findings in the previous chapter, where no significant difference in concentrations between basal and flush tears were detected, has raised questions about the validity of the ELISA kits used. The purpose of this chapter was focused on eliciting information regarding the nature and validity of the ELISA kits.

4.2 Methods

A total of 3 healthy non-CL wearers (2 males, 1 female) with mean age of 28.6 ± 1.6 were enrolled in this study. This study received ethics approval by the Office of Research Ethics at the University of Waterloo prior to participant enrollment.

Participants were asked to blink three times and 5 µL of basal tears were collected from the temporal canthus of each eye, using a 10 µL glass microcapillary tube (Wiertrol II, Thermo Fisher Scientific, Ottawa, ON, CA). The collected tears were pooled and immediately transferred to a microtube (Thermo Fisher Scientific, Ottawa, ON, CA) and stored at -80°C. This was repeated the following day.

4.2.1 Spike and Recovery

The standards from ELISA kits for quantifying CGRP, SP, and NPY (Phoenix Pharmaceuticals, Burlingame, CA, USA) were formulated into three different concentrations: 100 pg/mL, 10 pg/mL, and 1 pg/mL. A volume of 108 µL of each neuropeptide concentration was added (spiked) to three different collected tear samples (2 µL) from each participant. Non-spiked tear samples were used as control. The three spiked samples for each concentration and unspiked samples were added to the ELISA plate and incubated for 15 minutes. Then, 25 µL of the antibody-enzyme conjugate was added to each well and the plate was then incubated for 24 hours at 4°C. Excess

reagent was removed by washing the plate three times with buffer. The substrate solution was then added to each well. After 20 minutes of incubation, the reaction was stopped and the fluorescence of each well was read at 325 nm for excitation and 420 nm for emission using SoftMax Pro 5.4.1 on SPECTRAmax M5e ROM v2.1.35 (Molecular Devices, San Jose, CA 95134, USA). The percent recovery of the spiked sample was calculated using the following equation:

$$\text{Observed} = \frac{\text{Spike sample}}{\text{Sample used for spike (1 or 10 or 100 pg/mL)}} - \frac{\text{Unspiked sample}}{\text{Sample used for spike (1 or 10 or 100 pg/mL)}}$$

$$\text{Recovery} = \frac{\text{Observed}}{\text{Expected}} \times 100$$

4.2.2 Serial Dilution Response

For this experiment 5 µL basal tear were collected from both eyes from a single healthy non-CL wearer (same participant from the previous experiment) using a 10 µL glass microcapillary tube (Wiertrol II, Thermo Fisher Scientific, Ottawa, ON, CA). The collected tears were immediately transferred to a microtube (Thermo Fisher Scientific, Ottawa, ON, CA) and stored at -80°C. Only neuropeptide NPY was quantified. The NPY concentrations in the tears were determined using an ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). The standards were prepared in

accordance with the manufacturer instructions.[73] A concentration of 100 pg/mL NPY was used to spike 4 µL of tear sample. After spiking the spiked sample was diluted in 205 µL of buffer.

a) 11 µL of 100 pg/mL NPY was added to 4 µL of the tear sample. The spiked sample was then diluted in 205 µL of buffer.
b) 110 µL from previous dilution was diluted in 110 µL of buffer to obtain a 1:2 dilution
c) 110 µL from 1:2 dilution was again diluted in 110 µL of buffer to obtain a 1:4 dilution
d) 110 µL from 1:4 dilution was again diluted in 110 µL of buffer to obtain a 1:8 dilution

All the diluted samples were added to the plate in duplicates. Then, 25 µL of the antibody-enzyme conjugate was added to each well and the plate was then incubated for 24 hours at 4°C. Excess reagent was removed by washing the plate three times with buffer and a substrate solution was added. After 20 minutes of incubation, the reaction was stopped and the fluorescence of each well was read at 325 nm for excitation and 420 nm for emission using SoftMax Pro 5.4.1 on SPECTRAmax M5e ROM v2.1.35 (Molecular Devices, San Jose, CA 95134, USA). The percentage difference between observed and expected values were calculated using the following equation:

$$\text{Percentage difference (\%)} = \frac{\text{Observed value in dilution (1:2 or 1:4 or 1:8)}}{\text{Expected value / (2 or 4 or 8)}} \text{ X } 100$$

4.3 Results

4.3.1 Recovery of neuropeptides

The results (Tables 4-1, 4-2, 4-3) show the percent recovery of the spiked samples for neuropeptides CGRP, SP, NPY. These results were calculated using the % recovery equation described above. The neuropeptide concentrations in unspiked tear samples were deducted from the spiked samples. Although the recovery was within 80-120% of the expected values, there were a considerable number of samples that were greater than or less than this range.

Table 4-1. Spike and recovery of **CGRP** in tear film samples

Participants	Spiked samples (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	1 pg/mL	0.982 pg/mL	8.38 pg/mL	853.36
	10 pg/mL	9.82 pg/mL	1.94 pg/mL	19.75
	100 pg/mL	98.2 pg/mL	Not measureable	Not measureable
2	1 pg/mL	0.982 pg/mL	628.02 pg/mL	63,953.15
	10 pg/mL	9.82 pg/mL	1.35 pg/mL	13.74
	100 pg/mL	98.2 pg/mL	Not measureable	Not measureable
3	1 pg/mL	0.982 pg/mL	4.23 pg/mL	430.75

	10 pg/mL	9.82 pg/mL	10.48 pg/mL	106.72
	100 pg/mL	98.2 pg/mL	Not measureable	Not measureable

Table 4-2. Spike and recovery of **SP** in tear film samples

Participants	Spiked samples (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	1 pg/mL	0.982 pg/mL	6.64 g/mL	676.17
	10 pg/mL	9.82 pg/mL	1.29 pg/mL	13.13
	100 pg/mL	98.2 pg/mL	5.12 pg/mL	5.21
2	1 pg/mL	0.982 pg/mL	4.37 pg/mL	445.01
	10 pg/mL	9.82 pg/mL	1.39 pg/mL	14.15
	100 pg/mL	98.2 pg/mL	Not measureable	Not measureable
3	1 pg/mL	0.982 pg/mL	Not measureable	Not measureable
	10 pg/mL	9.82 pg/mL	5.27 pg/mL	53.66
	100 pg/mL	98.2 pg/mL	Not measureable	Not measureable

Table 4-3. Spike and recovery of **NPY** in tear film samples

Participants	Spiked samples (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	1 pg/mL	0.982 pg/mL	0.76 pg/mL	77.39
	10 pg/mL	9.82 pg/mL	1.10 pg/mL	11.20
	100 pg/mL	98.2 pg/mL	22.9 pg/mL	23.31
2	1 pg/mL	0.982 pg/mL	4.05 pg/mL	412.42
	10 pg/mL	9.82 pg/mL	1.89 pg/mL	19.24
	100 pg/mL	98.2 pg/mL	7.38 pg/mL	7.51
3	1 pg/mL	0.982 pg/mL	3.48 pg/mL	354.37
	10 pg/mL	9.82 pg/mL	2.21 pg/mL	22.50
	100 pg/mL	98.2 pg/mL	Not measureable	Not measureable

4.3.2 Dilution response

The initial concentration of NPY was 208.00 pg/mL. For the dilutions; 1:2 yielded 188.40 pg/mL, 1:4 yielded 153.60 pg/mL, and 1:8 yielded 204.28 pg/mL. The percentage of expected recovery for 1:2 was 181%, 1:4 was 295% and the difference for 1:8 was 785%.

Table 4-4. % recovery of **NPY** in tear film sample dilutions

Dilutions			
	Expected recovery	**Observed recovery**	**% of expected value**
1:2	104.00 pg/mL	188.40 pg/mL	181
1:4	52.00 pg/mL	153.60 pg/mL	295
1:8	26.00 pg/mL	204.28 pg/mL	785

4.4 Supplemental Troubleshooting Experiments

Both the spike and recovery and serial dilution response experiments yielded results which were unexpected, bringing into question the validity of the ELISA kits. This also suggested the possibility that other components in the tear film were interfering with the assay. To determine if there were other components in the tear film that were interfering with the assay, and to

further evaluate validity and the nature of the assay, the following experiments were conducted, and their summaries are described below.

4.4.1 Use of a blocking agent

The tear film have many components such as lipids, proteins, and mucins, [75] in addition to the analyte of interest. Therefore, there is a possibility that some of these extraneous components can interact with the antibodies and confound results. In the previous study, the results of the spike and recovery and serial dilution response experiments suggested the possibility of interference occurring within the ELISA assay. One possibility is the presence of non-specific binding and interference with antibody binding. To minimize this interference, a blocking agent was used reduce non-specific binding in the ELISA plate.

Purpose: To determine the effect of a blocking agent on neuropeptide quantification.

Method: Block ACE (Bio-Rad laboratory, Mississauga, Canada) was used to block non-specific binding sites of an SP ELISA plate (Phoenix Pharmaceuticals, Burlingame, CA, USA). Block ACE was dissolved in water and two concentrations of Block ACE were formulated: 0.4 g/mL and 0.1 g/mL. The Block ACE was then added to specified wells of an SP ELISA plate 30 minutes prior to conducting the assay. After 30 minutes, the contents of the wells were discarded and the ELISA was performed to quantify two known concentrations of SP standards: 1000 pg/mL, and 100

pg/mL prepared by diluting SP in assay buffer. Quantification of the two SP control concentrations without Block ACE was conducted as a control. The concentrations of SP associated with 0.4 g/mL, 0.1 g/mL no blocking, was compared.

Results: For the 1000 pg/mL control concentration of SP, the amount recovered from the well incubated with 0.1 g/mL Block ACE was 1018.21 pg/mL. The amount of SP recovered from 0.4 g/mL Block ACE was 981.78 pg/mL. The amount recovered from the blank well was 1039.73 pg/mL (Fig 4-1). For the 100 pg/mL control concentration of SP, the recovered concentration at 0.1 g/mL Block ACE was 21.18 pg/mL SP and 178.81 pg/mL SP for 0.4 g/mL Block ACE. The amount of SP recovered from the blank well was 104.50 pg/mL (Fig 4-1)

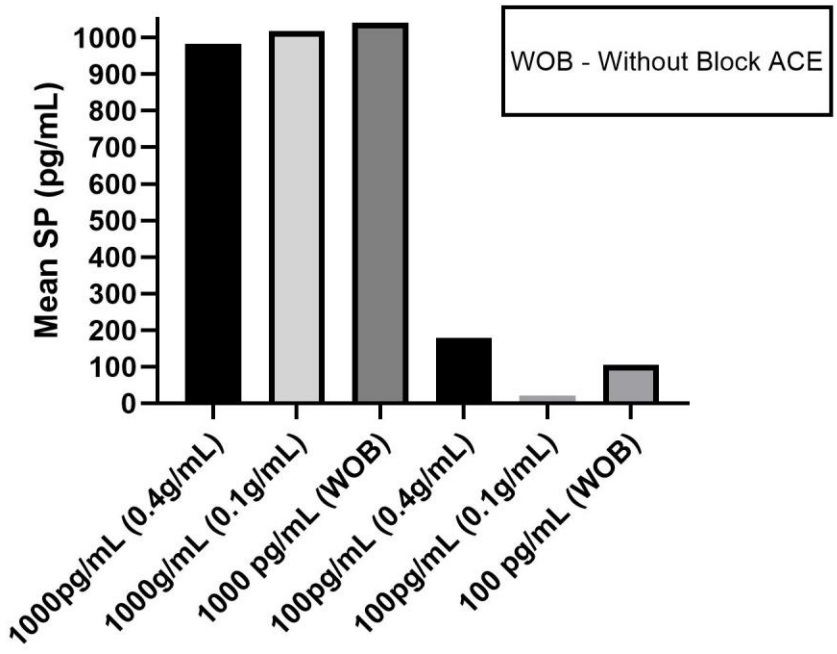

Figure 4-1. Amount of SP recovered from using 0.4 g/mL and 0.1 g/mL Block ACE.

Conclusion: The results suggest that using a blocking agent does not have any influence on the quantification of SP.

4.4.2 C18 column tips

The tear film contains a large amount of proteins[75] and it is possible that protein were interfering with the ELISA assay. To reduce the amount of protein in the tear samples prior to conducting an ELISA assay, passing tear samples through a C18 column may be helpful. A C18 column consists of silica particles with carbon chains attached. When the sample is passed through the column the carbon chains strongly retain non-polar solutes and the polar solutes are eluted

Purpose: The purpose of this experiment was to pre-separate other components in the tear film using C18 column tips (as per manufacturer instructions) and determine if pre-separation influences VIP quantification.

Method: A total of 50 µL of basal tears were collected using glass capillary tubes and pooled from both eyes of 5 healthy participants (3 females, 2 males) with mean age of 29.4 ± 2.8. C18 pipette tips (Thermo Fischer Scientific, Mississauga, ON, CA) were used to process the tears. First, 10 µL of tears were diluted with 10 µL of Buffer A provided with the VIP ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). Then 10 µL C18 pipette tips were prewashed using 50 µL of buffer A, followed by 150 µL of Buffer B of the ELISA kit. 10 µL of the tear sample was loaded into the tip and slowly washed with 150 µL of Buffer A and discarded. Then the peptide was eluted slowly with 150 µL Buffer B into a microtube. The eluent was dried and stored at -20°C prior to further analysis.

Known concentrations of VIP 1000 pg/mL and 100 pg/mL were prepared by diluting VIP in assay buffer and passed through the C18 pipette tips as described earlier. The eluent was dried and stored at -20°C. An ELISA was performed to quantify the amount of VIP within the eluents. A separate set of 1000 pg/mL and 100 pg/mL of VIP were formulated but were not passed through the C18 pipette tips and were used as control.

Results: The VIP concentration from the tear eluent was beyond the lower range of detection of the ELISA kit. Overall, a reduction was observed for the concentrations of VIP that were passed through the C18 pipette tip. For the 1000 pg/mL VIP, the amount recovered after using the C18 pipette tip was 265.28 pg/mL, and 1048.39 pg/mL was recovered from not using the C18 pipette tip (Fig 4-2). For the 100 pg/mL VIP concentration, the amount recovered from passing through the C18 pipette was beyond the lower range of detection of the kit, whereas not using the C18 pipette tip recovered an amount of 74.76 pg/mL (Fig 4-2).

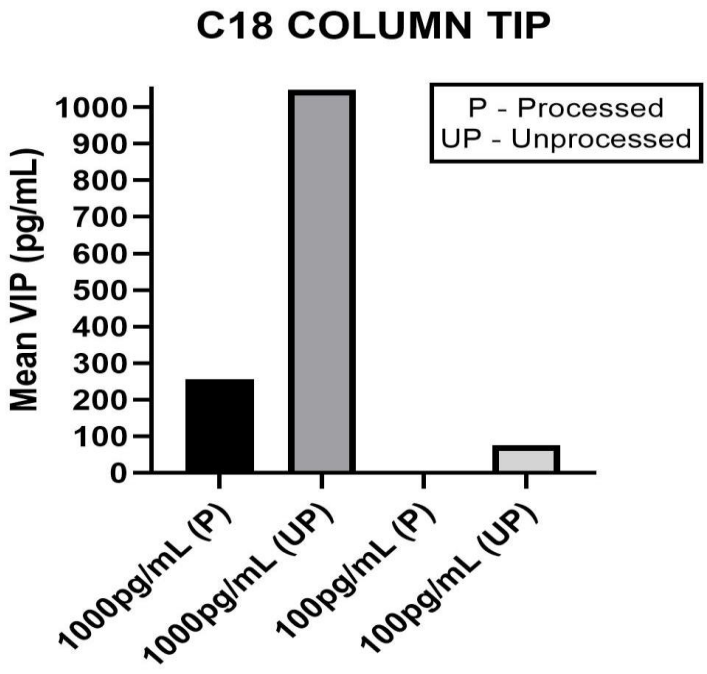

Figure 4-2. Difference between processed and unprocessed VIP samples of 1000 pg/mL and 100 pg/mL concentrations through C18 column tips

Conclusion: The results suggest that C18 pipette tips reduced VIP concentrations and that C18 may not be helpful in separating VIP neuropeptides from the small quantity of tear sample.

4.4.3 Interference of Albumin

One protein found in high concentrations in the tear film is albumin.[76] It is possible that albumin may interact with the ELISA assay quantification.

Purpose: To evaluate the effect of albumin on ELISA assay performance.

Method: Three solutions of VIP (1000 pg/mL, 100 pg/mL, and 10 pg/mL) were prepared by serially diluting 100,000 pg/mL VIP in bovine serum albumin solution (Sigma-Aldrich, Oakville, ON, Canada). The bovine serum albumin solution was prepared by mixing 1 mg bovine serum albumin in 1 mL of MilliQ water. Another three solutions of VIP (1000 pg/mL, 100 pg/mL, and 10 pg/mL) were prepared in buffer as a control. The concentrations of VIP were determined using an ELISA kit as previously described, as per manufacturer instructions.

Results: Only data for 1000 pg/mL of VIP with and without albumin was available. VIP concentration associated with albumin was 535.06 pg/mL, and VIP without albumin was 939.72 pg/mL (Fig 4-3). The other two experimental conditions 100 pg/mL and 10 pg/mL VIP yielded results which were below the detection limit of the kit.

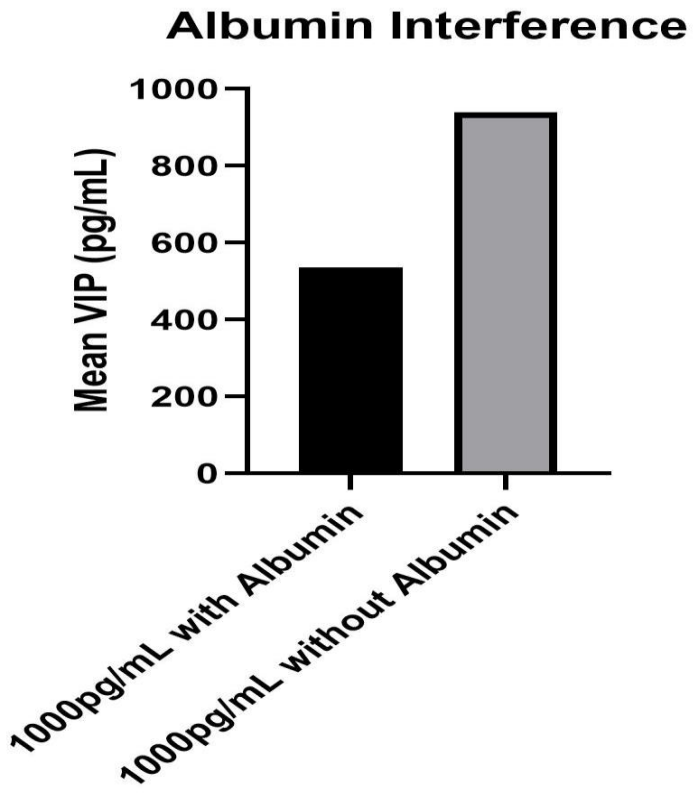

Figure 4-3. Comparison between 1000 pg/mL of VIP diluted in albumin and 1000 pg/mL of VIP diluted in diluent buffer.

Conclusion: The results suggest that albumin may interfere with the ELISA quantification of VIP.

4.4.4 Using Protease Inhibitors and Fresh tears

A potential reason for reduced neuropeptide concentrations may be due to freeze storage and degradation of tear samples over time. The half-life of neuropeptides is no more than 10 minutes[10] and there may already be neuropeptides lost by the time the samples arrive at the freezer. Another possible cause for degradation can be freeze-thaw cycles. Further, proteases and peptidases in the tear film may also contribute a role in reducing the concentration of neuropeptides.

Purpose: The purpose of this experiment was to test the effect of freeze storage on neuropeptide concentrations, and to test the effect of a protease inhibitor on SP quantification.

Methods: Protease inhibitor (Thermo Fisher Scientific, Ottawa, Canada) was prepared by dissolving 10 g of protease inhibitor into 100 mL of buffer. 10 µL of basal tears were collected from each eye and pooled to obtain a total of 20 µL from a single healthy participant using a glass microcapillary tube as previously described. 10 µL of tears were diluted in the protease inhibitor buffer within 10 minutes of collection. The remaining 10 µL of tears were diluted in assay buffer. Both diluted tear samples were each divided in 2 µL and 4 µL aliquots. A four-month old tear sample stored at -80°C from the same participant was retrieved and was aliquoted and treated with protease inhibitor in the same manner described above. The rational for dividing diluted

tear samples into 2 µL and 4 µL was to test if 4 µL of tears yield double the amount of SP as the 2 µL aliquot. In addition, 1000 pg/mL and 10 pg/mL of SP were also prepared with and without the protease inhibitor to test the effect of the protease inhibitor on pure SP. The SP ELISA was conducted as previously described.

Results: The 2 µL of freshly collected tears diluted in protease inhibitor showed a concentration of 1.15 pg/mL (Fig 4-4), while the 2 µL of freshly collected tears diluted in assay buffer showed a concentration of 2.50 pg/mL (Fig 4-4). 4 µL of freshly collected tears diluted in assay buffer yielded double (4.20 pg/mL) the amount of SP as in 2 µL tears (Fig 4-4). Similarly, 2 µL of frozen tears diluted in protease inhibitor showed a concentration of 0.65 pg/mL and 2 µL of frozen tears diluted in assay buffer showed a concentration of 1.20 pg/mL (Fig 4-5).

There was a decrease in concentration of SP tears which were frozen compared to that which were freshly collected. 2 µL of freshly collected tears diluted in assay buffer showed a concentration of 2.50 pg/mL (Fig 4-4), and tears which were frozen showed a concentration of 1.20 pg/mL (Fig 4-5).

The amount recovered from 1000 pg/mL SP diluted in protease inhibitor was 753.99 pg/mL, and without protease inhibitor was 998.25 pg/mL. The amount recovered from the 10

pg/mL SP diluted in protease inhibitor was 9.17 pg/mL, and without protease inhibitor was 10.85 pg/mL (Fig 4-6).

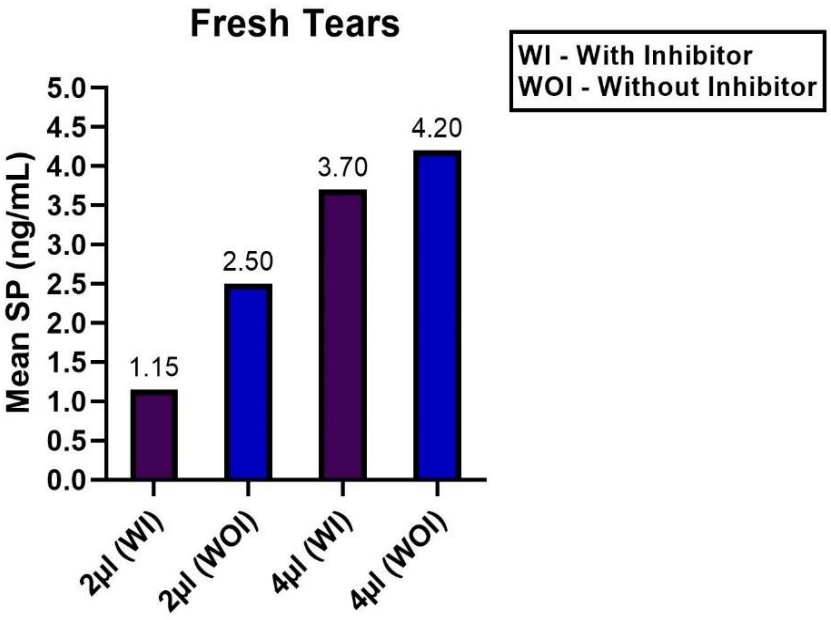

Figure 4-4. Effect of protease inhibitors on the concentration of SP in freshly collected tears.

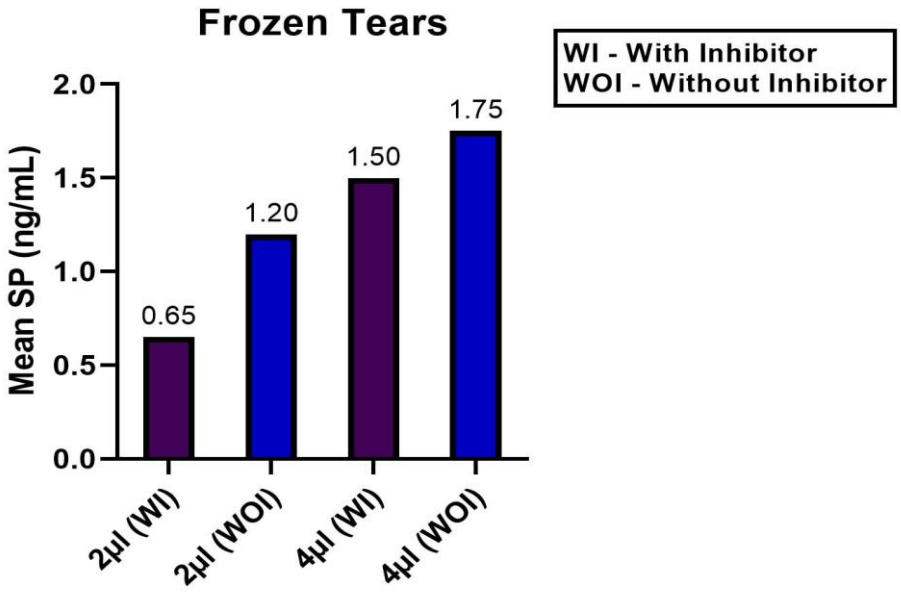

Figure 4-5. Effect of protease inhibitors on the concentration of SP in tears stored at -80°C.

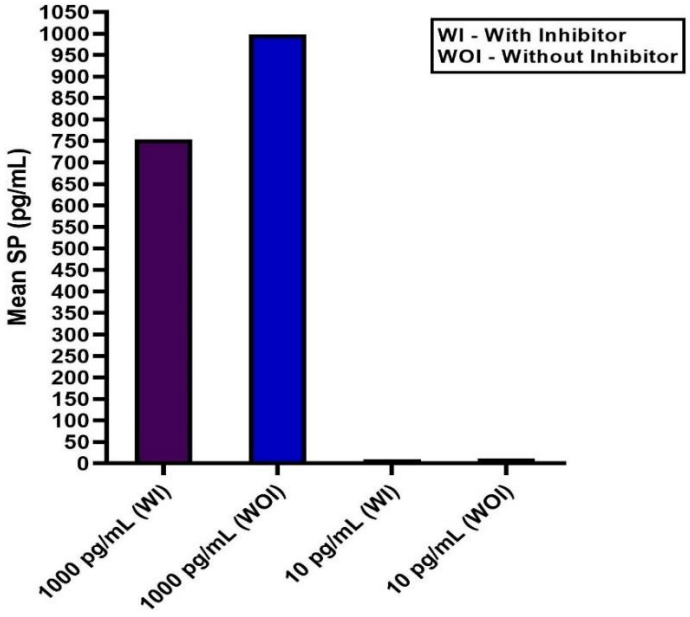

Figure 4-6. Effect of protease inhibitors on the 1000 pg/mL and 10 pg/mL SP.

Conclusion: Tears stored at -80°C yielded a lower concentration of SP compared to freshly collected tears and the effect appeared to be independent of volume. Adding protease inhibitors reduced SP concentrations and the effect also appeared to be independent of volume.

4.5 Discussion and Conclusion

The focus of this chapter was to elicit further information on the validity of the ELISA kits. The spike and recovery and serial dilution response experiments supplied highly variable results. Some possible reasons for this may be due to non-specific binding on the ELISA plate, interference from tear film proteins and protease, and sample storage conditions. After subsequent experimentation with adding blocking agents, employing C18 separation, testing the interference of albumin, the use of protease inhibitors, and testing at different storage conditions, it was determined that freshly collected tears were associated with the greatest recovery in neuropeptide concentration. It was also determined that blocking agents did not appear to impact the quantification, that albumin may reduce the amount of signal, and that C18 pipette tips, protease inhibitors, and freezing storage reduced neuropeptide concentrations in the tear film sample.

The ELISA kits were typically designed to quantify analytes of interest in large-volume samples, such as blood, plasma/serum, tissues and cerebrospinal fluid with no less than 1 mL in volume. The kit protocol describes the procedure of extraction of peptides from the blood, plasma/serum, tissues and cerebrospinal fluid. However, when the same protocol was followed for the extraction of neuropeptides from tear samples, almost no peptides were detected. This may be due to the small quantity of tear samples used. Larger quantities of tears samples can be

used in future to test if neuropeptides can be quantified reliably from the tear film. Another method that can be used to test the influence of other proteins on the assay is to dilute the tear samples in BSA and in assay buffer and compare the concentration of neuropeptides between the preparations. Further experiments to be conducted can be to test the concentrations of CGRP, NPY and VIP between tears which were frozen, and freshly collected tears from the same participant.

These experiments have provided some optimization steps to consider for tear sample collection and processing for the detection for neuropeptides. However, the highly variable results from the spike and recovery and serial dilution experiments suggest that further work is needed to optimize the ELISA assay.

Chapter 5 – Evaluation of Substance P Neuropeptide ELISA Kits

5.1 Introduction

The Substance P (SP) neuropeptide ELISA kit from Phoenix Pharmaceuticals (Burlingame, CA, USA) has been used in previous studies evaluating the concentration of SP in the tear film.[19,21,60] This kit was used for measuring the concentration of SP in the tear film in Chapter 3, but the results of the troubleshooting experiments in Chapter 4 has raised some questions about its validity. This chapter builds on the previous data through continued investigations into the nature of the kit. In addition, the performance of another commercially available SP ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA) was compared.

5.1.1 Absorbance Spectrum

Each chemical has a unique spectral absorbance profile and the absorbance profile may be helpful in determining the integrity of SP from various sources.[77,78]

5.1.1.1 Purpose

The purpose of this experiment was to test the similarity of the SP obtained from Phoenix Pharmaceuticals, Cayman Chemicals, and Sigma Aldrich.

5.1.1.2 Method

For this experiment, SP from Phoenix Pharmaceuticals (Burlingame, CA, USA), SP from Cayman Chemicals (Ann Arbor, Michigan, USA) and SP from Sigma-Aldrich (Oakville, ON, Canada) were each formulated at 0.5 mg/mL in 0.9% saline. Their UV absorbance profile from 200 nm to 300 nm was obtained using SoftMax Pro 5.4.1 software on a SPECTRAmax M5e ROM v2.1.35 (Molecular Devices, San Jose, CA 95134, USA). After each measurement, the cuvette was washed with saline, dried with sterile Q tips and aerosol spray prior to the next measurement.

5.1.1.3 Results:

Fig 5-1 shows the similar absorbance profile of SP from Phoenix Pharmaceuticals and SP from Sigma Aldrich while, the absorbance profiles for SP from Cayman Chemicals is different than other two.

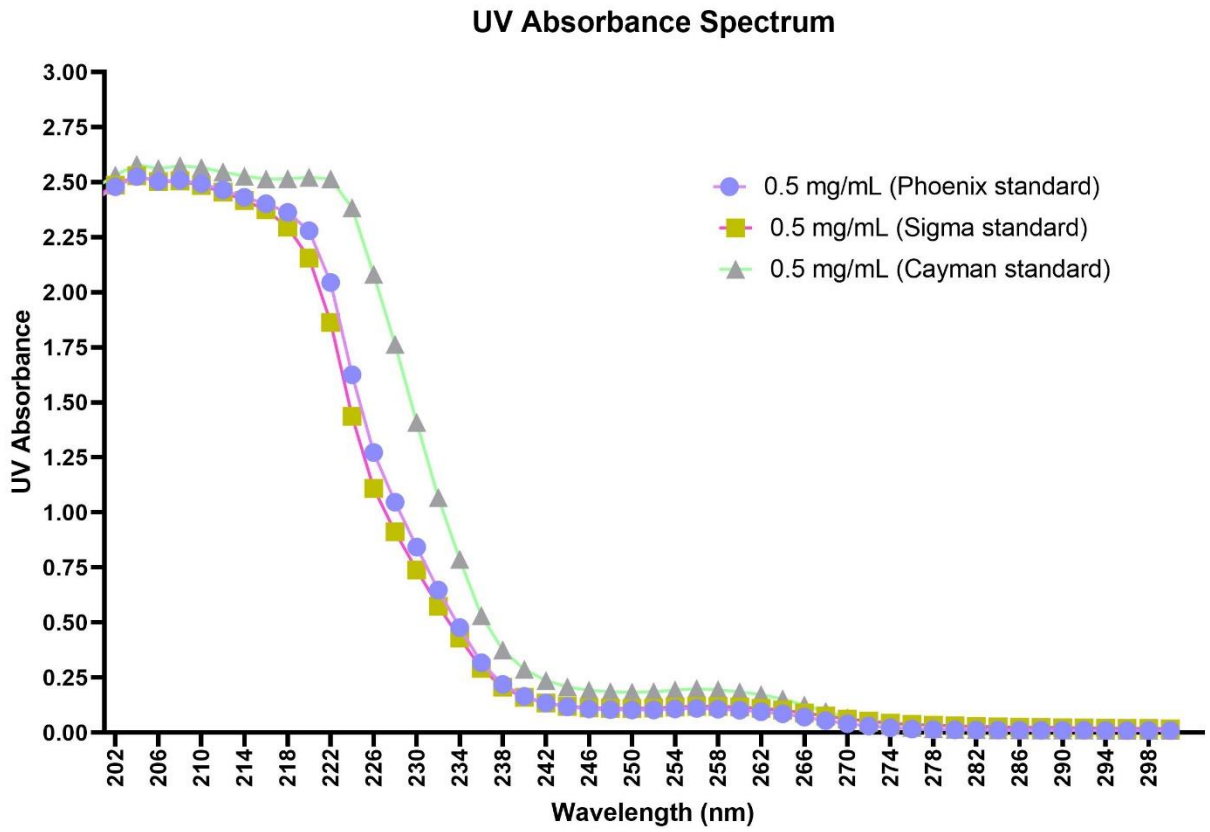

Figure 5-1. Absorbance spectrum compared between the SP obtained from Phoenix Pharmaceuticals, Cayman Chemicals and Sigma Aldrich

5.2 Agreement between SP from Phoenix Pharmaceuticals and external SP in Phoenix Pharmaceuticals SP ELISA kit and Cayman Chemicals SP ELISA kit

Since the SP from Phoenix Pharmaceuticals and the SP from Sigma Aldrich have nearly identical spectral absorbance profiles, they could serve as two independent controls for eliciting the internal variability of the Phoenix Pharmaceuticals and Cayman Chemicals ELISA kits.

5.2.1 Purpose

The purpose of this experiment was to determine the quantity of known concentrations of two similar SP substances and determine their agreement with both the Phoenix Pharmaceuticals and Cayman Chemicals ELISA kit.

5.2.2 Method

5.2.2.1 SP ELISA kit from Phoenix Pharmaceuticals

SP from Phoenix Pharmaceuticals and the SP from Sigma Aldrich were serially diluted to obtain concentrations of 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL, 7.8 pg/mL, 3.9 pg/mL. The plate was pre-coated with the capture antibody. 50 µL of each of the prepared concentrations were added to each well in triplicates. 25 µL of the antibody-enzyme conjugate was added to each well and the plate was then incubated for 24 hours at 4°C. Excess

reagent was then removed by washing the plate three times with buffer and a substrate solution was added and incubated on an orbital shaker. After 20 minutes of incubation, the reaction was stopped and the fluorescence of each well was read at 325 nm for excitation and 420 nm for emission using SoftMax Pro 5.4.1 software on a SPECTRAmax M5e ROM v2.1.35 (Molecular Devices, San Jose, CA 95134, USA). Each concentration was plated in triplicate and the experiment was repeated three times.

5.2.2.2 SP ELISA kit from Cayman Chemicals

SP from Phoenix Pharmaceuticals and SP from Sigma Aldrich were serially diluted to obtain solutions with concentrations of 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL, 7.8 pg/mL. 50 µL of each of the solutions were added to each well in triplicates, followed by 50 µL of SP AChE (acetylcholinesterase) tracer to each well. Then 50 µL of SP antiserum was added and the ELISA plate was incubated for 16 hours at 4°C. After 16 hours the content in the wells were discarded and the wells were washed five times with wash buffer provided with the kit. Ellman's reagent (200 µL) was added to each plate and incubated for 2 hours on an orbital shaker. After 2 hours of reaction, the absorbance intensity was determined at 412 nm using SoftMax Pro 5.4.1 software on a SPECTRAmax M5e ROM v2.1.35 (Molecular

Devices, San Jose, CA 95134, USA). Each concentration was plated in triplicate and the experiment was repeated three times.

5.2.3 Statistical Analysis

The results were analyzed using GraphPad Prism version 8.3.0. (San Diego, CA, USA). Bland-Altman plots were used to determine the agreement of the SP from Phoenix Pharmaceuticals with the SP from Sigma Aldrich, for both the Phoenix Pharmaceuticals and Cayman Chemicals ELISA kit. Since the Phoenix Pharmaceuticals ELISA kit reported in fluorescence units and the Cayman Chemicals ELISA kit reported in absorbance units, the results were harmonized by reporting percentage difference against the average for the Bland Altman plots.

5.2.4 Results

Tables 5-1 and 5-2 describe the absorbance and fluorescence units (mean ± SD), and returned concentrations for each corresponding control concentration of SP from both Phoenix Pharmaceuticals and Sigma Aldrich.

Table 5-1 A summary of the absorbance units reported for each SP concentration measured using the Cayman Chemicals SP ELISA kit.

Control Concentrations	Phoenix Pharmaceuticals SP (mean ± SD)	Returned Concentration (pg/mL)	Sigma Aldrich SP (mean ± SD)	Returned Concentration (pg/mL)
500 pg/mL	0.289 ± 0.073	455.21 ± 108.15	0.313 ± 0.058	490.24 ± 96.67
250 pg/mL	0.341 ± 0.081	218.15 ± 175.16	0.366 ± 0.070	334.54 ± 169.48
125 pg/mL	0.396 ± 0.105	112.88 ± 73.47	0.432 ± 0.069	192.61 ± 71.41
62.5 pg/mL	0.459 ± 0.105	80.90 ± 22.32	0.529 ± 0.100	52.42 ± 8.81
31.2 pg/mL	0.559 ± 0.149	30.79 ± 13.46	0.607 ± 0.113	27.35 ± 17.01
15.6 pg/mL	0.613 ± 0.175	38.74 ± 30.22	0.679 ± 0.126	10.31 ± 3.71
7.8 pg/mL	0.652 ± 0.171	13.41 ±6.69	0.734 ± 0.141	11.38 ± 8.37

Table 5-2 A summary of the fluorescence units reported for each SP concentration measured using the Phoenix Pharmaceuticals SP ELISA kit.

Control Concentrations	Phoenix Pharmaceuticals SP (mean ± SD)	Recovered Concentration (pg/mL)	Sigma Aldrich SP (mean ± SD)	Recovered Concentration (pg/mL)
500 pg/mL	3055.881 ± 1259.030	593.12 ± 207.11	3223.587 ± 2525.388	413.64 ± 318.94
250 pg/mL	4541.588 ± 2194.499	253.39 ± 80.08	4774.970 ± 3239.348	303.84 ± 218.99
125 pg/mL	5836.092 ± 2812.233	113.64 ± 36.29	5857.764 ± 3758.113	134.09 ± 36.64
62.5 pg/mL	6985.764 ± 3178.217	54.59 ± 23.96	6682.423 ± 3845.265	105.56 ± 55.34
31.2 pg/mL	8023.917 ± 3272.647	27.53 ± 19.22	8257.590 ± 4924.259	26.25 ± 9.05
15.6 pg/mL	8712.723 ± 3964.394	15.87 ± 5.92	9120.351 ± 4599.981	11.53 ± 3.58
7.8 pg/mL	9107.385 ± 5236.212	14.09 ± 11.77	9707.319 ± 4346.209	6.69 ± 0.68
3.9 pg/mL	9633.451 ± 4835.918	7.15 ± 3.23	10314.618 ± 3989.103	4.70 ± 1.82

Fig 5-2 (a) and 5-2 (b) show the agreement between of SP from Phoenix Pharmaceuticals with the SP from Sigma Aldrich, for the Phoenix Pharmaceuticals ELISA kit and the Cayman Chemicals ELISA kit, respectively. The mean difference was -3.36%, and the 95% limits of agreement were [-10.75, 4.01], for the Phoenix Pharmaceuticals kit. The mean difference for the Cayman Chemical kit was -9.70%, and the 95% limits of agreement were [-14.61, -4.79].

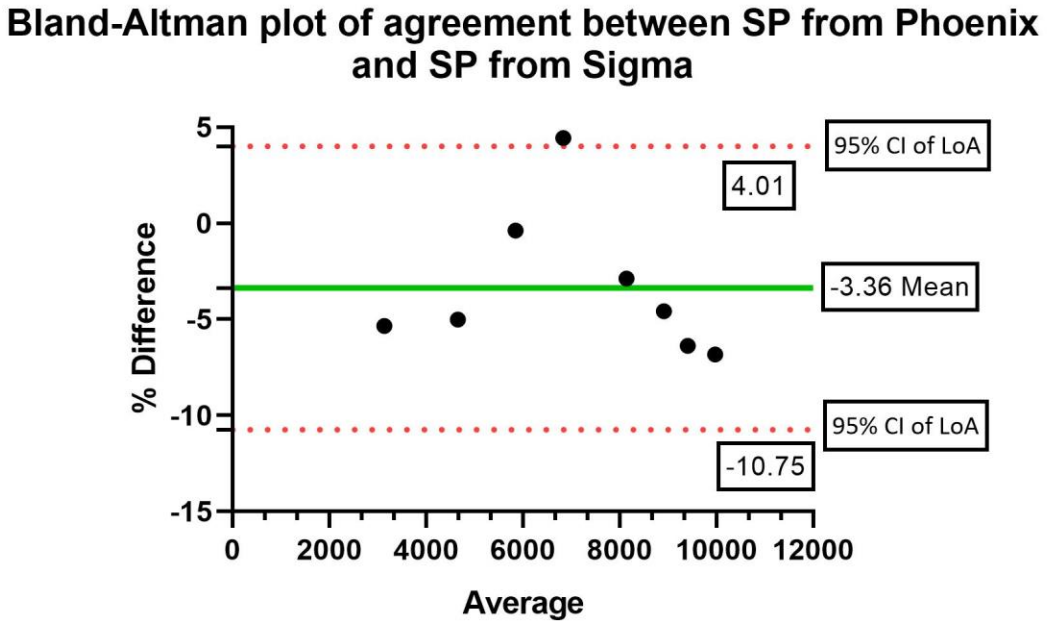

Figure 5-2 (a) Bland-Altman Plot reporting the agreement between SP from Phoenix Pharmaceuticals and SP from Sigma Aldrich in the Phoenix Pharmaceuticals SP kit. The green line represents the mean difference and the red dotted lines (above and below) represent the upper and lower 95% limits of agreement.

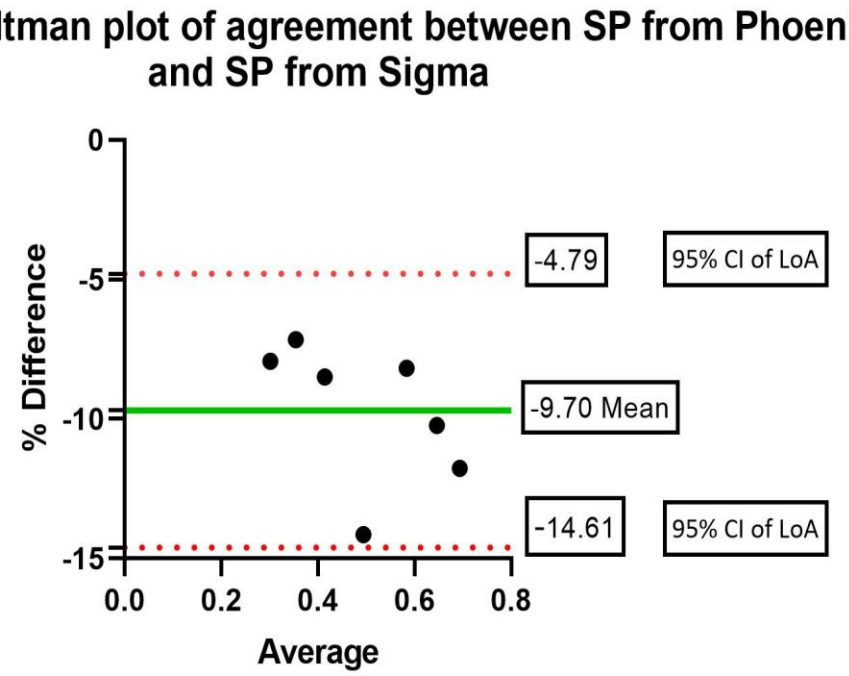

Figure 5-2 (b) Bland-Altman Plot reporting the agreement between SP from Phoenix Pharmaceuticals and SP from Sigma Aldrich, using the Cayman Chemicals ELISA kit. The green line represents the mean difference and the red dotted lines (above and below) represent the upper and lower 95% limits of agreement.

5.3 Discussion and Conclusion

The results from this chapter show that the Cayman Chemicals ELISA kit has a smaller 95% limits of agreement interval compared to the Phoenix Pharmaceuticals ELISA kit, despite a slightly higher mean difference. The detection of bias (mean difference) in both the kits suggests that overall, SP from both sources were not being quantified equally. This may be due to small differences with the chemicals that could affect the quantification from ELISA kits. Another reason for this difference may be due to the difference in the unit of quantification.

The ELISA kit from Phoenix Pharmaceuticals measured SP in fluorescence units and the ELISA kit from Cayman Chemicals measured SP in absorbance units. Fluorescence is based on the emission of the light by a substance that was excited by a photon, while absorbance measures the amount of light absorbed by a substance at a given wavelength.[79] While an assumption was made that the SP from Phoenix Pharmaceuticals and the SP from Sigma Aldrich were identical, they were not based on the spectral absorbance plots. This minor difference may explain the mean bias observed in the Bland Altman plots and overall poor agreement between the two SP substances.

When examining the variability of the absorbance and fluorescence values corresponding to the SP control concentrations for both the Phoenix Pharmaceuticals and Cayman Chemicals SP

ELISA kits (Tables 5-1 and 5-2) the variability in both absorbance and fluorescence increased towards lower control concentrations, while variability of the returned concentrations showed an opposite trend. This observation can be explained by the lower control concentrations returning concentrations which are likely outside the linear region of the standard curve. The high variability in fluorescence and absorbance units associated with low concentrations would also explain the poor agreement observed in the Bland Altman plots, in either kit. Since the working range of concentrations in this study was towards the lower end reported by some existing studies,[17,18,20,22] future work to investigate a higher range of concentrations (e.g., greater than 1 ng/mL) may be valuable.

Chapter 6– General Discussion

The objective of this thesis was to develop and optimize a method for the quantification of neuropeptides (CGRP, SP, NPY, VIP) in the human tear film. While many of the studies in the literature only measured the concentration of SP and or CGRP [17,18,20-22,56] there are very few studies that have measured the concentration of all four neuropeptides in the tear film.[19,60] The reported studies all have different approaches to tear film collection and neuropeptide quantification, which are likely the major reasons explaining the large variation observed in the reported quantities of these neuropeptides in the literature (summarized in Table 3-1). This large variability poses challenges in detecting meaningful changes in physiological levels. Thus, there is a need to determine methods of tear collection and optimize sample processing to reduce the sources of variability. The development and optimization steps of the neuropeptide ELISA are summarized in the following sections.

The first objective of this study was to develop a method of tear collection and measure the concentration of neuropeptides (CGRP, SP, NPY, VIP) using a commercially available ELISA kit. The results from Chapter 3 showed that there was no significant difference in neuropeptide concentrations between basal and flush tear collection techniques, and no significant difference in neuropeptide concentrations on two consecutive days. It was anticipated that flush tears

would yield a lower concentration than basal tears due to dilution, however, there was no significant difference detected between the two collection techniques. This finding raised fundamental questions about the validity of the ELISA technique for the quantification of neuropeptides in the tear film.

The second objective of this study was to develop learnings (and test potential deficiencies) around the neuropeptide ELISA kits that had been used in previous studies. The results of the two tests (spike and recovery of neuropeptides and the response to dilution) suggested the possibility of tear film proteins interfering with the ELISA process. After conducting additional troubleshooting steps, it was concluded that a) blocking agent did not improve assay results, b) albumin showed interference with the ELISA assay, c) neuropeptides were lost from using C18 column separation, d) neuropeptides were lost using protease inhibitors. Additionally, freshly collected tears yielded higher neuropeptide concentrations than tears stored at -80°C.

The final aim of this study was to optimize an ELISA kit to measure the concentration of SP neuropeptide in the tear film. The variability two commercially available ELISA kits were measured and compared. The results from this experiment suggest that both SP ELISA kits from Cayman Chemicals and Phoenix Pharmaceuticals showed significant variability.

Of note, there were several limitations to our clinical study population investigated using these kits, including participant recruitment, sample size and tear collection method.

- Recruitment classification: The participants recruited for this study were included based solely on the OSDI questionnaire score to rule out symptoms of dry eye. This may have resulted in inclusion of participants with mild DED for this study, potentially influencing the amount of neuropeptides collected. Clinical tests to detect DED could have been used to fine-tune the selection of participants. A more rigorous approach to selecting participants will be the focus for future work.
- Sample size: We recruited only 10 participants and only 8 completed the study. Given the variation between basal and flush tears between day one and day two in individual participants, a larger sample size could have been used to detect smaller differences.
- Tear collection method: This study used microcapillary glass tubes for collecting tears, which may have induced reflex tearing and diluted the concentrations of the neuropeptides collected from the tear film. This may be one of the reasons for failing to detect any significant difference between basal and flush tears.

Future research efforts should be directed at continuing to optimize ELISA kits for measuring the concentration of neuropeptides in the tear film. Once that is accomplished, we

could begin to explore the role of neuropeptides in various population groups, such as CL wearers, CL wearers with discomfort, and those with dry eye disease.

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